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Characterization of Nonattaching Mutants of *Agrobacterium tumefaciens*

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The first step in tumor formation by *Agrobacterium tumefaciens* is the site-specific binding of the bacteria to plant host cells. Transposon mutants of the bacteria which fail to attach to carrot suspension culture cells were isolated. These mutants showed no significant attachment to carrot cells with either microscopic or viable cell count assays of bacterial binding. The nonattaching mutants were all avirulent. When revertants of the mutants were obtained by enriching for bacteria which do bind to carrot cells, the bacteria were found to have regained the ability to bind to carrot cells and virulence simultaneously. These results suggest that the ability of the bacteria to bind to plant cells is required for virulence. Like the parent strain, all of the nonattaching mutants synthesized cellulose, but unlike the parent strain, they failed to aggregate carrot suspension culture cells. The transposon Tn*5*, which was used to obtain the mutants, was located on a 12-kilobase *Eco*RI fragment of the bacterial chromosomal DNA in all of the nonattaching mutants from strain C58. That the mutant phenotype was due to the Tn*5* insertion was shown by cloning the Tn*5*-containing DNA fragment from the mutant bacteria and using it to replace the wild-type fragment in the parent strain by marker exchange. The resulting bacteria had the same mutant phenotype as the original Tn*5* mutants; they did not attach to carrot cells, they did not cause the aggregation of carrot cells, and they were avirulent. No difference was seen between the parent strain and the nonattaching mutants in hydrophobicity, motility, flagella, fimbriae, β-2-glucan content, size of lipopolysaccharide, or ability of the lipopolysaccharide to inhibit bacterial attachment to tissue culture cells. Differences were seen between the parent strain and the nonattaching mutants in the polypeptides removed from the bacteria during the preparation of spheroplasts. Three of the mutants were lacking a polypeptide of about 34 kilodaltons (kDa). One mutant was lacking the 34-kDa polypeptide and another polypeptide of about 38 kDa. The fifth mutant was lacking a polypeptide slightly smaller than the 34-kDa polypeptide missing in the other four mutants. These missing polypeptides all reappeared in the revertants of the mutants. Thus, bacterial binding to plant cells appears to require the presence of these polypeptides.

The initial step in tumor formation by *Agrobacterium tumefaciens* in wounded plants is the site-specific attachment of the bacteria to the host cell (5). Bacterial attachment to plant cells growing in suspension culture has also been observed (9, 13, 16, 18). Both attachment to wound sites in whole plants and attachment to tissue culture cells are specific for strains of *A. tumefaciens*. Related bacteria such as rhizobia and many strains of avirulent *Agrobacterium radiobacter* generally do not show such attachment to tissue culture cells and do not compete with *A. tumefaciens* for receptor sites on wounded bean leaves (5, 9). These observations suggest that the bacteria attach to the plant cells by some binding site(s). Carrot cells are not active participants in the binding interaction with *A. tumefaciens*. The bacteria bind with only slightly altered kinetics to dead carrot cells (8). This paper examines the role of the bacteria and the bacterial surface in the attachment interaction.

In a study of *A. tumefaciens* mutants that were avirulent and in which the transposon was located in the bacterial chromosome, Douglas et al. (2) described mutants that were unable to bind to plant cells. This paper describes the isolation and characterization of additional transposon mutants that failed to bind to carrot suspension culture cells. These mutants were not originally chosen for their avirulence; their properties, including virulence, are also described. The surface components of these mutants were examined in an attempt to determine the components involved in the attachment of *A. tumefaciens* to plant cells. That the mutants resulted from the insertion of Tn*5* was determined by cloning the DNA containing the Tn*5* insertion and replacing the wild-type gene in the parent bacterial strain with the Tn*5*-containing DNA by marker exchange. The phenotype of the resultant bacteria was compared with that of the original mutants and found to be identical.

MATERIALS AND METHODS

Strains and construction of mutants. Bacteria were grown, and viable counts were determined as previously described (9). Sources of virulent strains of *A. tumefaciens* A6 and C58, the avirulent strain NT1, and *Escherichia coli* 1830(pJB4JI) were the same as previously listed (7). Avirulent *A. tumefaciens* IIBNV6 was obtained from B. B. Lippincott and J. A. Lippincott, Northwestern University, Evanston, Ill. *E. coli* HB101(pRK2013) was obtained from Paul Bishop, North Carolina State University, Raleigh. *E. coli* JM83 and JM83(pUC9) were obtained from Rosemarie Spencer, Purdue University, West Lafayette, Ind. Suspension cultures of *Daucus carota* were grown in Murashige and Skoog (M&S) medium (12) as previously described (8).

The transposon Tn*5* was introduced into *A. tumefaciens* by conjugation from *E. coli*(pJB4JI) as previously described (7). Transconjugants were selected for growth on minimal medium with 60 μg of neomycin per ml. Neither parent grew under these conditions. The resulting isolates were picked, restreaked on minimal medium with neomycin, inoculated into a tube with carrot cells in 1 ml of M&S medium, and incubated overnight to test for their ability to attach to carrot suspension culture cells by using a microscopic assay for

attachment (8, 9). The colonies were also inoculated on *Bryophyllum* leaves with toothpick wounds to test for virulence. Isolates which showed no attachment in the microscopic assay or were avirulent on *Bryophyllum* leaves were grown in Luria broth and retested for virulence and attachment by using the viable cell count assay (8, 9). Five isolates which failed to bind to carrot suspension cells were obtained from four independent crosses introducing Tn*5* into *A. tumefaciens* C58. Two isolates which failed to bind to carrot cells were obtained from the introduction of Tn*5* into *A. tumefaciens* A6. These isolates were characterized further. These isolates grew at the same rate as did the parent strain in Luria broth. They also grew in minimal medium (9) containing 20 μg of neomycin per ml. However, they grew very slowly in Luria broth containing neomycin.

Bacterial treatments. Bacteria were grown in Luria broth to stationary phase. They were collected by centrifugation at 10,000 × *g* and suspended in phosphate-buffered saline as previously described (9). The bacteria were killed by exposure to 65°C for 30 min, UV light, or 0.1% glutaraldehyde for 10 min. Each of these treatments caused a decrease in the number of viable bacteria of between 10^{-5} and 10^{-7}. The dead bacteria were collected by centrifugation and added to a suspension of 10^5 carrot cells per ml to give a final bacterial concentration of 10^7 bacteria per ml. The attachment of the bacteria to the carrot cells was monitored with a light microscope after 60 and 120 min of incubation. In addition to killing the bacteria, these treatments may alter the surface of the bacteria by denaturing or cross-linking surface proteins.

To inhibit bacterial protein synthesis during attachment, bacteria were incubated with 5 μg of tetracycline per ml or 200 μg of chloramphenicol per ml for 5 min before adding the bacteria to carrot cells in M&S medium containing the same concentration of the antibiotic. Bacterial protein synthesis was monitored by measuring the incorporation of [^{3}H]leucine into acid-insoluble material during incubation. Incorporation of [^{3}H]leucine in the presence of the antibiotics was about 1% of that observed in the absence of the antibiotics. Bacterial attachment was monitored with the light microscope after 60 min of incubation of 10^7 bacteria per ml with 10^5 carrot cells per ml. Since live carrot cells are not required for the attachment of *A. tumefaciens* (8), these experiments were carried out with both live and heat-killed carrot cells. There was no difference in the results.

Bacteria were collected, suspended in phosphate-buffered saline, pH 7.5, and treated with 1 mg of trypsin per ml for 60 min. The bacteria were diluted 1 to 10^5 into M&S medium (pH 5.5) containing 10^5 carrot cells per ml and 10 μg of soybean trypsin inhibitor per ml. The rate of attachment of the bacteria to the carrot cells was measured as previously described (9) and compared with the rate of attachment of control bacteria, which were treated exactly as the experimental bacteria except for the omission of the trypsin from the phosphate-buffered saline.

Preparation and use of bacterial spheroplasts. Bacterial spheroplasts were prepared by the method of Sonoki and Kado (17). They were suspended in 0.45 M sucrose. Measurements of bacterial attachment were made in 0.45 M sucrose, and the bacteria were plated on Luria broth agar containing 0.2 M sucrose. The use of this medium did not affect the rate of attachment of intact bacteria. As a rough measure of the resynthesis of the bacterial cell wall, bacteria were diluted in sterile water, suspended by vortexing, and plated on Luria broth agar plates. Viable cell counts obtained in this way were compared with those obtained by diluting the bacteria in 0.4 M sucrose and plating them on Luria broth agar plates containing 0.2 M sucrose. The plating efficiency of spheroplasts as judged by comparison with bacterial counts made with the light microscope was >98%.

Interaction of bacteria with plant cells. Measurements of the ability of the bacteria to attach to carrot suspension culture cells and to cause aggregation of the carrot cell cultures were made as previously described (8, 9). Both microscopic and viable cell count measurements of attachment were made. As viewed under the microscope, bacteria were considered to be attached if they no longer showed Brownian motion and moved with the carrot cells when the slide was tapped so that the carrot cells rolled. Viable cell count measurements of attachment were made as previously described (8, 9). Samples of the culture were filtered through Miracloth, which retains carrot cells and attached bacteria. Free bacteria pass through the filter. The number of free bacteria was determined by viable cell counts. Carrot cells and attached bacteria were suspended in phosphate-buffered saline and ground in a Waring blender, and the number of attached bacteria was determined by viable cell counts. The initial screening for bacteria which failed to bind to carrot cells was made by inoculating about 10^5 bacteria into small tubes containing 1 ml of M&S medium with 10^5 carrot cells. The tubes were incubated for 24 h, and the binding of the bacteria to the carrot cells was determined by examination with the light microscope. Bacteria which showed no binding in this test were kept for further testing, including the ability of the bacteria to bind to carrot cells after 60 min of incubation of 3×10^3 bacteria per ml with 10^5 carrot cells per ml of M&S medium.

The ability of the mutant bacteria to compete with the wild-type parent strain for binding sites on wounded bean leaves was determined by the method of Lippincott and Lippincott (5), except that Top Crop rather than pinto beans were used in the assay. Avirulent *A. tumefaciens* IIBNV6, which is reported (5) to compete for binding sites in this system, was used as a positive control in these assays.

Bacterial virulence was determined by infecting *Bryophyllum diagremontiana* and carrot root discs. All plants were grown in a greenhouse. Carrots were purchased in a supermarket. *B. diagremontiana* leaves were infected by wounding the plant with a toothpick containing a colony of *A. tumefaciens*. Carrot discs were infected with 0.05 ml of a suspension of bacteria as described by Klein and Tenenbaum (3). Plants and discs were scored for the presence of tumors for the succeeding 6 weeks.

Characterization of bacterial mutants. Bacterial isolates were tested for the ability to synthesize cellulose fibrils as previously described (7). Bacterial motility was observed during examination of the interaction of the bacteria and carrot cells with the light microscope. Bacteria were examined for pili and flagella by using negative stain with phosphotungstic acid or with chromium and the transmission electron microscope. Bacterial spheroplasts were prepared by the method of Sonoki and Kado (17). The proteins removed from the bacteria during this procedure were examined by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) by the method of Laemmli (4) and the silver stain protocol of Merril et al. (10) to visualize the resulting bands. Running gels of 11 and 15% acrylamide with a 3% stacking gel were used. Lipopolysaccharide (LPS) was extracted from the bacteria as previously described (9) and examined by SDS-PAGE by the method of Tsai and Frasch (19). The concentration of LPS was determined by measuring the amount of ketodeoxyoctonate per milliliter as previously described (9). Bacterial hydrophobicity was ex-

amined by observing the tendency of the bacteria to accumulate at the interface between water and hexadecane droplets (15). Bacterial extracellular polysaccharide was prepared from the medium after the bacteria were grown to stationary phase in nutrient broth by the protocol of Carlson and Lee (1). Bacterial β-2-D-glucan was extracted and quantitated by the method of Puvanesarajah et al. (14).

DNA isolation, hybridization, and cloning. The determination of whether the Tn*5* was located in the bacterial chromosome or in a plasmid was carried out as previously described (7). Tn*5* was located in the bacterial chromosome in all the nonattaching mutants. The Tn*5* mutants were tested for the presence of Mu DNA (resulting from the presence of Mu in pJB4JI) by DNA hybridization as previously described (7). No Mu DNA was detected in any nonattaching mutant derived from *A. tumefaciens* C58. The two nonattaching mutants derived from strain A6 contained Mu DNA as well as multiple insertions of Tn*5*. These mutants were not characterized further. Bacterial DNA was prepared from wild-type and mutant C58 *A. tumefaciens* and digested with *Eco*RI; the fragment carrying the Tn*5* insertion was identified by hybridization to Southern transfers of DNA fragments separated by agarose gel electrophoresis as previously described (7). *A. tumefaciens* DNA from the nonattaching mutants was digested with *Eco*RI and cloned into the *Eco*RI site of pUC9 by the methods of Maniatis et al. (6). The resulting plasmids were transformed into *E. coli* JM83, and the transformants were selected for neomycin resistance and subsequently for ampicillin resistance.

To determine whether the Tn*5* insertion was responsible for the mutant phenotype, the cloned *A. tumefaciens* DNA containing the Tn*5* insertion was introduced into wild-type strains including the parent strain by marker exchange. A triparental mating was carried out by using *E. coli*(pRK2013) to mobilize the transfer of the pUC9 DNA clones from *E. coli* JM83 to *A. tumefaciens*. Cultures of *E. coli* JM83 containing the plasmid with the cloned DNA fragment and *E. coli* HB101(pRK2013) were grown to mid-log phase at 37°C in Luria broth. *A. tumefaciens* C58 was grown to mid-log phase at 25°C in Luria broth. Volumes of the bacterial cultures containing approximately equal numbers of bacteria were mixed on Luria agar plates and incubated overnight at 25°C to allow conjugation to occur. Bacteria were harvested by scraping the plates and washed once with 0.9% NaCl. Transconjugants were selected for the ability to grow on minimal medium in the presence of 60 μg of neomycin per ml. None of the parent strains grew under these conditions, nor did any transconjugants from matings of any two parent strains without the third. Transconjugants were tested for the retention (presumably from integration into the bacterial chromosome by a single homologous recombination event) of the entire pUC9 by testing for the presence of the ampicillin resistance gene from pUC9 by using an Amp Screen (Bethesda Research Laboratories). The use of Amp Screen was necessary since wild-type *A. tumefaciens* is not very sensitive to ampicillin. Amp Screen allows screening of the colonies for the production of β-lactamase, which was not detected in the wild-type parent. At least five transconjugants from each cross that did and that did not show production of β-lactamase were tested for the ability to bind to carrot cells with the microscopic attachment assay. All of the isolates which appeared to contain all of pUC9 (and thus would be expected to contain a wild-type and a mutant copy of the attachment genes) were able to bind to carrot cells. All the isolates tested which were neomycin resistant but did not appear to contain the pUC9 ampicillin resistance gene (and which had presumably arisen by a double homologous recombination event and would be expected to contain only the mutant copy of the gene) were unable to attach to carrot cells. The properties of these transconjugants were characterized further as described above for the original mutants.

Revertants of the nonattaching mutants. Cultures of each of the bacterial mutants were grown in the absence of neomycin. A large excess of bacteria (10^9 bacteria per ml) was then incubated with 10^5 carrot cells per ml for 24 h. The carrot cells were collected by filtration through Miracloth, and most of the trapped, but not attached, bacteria were removed by washing three times with 10 volumes of M&S medium. Although mechanical trapping of bacteria was not a problem in ordinary attachment assays with 10^3 to 10^4 bacteria per ml, mechanical trapping of bacteria was observed with the light microscope for high bacterial concentrations (e.g., 10^9 bacteria per ml). The carrot cells and any attached or mechanically trapped bacteria were then plated on Luria broth agar plates. The suspension of carrot cells and bacteria contained about 5×10^9 bacteria per ml after 24 h of incubation. After washing, the carrot cells were resuspended in 0.1 of the original volume. The number of bacteria recovered from the plating of the carrot cells was <100/ml, of which about half appeared to be revertants which had recovered the ability to bind to carrot cells (the remainder appeared to be mechanically trapped mutant bacteria). Thus, this selection technique appeared to be relatively powerful; the reversion frequency for the mutants can be estimated to be about 1 in 10^9. The revertants were neomycin sensitive and thus presumably no longer contained Tn*5*. One arbitrarily chosen revertant from each mutant was characterized further as described above for the characterization of the original mutants.

RESULTS

Characteristics of bacterial attachment to carrot suspension culture cells. Live bacteria of virulent strains of *A. tumefaciens* attach to carrot suspension cultures within 60 min of the addition of the bacteria to the plant cell cultures (8). The binding of live bacteria and bacteria killed by exposure to heat, glutaraldehyde, or UV light to live carrot cells was examined under the light microscope with 10^7 bacteria per ml and 10^5 carrot cells per ml. Live bacteria were observed bound to the plant cells after 40 min of incubation (Fig. 1). No bacteria were attached to the carrot cells when bacteria killed by any of the treatments were incubated with the plant cells. To determine whether the requirement for live bacteria represented a requirement for bacterial protein synthesis, the bacteria were treated with chloramphenicol or tetracycline for 5 min before their addition to the carrot cell culture in the continued presence of the antibiotic. Although each antibiotic was present in sufficient concentration to prevent the incorporation of [^{3}H]leucine into acid-insoluble material by the bacteria, the antibiotics had no effect on the ability of the bacteria to bind to carrot cells (Fig. 1). This result suggests that bacterial protein synthesis is not required for the attachment of *A. tumefaciens* to carrot cells. Whether the inability of dead bacteria to bind to carrot cells is due to a real requirement for live bacteria or whether it is a result of an alteration in the bacterial surface caused by the treatments used to kill the bacteria remains undetermined.

To examine the role of the bacterial surface in the attachment interaction, the attachment of bacterial spheroplasts to carrot cells was examined. Since live bacteria are required

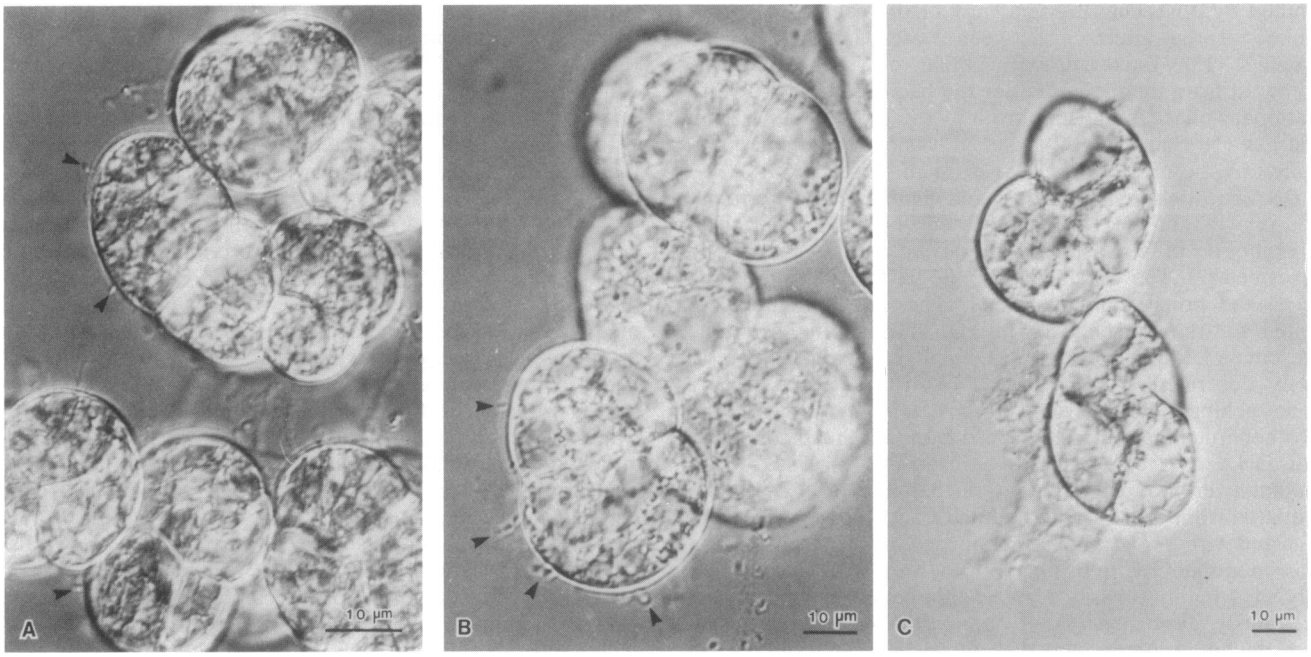

FIG. 1. Effects of treatments of the bacteria on bacterial attachment to carrot cells. Attachment of *A. tumefaciens* A6 to living carrot suspension culture cells viewed in the light microscope with Nomarski optics. (A) Carrot cells incubated with untreated bacteria for 40 min; (B) Carrot cells incubated with tetracycline-treated bacteria in the presence of 5 μg of tetracycline per ml for 60 min; (C) Carrot cells incubated with heat-killed bacteria (56°C for 30 min) for 75 min. Note the attached bacteria at the arrowheads in panels A and B. No attached bacteria were observed in panel C. Carrot cells (10^5/ml) and bacteria (2×10^7 to 6×10^7/ml) were used.

for bacterial attachment, it was not possible to kill the bacteria after spheroplast formation. Instead, live spheroplasts were used immediately after preparation, and the time course of attachment of intact bacteria and of spheroplasts was examined (Fig. 2). The attachment of bacterial spheroplasts to carrot cells was delayed about 15 to 20 min when compared with the attachment of intact bacteria (Fig. 2). Immediately after preparation, the viability of the spheroplasts was reduced by more than 10^5 by dilution in sterile water. After about 20 min of incubation, the spheroplasts began to lose their sensitivity to dilution in water, indicating that the regeneration of the bacterial cell wall had begun; the bacteria also began to bind to carrot cells at this time. Thus, the outer surface of the bacteria appears to be required for bacterial binding to carrot cells. In an attempt to examine which components of the outer surface are required, bacteria were treated with trypsin (pH 7.5) and immediately diluted 1 to 10^5 in M&S medium (pH 5.5) containing soybean trypsin inhibitor; their rate of attachment to carrot cells was compared with that of bacteria treated identically except for the omission of the trypsin in the incubation at pH 7.5. Soybean trypsin inhibitor had no effect on the rate of bacterial attachment. The trypsin-treated bacteria showed a small delay in attachment (5 to 10 min) when compared with the control bacteria, suggesting that protein components exposed on the bacterial surface may be important in bacterial attachment (Fig. 3).

Isolation of nonattaching bacterial mutants and their interaction with carrot cells and with plants. Five mutants of *A. tumefaciens* C58 which did not attach to carrot suspension culture cells were obtained as a result of the introduction of Tn*5* into C58 by conjugation from *E. coli* 1830(pJB4JI). None of the *A. tumefaciens* transconjugants contained the plasmid pJB4JI as judged by their sensitivity to gentamicin (pJB4JI carries a gene for gentamicin resistance) and by the absence of any new plasmid bands when bacterial DNA was examined by using lysis in the well as described below. None of the transconjugants showed any DNA hybridization with Mu DNA. The five nonattaching mutants were the products of four independent crosses. The mutants showed no attachment to carrot cells when 10^7 bacteria per ml were incubated for 24 h with 10^5 carrot cells and the mixture was examined with the light microscope (Fig. 4). They also showed no significant attachment to carrot cells when 3×10^3 bacteria per ml were incubated with 10^5 carrot cells per ml for 1 h,

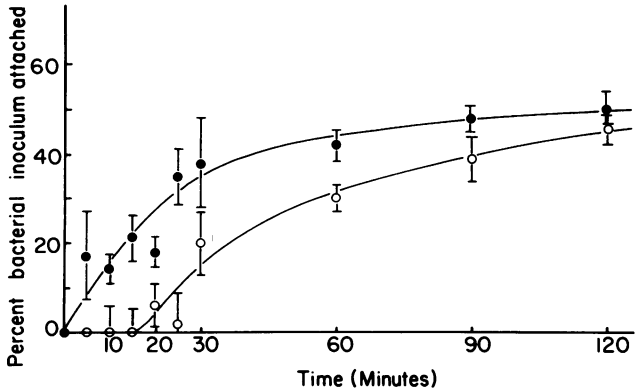

FIG. 2. Time course of attachment of *A. tumefaciens* A6 to carrot cells. Symbols: ●, untreated A6; ○, A6 spheroplasts. Bars indicate standard deviations of a minimum of three experiments. Attachment of bacterial spheroplasts was delayed 15 to 20 min when compared with untreated bacteria. Attachment was measured in the presence of 0.45 M sucrose.

and the number of attached bacteria was determined by separation of the attached bacteria from the free bacteria by filtration as previously described (9) (Table 1).

Two mutants of *A. tumefaciens* A6 that did not attach to carrot cells were obtained as a result of crosses similar to those described for strain C58 introducing Tn*5* into strain A6. Both mutants showed hybridization with Mu DNA and appeared to contain multiple insertions of Tn*5* as judged by hybridization of Tn*5* to Southern blots of *Eco*RI-digested mutant DNA (data not shown). Neither of these mutants showed binding to carrot cells (Fig. 1). They also did not show any attachment to carrot cells when the kinetic assay was used (0 ± 10% of the original inoculum bound in 1 h; the parent strain bound 60 ± 10% of the original inoculum). Because these strains might be multiple mutants, they were not characterized in detail.

During the screening of the *A. tumefaciens* transconjugants that were neomycin resistant, bacteria were screened independently for the ability to attach to carrot cells and for virulence on *B. diagremontiana* leaves. All isolates which were unable to attach to carrot cells were avirulent on *B. diagremontiana*. About 5% of the transconjugants showed reduced or no virulence on *B. diagremontiana*. The nonattaching mutants constituted about 15% of the avirulent isolates. The failure to obtain any mutants that retained virulence and were unable to attach to carrot cells suggests that the ability to attach to carrot cells is required for virulence of *A. tumefaciens*. The nonattaching mutants were also found to be avirulent on carrot discs (Table 1).

The ability of avirulent agrobacteria to inhibit tumor formation by virulent *A. tumefaciens* when the avirulent bacteria are inoculated before, but not after, the virulent bacteria has been used as a measure of the ability of the avirulent bacteria to compete for receptor sites on the plant cell surface (5). This assay was used to measure the ability of the mutants that failed to attach to carrot suspension culture cells to bind to cells in the whole plant. Avirulent strain IIBNV6, which has been reported to show binding in this assay, was used as a control (5). None of the nonattaching mutants showed any ability to inhibit tumor formation by strain C58. However, inoculation of strain IIBNV6 before, but not after, strain C58 resulted in a 50% inhibition of tumor formation (Table 2). Thus, the mutants of strain C58 which failed to bind to carrot suspension culture cells also failed to inhibit tumor formation on wounded bean leaves.

Wild-type *A. tumefaciens* causes the aggregation of carrot suspension cells when incubated with them for 20 h (8). This ability to aggregate carrot suspension cells is dependent on the ability of the bacteria to synthesize cellulose fibrils (7). The nonattaching mutants were examined for the ability to produce cellulose by testing for their ability to produce material which showed a fluorescent stain with Calcofluor or Cellufluor when grown in the presence of Soytone. All the nonattaching mutants produced material which fluoresced with these stains. The ability of the nonattaching mutants to cause the aggregation of carrot suspension cells was examined by incubating the bacteria with carrot cells in M&S medium for 20 h. None of the mutants caused the aggregation of the carrot cells although the parent strain caused visible aggregation of the carrot cells under these conditions (Table 1; Fig. 5). This result suggests that carrot cell binding as well as cellulose synthesis is required for aggregation and that the mutants failed to bind to the carrot cells.

Characterization of surface components of nonattaching mutants. Since bacterial spheroplasts do not bind to carrot cells until sufficient time has elapsed so that they have begun to resynthesize surface components, it appears that some surface constituents removed from the bacteria during spheroplast preparation may be involved in bacterial attachment to carrot cells. Therefore, these surface components were compared from the nonattaching mutants and the parent strain. In addition, general comparisons of the surface properties of the parent and the mutants were carried out.

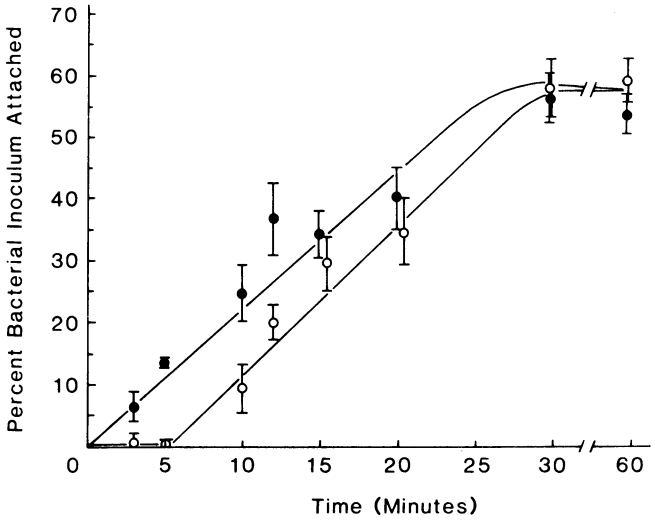

FIG. 3. Time course of attachment of *A. tumefaciens* A6 to carrot cells. Symbols: ●, untreated A6; ○, trypsin-treated A6. Bars indicate standard deviations of a minimum of three experiments. Attachment of trypsin-treated bacteria was delayed 5 min when compared with untreated bacteria. Attachment was measured in M&S medium (pH 5.5) with 10 μg of soybean trypsin inhibitor per ml.

The parent strain of *A. tumefaciens* is weakly hydrophobic as judged by its tendency to accumulate at the interface between water and hexadecane droplets. All the nonattaching mutants appeared to be unaltered in hydrophobicity when examined under the same conditions as the parent strain (data not shown). The parent strain is motile. All of the nonattaching mutants were seen in the light microscope to be motile in M&S medium in the presence of carrot cells (Table 1). The parent strain was observed to have fimbriae when examined with negative stain and the transmission electron microscope. All nonattaching mutants had fimbriae which appeared to be identical to those of the parent strain (data not shown). No difference was observed in the amount of extracellular polysaccharide produced by the parent strain and by the mutants (data not shown). β-2-D-Glucan is reported to be produced by the agrobacteria and to be absent in one class of the nonattaching mutants described by Douglas et al. (2, 11, 14). No significant difference was observed in the amount of β-2-D-glucan produced by the parent strain and the mutants Att-C43, Att-C539, and Att-C69 (the other two mutants were not examined). The relative amounts of β-2-D-glucan and the two higher-molecular-weight polysaccharides eluted from the Bio-Gel column by the procedure of Puvanesarajah et al. (14) were approximately the same for the parent strain and the mutants and were similar to those reported by Puvanesarajah et al. (14).

LPS from virulent strains of *A. tumefaciens* inhibits tumor formation by the bacteria if applied to wounded bean leaves before, but not after, the inoculation of the bacteria (20). This inhibition is believed to result from blocking the plant

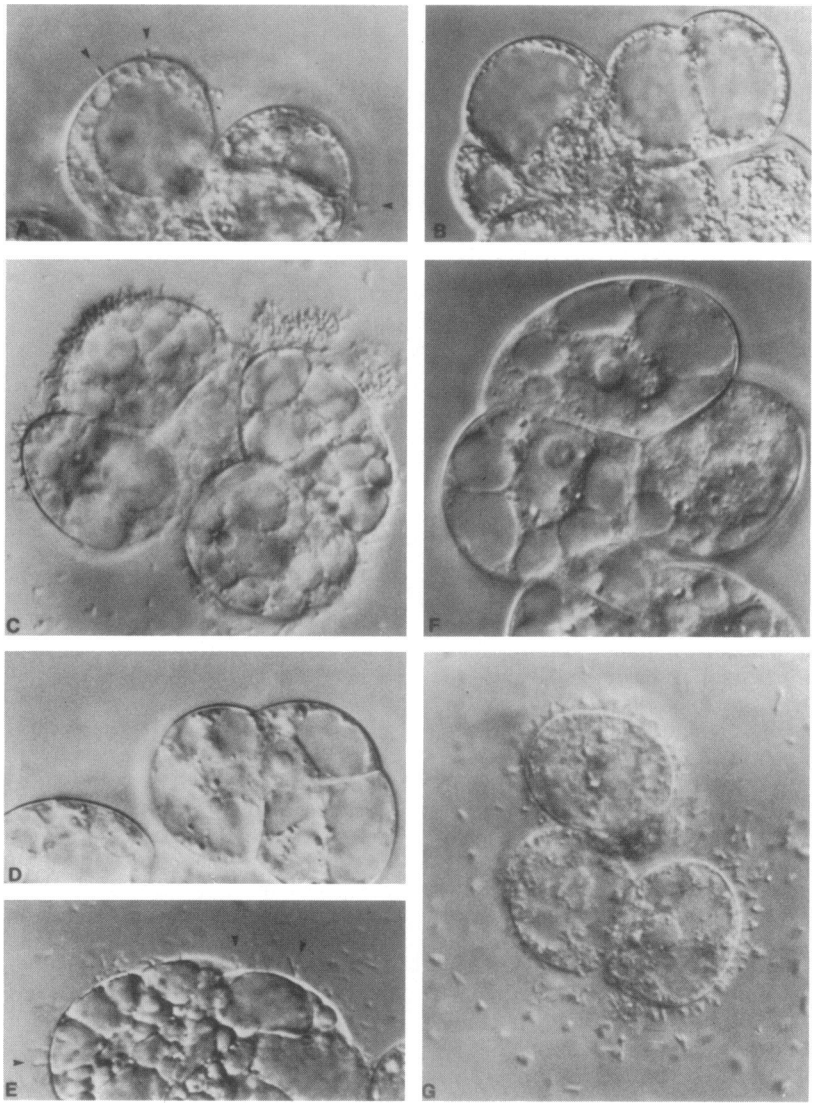

FIG. 4. Attachment of *A. tumefaciens* to carrot suspension culture cells as seen in the light microscope with Nomarski optics. (A) Strain A6 (virulent parent); (B) Att-A339 (nonbinding mutant of A6); (C) strain C58 (virulent parent); (D) Att-C43 (nonbinding mutant of C58); (E) Att-C43 revertant (binding revertant); (F) Att-C69 (nonbinding mutant of C58); (G) Att-C69 revertant (binding revertant). Similar results were obtained with the other nonbinding mutants and the revertants of those mutants. All cultures contained 10^8 bacteria per ml and 10^5 carrot cells per ml. The bacteria were incubated with the carrot cells for 20 h. Arrows indicate attached bacteria. Note that the pattern of attached bacteria is different for strains A6 and C58. This difference is reproducible and characteristic of these strains.

receptors for the attachment of *A. tumefaciens* by the LPS. Attachment of *A. tumefaciens* to tobacco tissue culture cells is also inhibited by the prior incubation of the plant cells with LPS from virulent *A. tumefaciens* (9). LPS from at least one strain of avirulent *A. radiobacter* is ineffective in preventing bacterial attachment (9). LPS was prepared from the parent strain and the nonattaching mutants of *A. tumefaciens*, and its ability to inhibit the attachment of the virulent strain A6 of *A. tumefaciens* to carrot suspension culture cells was examined by using the kinetic method of assaying bacterial attachment. LPS from the parent strain, C58, was more effective in inhibiting attachment to carrot cells than in inhibiting attachment to tobacco cells (0.2 μg of ketodeoxyoctonate per ml gave 50% inhibition of attachment to tobacco cells [9], while 0.02 μg of ketodeoxyoctonate per ml gave 50% inhibition of attachment to carrot cells.). The reasons for this difference are not known. Similar concentrations of LPS from the parent strain and from each nonattaching mutant were required to obtain ca. 50% inhibition of bacterial attachment to carrot cells (Table 3). When LPS from the parent strain and the nonattaching mutants was examined with PAGE, all the LPS preparations were found to contain rough LPS. No difference in LPS size was seen for any of the mutants when compared with that of the parent strain (Fig. 6).

Since bacterial spheroplasts did not bind to carrot suspension culture cells, the proteins removed during the preparation of the spheroplasts were examined with SDS-PAGE. These proteins, which presumably include the outer membrane and periplasmic-space proteins, differed between the parent strain and the nonattaching mutants. Three of the nonattaching mutants (Att-C28, Att-C234, and Att-C539) lacked a polypeptide band of about 34,000 dalton (Da) which was present in extracts of the parent strain C58 (Table 1; Fig.

TABLE 1. Characteristics of nonattaching bacterial strains

Bacterial strain	Attachment to carrot cells		Virulence on:		Ability to aggregate carrot cells	Tn5 Location	Motility	Surface component		
	Microscopic[a]	Kinetic[b]	*B. diagremontiana*	Carrot discs				LPS	Ability of LPS to inhibit attachment	Proteins released during spheroplast prepn
Parent C58	+	36 ± 5	+	+	+	−	+	Rough	+	Standard
Mutants										
Att-C28	−	0 ± 5	−	−	−	12-kb fragment[c]	+	Rough	+	−34 kDa[d]
Att-C234	−	10 ± 10	−	−	−	12-kb fragment	+	Rough	+	−34 kDa
Att-C43	−	0 ± 5	−	−	−	12-kb fragment	+	Rough	+	−34 kDa, −38 kDa
Att-C539	−	0 ± 10	−	−	−	12-kb fragment	+	Rough	+	−34 kDa
Att-C69	−	0 ± 5	−	−	−	12-kb fragment	+	Rough	+	−33 kDa
Revertants										
Att-C28-r	+	21 ± 6	+	+	+	NT	+	Rough	NT	All present
Att-C234-r	+	NT[e]	+	+	+	NT	+	Rough	NT	All present
Att-C43-r	+	NT	+	+	+	NT	+	NT	NT	All present
Att-C539-r	+	NT	+	+	+	NT	+	NT	NT	All present
Att-C69-r	+	NT	+	+	+	NT	+	NT	NT	All present

[a] Bacteria (10^7/ml) incubated with carrot cells for 24 h.
[b] Bacteria (3×10^3/ml) incubated with carrot cells for 1 h. Values given are the mean and standard deviation of percent attached bacterial inoculum for a minimum of three separate experiments.
[c] 12-kb fragment, Chromosomal 12-kilobase *Eco*RI fragment.
[d] Minus sign indicates that the strain is missing a polypeptide band of the indicated molecular mass on SDS-PAGE when compared with that of the parent strain (standard).
[e] NT, Not tested.

7). One mutant (Att-C43) lacked this 34,000-Da polypeptide and a polypeptide of about 38,000 Da. The fifth mutant (Att-C69) lacked a polypeptide just slightly smaller than that missing in the other mutants (33,000 Da). The lack of these polypeptides was reproducible and was seen in several extracts prepared from each mutant bacterial strain grown in Luria broth or in M&S medium.

Isolation and characterization of revertants of the nonattaching bacterial mutants. Revertants of the nonattaching mutants were obtained by selecting for those bacteria which bound to carrot cells when the carrot cells were incubated with a large excess of bacteria (10^9 bacteria per ml and 10^5 carrot cells per ml). Revertants of all the nonattaching mutants were obtained. These revertants recovered the ability to attach to carrot cells (Table 1; Fig. 4). They simultaneously recovered virulence on *B. diagremontiana* and on carrot disks (Table 1; Fig. 8). The revertants also recovered the ability to aggregate carrot suspension culture cells (Table 1; Fig. 5). In addition, when the proteins released from the bacteria during the preparation of spheroplasts were examined with SDS-PAGE, the revertants were found to have recovered the polypeptide bands missing in the nonattaching mutants (Table 1; Fig. 7). These results suggest that the ability of the bacteria to bind to carrot suspension culture cells is required for virulence and that the polypeptide bands missing in the nonattaching mutants are involved in this attachment interaction.

TABLE 2. Effects of avirulent competing bacterial strains on tumor formation by virulent *A. tumefaciens* C58 on wounded bean leaves

Avirulent competing bacterial strain[a]	Mean no. of tumors/leaf when competing bacteria were inoculated[b]:	
	15 min before strain C58	15 min after strain C58
None (water)	23 ± 3	25 ± 2
IIBNV6	13 ± 2	25 ± 2
Att-C539	27 ± 3	28 ± 3
Att-C28	26 ± 2	22 ± 2
Att-C43	27 ± 3	25 ± 3
Att-C43 transconjugant	26 ± 2	20 ± 3
Att-C539 transconjugant	25 ± 4	20 ± 2

[a] Both strain C58 and the competing strain were inoculated at a concentration of 10^9 bacteria per ml and 0.05 ml per leaf. The protocol described by Lippincott and Lippincott (5) was used.
[b] Values given are the mean of two separate experiments involving a minimum of 10 leaves for each measurement.

Genetic characterization of the nonattaching bacterial mutants. All the nonattaching mutants contained Tn5 as judged by DNA hybridization to nick-translated Tn5 DNA. None of the mutants contained any Mu DNA as judged by DNA hybridization. To determine whether the Tn5 was located in the chromosome or in one of the plasmids present in *A. tumefaciens*, the mutant bacteria were lysed in the well of an agarose electropohresis gel, and the chromosomal and plasmid DNAs were separated by electrophoresis (7). Hybridization of Tn5 DNA to a Southern transfer of these gels indicated that the Tn5 was located in the chromosomal DNA in all the nonattaching mutants (*A. tumefaciens* Tn5 mutant Ce-12 in which the Tn5 is located in the cryptic plasmid [7] was used as a control in these experiments [data not shown]). To determine whether Tn5 was integrated in different or similar locations in the mutants, Tn5 was hybridized to Southern blots of bacterial DNA digested with *Eco*RI. *Eco*RI was chosen because Tn5 lacks any *Eco*RI sites. The size of the *Eco*RI fragment that hybridized with Tn5 DNA, 18 kilobases, was the same in all the nonattaching mutants (Fig. 9).

To determine whether the Tn5 insertion was indeed responsible for the mutant phenotype of the nonattaching mutants and whether all the various described properties of these mutants were due to the single Tn5 insertion, *A. tumefaciens* DNA containing the Tn5 insertion was cloned from three of the nonattaching mutants (Att-C43, Att-C539,

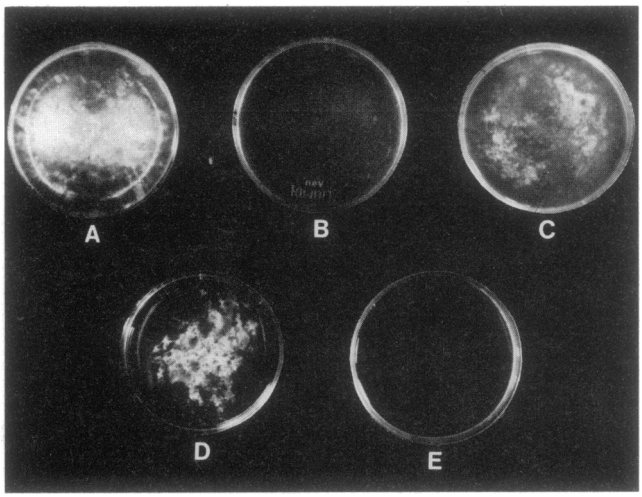

FIG. 5. Aggregation of carrot suspension cultures after incubation with *A. tumefaciens* for 20 h. Bacteria were incubated with carrot cells in a flask and poured into a Petri plate for photography. (A) Strain C58 (virulent parent); (B) Att-C43 (nonbinding mutant of C58); (C) Att-C43 revertant (binding revertant); (D) Strain A6 (virulent parent); (E) Att-A339 (nonbinding mutant of A6). Virulent strains of *A. tumefaciens* bound to carrot cells and caused their aggregation. Nonbinding mutants of *A. tumefaciens* did not form aggregates with carrot cells. Revertants of nonbinding mutants which bound to carrot cells also caused aggregation of the carrot cells. Parent, mutant, and revertant bacteria were all able to synthesize cellulose.

and Att-C69), and the Tn*5* insertion was introduced into the wild-type parent strain C58 by marker exchange. Two types of *A. tumefaciens* neomycin-resistant transconjugants resulted, those which expressed ampicillin resistance (presumably the ampicillin resistance gene of the cloning vector pUC9) and those which did not express ampicillin resistance. The first class of transconjugants probably resulted from a single homologous recombination event between pUC9 containing the cloned *A. tumefaciens* DNA with the Tn*5* insert and the *A. tumefaciens* chromosome. These bacteria contain pUC9 and two copies of the attachment genes, one of which has the Tn*5* insert. The second class of transconjugants result from a double homologous recombination event. These bacteria do not contain pUC9, and they contain only the copy of the attachment genes with the Tn*5* insertion. When the two classes of transconjugants were tested for their ability to attach to carrot cells, bacteria belonging to the first class were able to bind to carrot cells, while bacteria belonging to the second class could not (Table 4). One transconjugant of the second class was arbitrarily chosen for each of the three Att mutants whose DNA was used in the marker exchange experiments; these three transconjugants were characterized further. None showed any evidence of attachment to carrot cells as judged in the light microscope. One was examined for attachment with the kinetic method; no detectable attachment was observed (Table 4). The transconjugants failed to aggregate carrot cells. They were avirulent on *B. diagremontiana* leaves. In addition, when the proteins released from the bacteria during the preparation of spheroplasts were examined with SDS-PAGE, the transconjugants were found to be missing the polypeptide bands missing in the nonattaching mutants from which they were derived (Table 4). Hybridization of radioactive Tn*5* DNA to Southern transfers of *Eco*RI digests of

TABLE 3. Effects of LPS on the attachment of *A. tumefaciens* A6 to carrot tissue culture cells

Source of LPS	LPS concn (μg of KDO[a]/ml)	% Control attachment[b]
None	0	100% (Control)[c]
Wild-type parent strain C58	0.02	62 ± 4
Mutant strains		
Att-C28	0.01	44 ± 5
Att-C234	0.01	70 ± 3
Att-C43	0.01	62 ± 5
Att-C539	0.02	43 ± 8
Att-C69	0.02	50 ± 5

[a] KDO, Ketodeoxyoctonate.

[b] Bacterial attachment was measured after 60 min of incubation of 3×10^3 *A. tumefaciens* A6 per ml with 10^5 carrot cells per ml of M&S medium to which the appropriate LPS was added 15 min before the bacteria were. Values given are the mean of a minimum of three separate experiments.

[c] Attachment of bacteria after 60 min in the absence of LPS was 50 ± 5% of the bacteria inoculated.

DNA of these transconjugants separated by electrophoresis showed that the Tn*5* was contained in the same size *Eco*RI fragment (18 kilobases) as in the original mutant strains (Fig. 9). Thus the properties of the transconjugants were the same as those of the original mutants, suggesting that the mutant phenotype was indeed due to the Tn*5* insertion.

DISCUSSION

The isolation of *A. tumefaciens* mutants which fail to attach to carrot suspension culture cells allows us to examine the role of bacterial attachment in virulence. The previously described nonattaching mutants of *A. tumefaciens* were isolated from a collection of avirulent mutants. The nonattaching mutants described in this paper were isolated by screening for mutants which failed to bind to carrot suspension culture cells. All of the nonattaching mutants obtained were avirulent. Revertants of the nonattaching

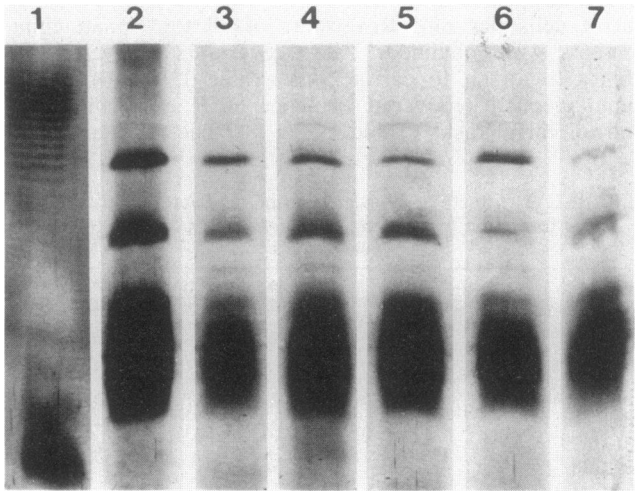

FIG. 6. SDS-polyacrylamide gel of LPS extracted from 10^{11} bacteria. LPS was stained with silver. Lanes: 1, 5 μg of LPS from *E. coli* O111:B4 (Sigma) used as a standard; 2 to 6, extracts of nonattaching bacterial mutants: 2, Att-C539; 3, Att-C234; 4, Att-C43; 5, Att-C28; 6, Att-C69; 7, LPS extracted from the wild-type parent strain C58. No differences in the size of LPS were seen on SDS-PAGE.

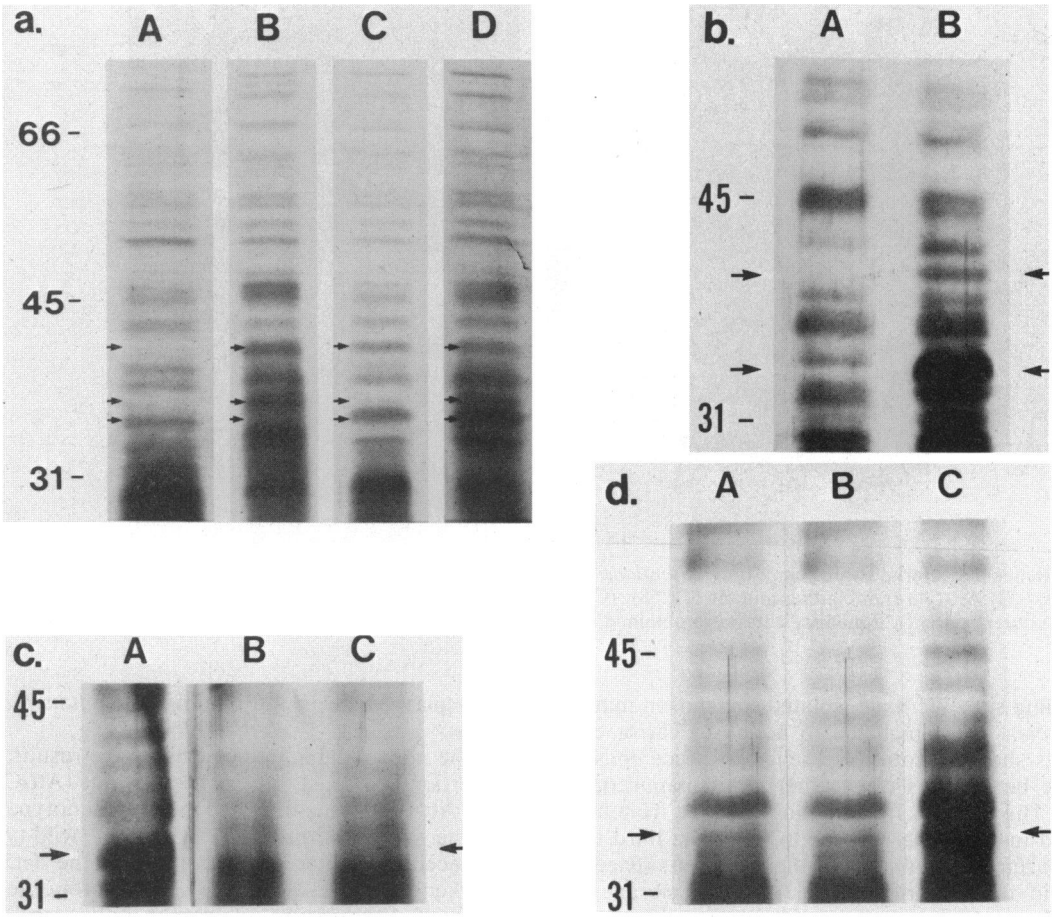

FIG. 7. Polypeptides removed from *A. tumefaciens* during the preparation of bacterial spheroplasts. The extract from 10^{10} bacteria was electrophoresed on SDS–11% polyacrylamide gels with a 3% acrylamide stacking gel. The resulting bands were silver stained. (a) Polypeptides extracted from wild-type and nonattaching mutant bacteria. Lanes: A, mutant bacterium Att-C43 (note that the bands at the position of the top and middle arrows are missing); B, Att-C69 (note that the band at the bottom arrow is missing); C, Att-C539 (note that the band at the middle arrow is missing); D, C58 wild-type parent (note that the bands at all three arrows are present). (b to d) Polypeptides extracted from nonattaching mutants, revertants of the mutants, and the transconjugants resulting from the introduction of the cloned DNA fragment containing the Tn*5* insertion into the wild-type parent bacteria. (b) Lanes: A, Att-C43 transconjugant (note that the two bands at the arrows are missing. Two other bands, one at 31-kilodalton molecular mass and one just above the top arrow, may be reduced in amount in the transconjugant. The significance of this change is unknown); B, revertant of Att-C43 (note that the bands at the arrows are present). (c) Lanes: A, Att-C539 revertant (note that the band at the arrow is present); B, Att-C539 transconjugant (note that the band at the arrow is missing); C, Att-C539 (note that the band at the arrow is missing). (d) Lanes: A, Att-C69; B, Att-C69 transconjugant; C, Att-C69 revertant. Note that the band at the arrow is missing in the mutant and the transconjugant but is present in the revertant. All the differences shown were reproducible on different gels and with several extracts of the bacteria. Numbers to the left of the gels represent the positions of molecular mass (kilodaltons) markers.

mutants were obtained by enriching for those bacteria which could bind to carrot cells. The bacteria that had recovered the ability to attach to carrot cells simultaneously recovered virulence. In addition, when the DNA containing the transposon insertion was cloned from the transposon mutants and the cloned DNA was introduced into the wild-type virulent parent bacteria by marker exchange, the resulting bacteria became nonattaching and avirulent at the same time. Thus the ability of *A. tumefaciens* to attach to carrot cells appears to be associated with, and probably required for, bacterial virulence.

Although I was unable to obtain binding of dead *A. tumefaciens* to carrot cells, bacterial protein synthesis was not required for bacterial attachment. Thus it appeared to be feasible to examine bacteria which had been grown in the absence of plant cells to determine the surface components involved in bacterial attachment and to ascertain which surface components were altered in the nonattaching mutants. Bacterial spheroplasts did not bind to carrot cells until sufficient time had elapsed for the resynthesis of some of the bacterial surface, indicating that some of the components required for bacterial binding were removed by the spheroplast formation procedure. Treatment of the bacteria with trypsin also delayed attachment, suggesting that surface proteins play a role in bacterial attachment.

Bacterial mutants which fail to bind to carrot cells could theoretically be of three types: (i) mutants which have an alteration in one of the fundamental constituents of the bacterial cell surface (such mutants would show alterations in many different surface components as secondary effects of the mutation), (ii) mutants which produce more or different extracellular material which would cover or mask the binding sites which recognize the plant cell surface, and (iii) mutants which lack some component which is part of the

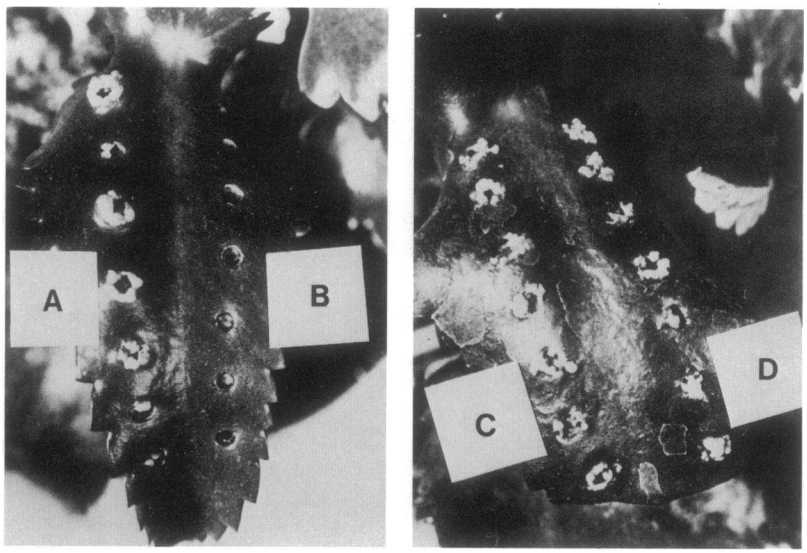

FIG. 8. Virulence of bacterial strains on *B. diagremontiana* leaves. A bacterial colony was inoculated into each wound site. (A) Strain C58 (virulent parent); (B) Att-C43 (nonbinding mutant of C58); (C) Att-C43 revertant (revertant of Att-C43 which bound to carrot suspension cells); (D) A6 (virulent parent). Similar results were obtained with the other nonbinding mutants and the revertants of those mutants.

bacterial binding site. We have isolated transposon mutants of *A. tumefaciens* A6 which fail to attach to carrot cells. These mutants show alterations in several surface constituents and may be mutants of the first type (manuscript in preparation). The nonattaching mutants derived from strain C58 described in this paper appear to be of the third type. Most of the surface components of these mutants (flagellae, pili, LPS, and the majority of the outer membrane and periplasmic-space proteins) appear to be unaltered. Although LPS has been implicated in both the reaction of *A. tumefaciens* with wounded bean leaves which leads to tumor formation (20) and in the attachment interaction of the bacteria with tissue culture cells (9), the nonattaching mutants described in this paper do not appear to have any alteration in their LPS. Both the parent and mutant bacteria have rough LPS. LPS from both the parent and mutant bacteria inhibits the attachment of wild-type *A. tumefaciens* to carrot suspension culture cells, and both types of LPS cause this inhibition at similar concentrations. β-2-D-Glucan has also been implicated in the binding of *A. tumefaciens* to plant cells (14). One of the two classes of nonattaching mutants characterized by Douglas et al. (2) lacks detectable β-2-D-glucan (14). However, these mutants are reported to be pleiotropic; they lack flagellae, are unable to conjugatively transfer the plasmid pAgK84, and overproduce extracellular polysaccharide (2, 14). β-2-D-Glucan appears to be localized in the periplasmic space in the agrobacteria and rhizobia and to have a role in the osmotic adaptation of these organisms (11). The absence of β-2-D-glucan may cause a change in the three-dimensional structure of the bacterial surface. The nonattaching mutants described in this paper, like the ChvA mutants described by Douglas et al. (2, 14), appear to contain unaltered amounts of β-2-D-glucan which thus may be necessary, but not sufficient, for bacterial attachment. The nonattaching mutants described here all lack one or more of the bands seen with SDS-PAGE of the polypeptides removed from the bacteria during the preparation of bacterial spheroplasts. These polypeptide bands reappear in revertants of the nonattaching mutants which are able to bind to carrot cells. Thus, these polypeptides may play a role in the binding of *A. tumefaciens* to plant cells.

The nonattaching mutant phenotype results from the Tn*5* insertion in at least three of the mutants (Att-C43, Att-C539, and Att-C69) since the same mutant phenotype was obtained in the parent strain (C58) when the wild-type gene was replaced by cloned DNA containing the Tn*5* insertion by marker exchange. All of the various properties (avirulence, lack of aggregation of carrot cells, and the missing polypeptides) associated with the original nonattaching mutants were transferred with the Tn*5* insertion.

The examination of the nonattaching mutants isolated in this study suggests that the ability of the bacteria to bind to plant cells is required for virulence in *A. tumefaciens*, and that two polypeptides found in the bacterial outer membrane or periplasmic space are required for bacterial attachment.

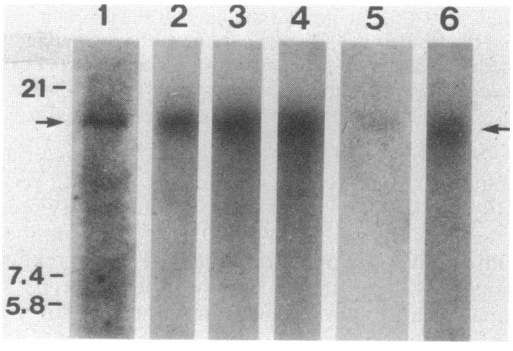

FIG. 9. Autoradiograph of 0.7% agarose gels containing *Eco*RI-digested DNA and hybridized with nick-translated ^{32}P-containing Tn*5* DNA. Lambda DNA digested with *Eco*RI was used to provide size markers. Only one band of hybridization was seen with each of the nonattaching mutants. The estimated size of this band is about 18 kilobases. There was no hybridization with the parent strain of *A. tumefaciens*. Lanes: 1, Att-C43; 2, Att-C539; 3, Att-C28; 4, Att-C234; 5, Att-C69; 6, Att-C539 transconjugant. Similar results were obtained with the transconjugants derived from Att-C43 and Att-C69 cloned DNA.

TABLE 4. Characteristics of nonattaching bacterial strains constructed by marker exchange

Bacterial strain	Attachment to carrot cells		Virulence on *B. diagremontiana*	Ability to aggregate carrot cells	Proteins released during spheroplast prepn
	Microscopic[a]	Kinetic[b]			
C58 parent	+	36 ± 5	+	+	Standard
C58 transconjugants					
From clone of Att-C43	−	0 ± 10	−	−	−38 kDa, −34 kDa[c]
From clone of Att-C539	−	NT[d]	−	−	−34 kDa
From clone of Att-C69	−	NT	−	−	−33 kDa

[a] Bacteria (10^7/ml) incubated with carrot cells for 24 h.
[b] Bacteria (3×10^3/ml) incubated for 1 h with 10^5 carrot cells. Values given are the mean and standard deviation of attached bacterial inoculum.
[c] Minus sign indicates that the strain is missing a polypeptide band of the indicated molecular mass on SDS-PAGE when compared with that of the parent strain (standard).
[d] NT, Not tested.

Thus the bacterial binding site for attachment to plant cells may be composed of LPS and protein.

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