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Agrobacterium tumefaciens Interaction with Suspension-Cultured Tomato Cells[1]

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ABSTRACT

Adherence of *Agrobacterium tumefaciens* to suspension-cultured tomato cells has been characterized using a quantitative binding assay. Saturable binding of radiolabeled *A. tumefaciens* to plant cells resulted in 100 to 300 bacteria bound per cell. Specificity of *A. tumefaciens* binding was also inferred from two additional results: (a) an initial incubation of plant cells with *A. tumefaciens* reduced subsequent binding of radiolabeled *A. tumefaciens* by 60% to 75%; (b) tomato cells bound less than three *E. coli* per cell. Protease treatment of plant cells had no effect on subsequent bacterial binding, but prior treatment of plant cells with pectinolytic enzymes increased binding 2- to 3-fold. Pectin-enriched and neutral polymer-enriched fractions were obtained from tomato cell walls. The soluble pectin-enriched fraction inhibited binding of bacteria to plant cells by 85% to 95%, whereas the neutral polymer fraction only partially inhibited binding. Preliminary characterization of the activity showed it is heat stable, partially inactivated by protease treatment, and substantially inactivated by acid hydrolysis.

Virulent strains of *Agrobacterium tumefaciens* cause tumor formation in a large number of dicotyledenous plants (8). Attachment of the bacterium to a site in a plant wound appears to be required for tumor induction (10, 16, 26). In subsequent events, a portion of the virulent bacterial Ti plasmid (the transferred DNA 'T-DNA') integrates into the plant cell genome via a process which is not understood.

Plant tissues transformed by *A. tumefaciens in vitro* or *in vivo* produce one or more novel (to the plant) metabolites called opines, and grow in culture without added hormones. Although there is an increased understanding of the *A. tumefaciens* T-DNA-plant genome interaction at a molecular level (for reviews, see 3, 4, 6), very little is known about this plant-pathogen interaction at a cellular level. For example, neither the nature of the bacterial-plant cell surface interaction nor the process by which the Ti plasmid (or a portion thereof) is transferred into the eucaryotic cell and integrated into the nuclear genome is known.

Several lines of evidence indicate that attachment of *A. tumefaciens* to a site on the plant cell surface appears to be required for tumor production. Chromosomal mutants of *A. tumefaciens* bearing the Ti plasmid but with no or very low plant cell surface attachment affinity did not induce tumors on susceptible plant tissue (10). Avirulent (plasmid-free) strains of *A. tumefaciens* bound to plant cell surfaces and competitively inhibited tumor formation by virulent strains presumably by occupying available binding sites (16). In contrast, unrelated or distantly related bacterial species apparently have low affinity for the postulated adherence site since they did not competitively inhibit tumor formation. When virulent *A. tumefaciens* were incubated with cell wall fractions from monocotyledonous, embryonic, or previously transformed plant material, *i.e.* tissues which are not transformed by *A. tumefaciens*, subsequent tumor formation was not inhibited on pinto bean leaves (18). Wall fractions from dicotyledonous plants sensititive to transformation by *A. tumefaciens*, however, substantially inhibited tumor formation (18, 19). The specific nature of the plant cell surface adherence site for *A. tumefaciens* has not been extensively characterized, but it has been reported to reside in the plant cell wall and not in the plasma membrane (17). A pectic moiety of the cell wall may be involved in *A. tumefaciens* binding (27). Taken together, these data suggest that susceptible plants have a specific binding site for *A. tumefaciens* that is necessary for transformation and that this site is either absent or altered in monocot, embryonic, or previously transformed cell walls.

Our present understanding of *A. tumefaciens*-plant interactions is derived to a large extent from results of bioassay experiments in which tumor formation is measured after inoculation of wounded plant tissue with *A. tumefaciens* (10, 16, 26). While the necessity and specificity of *A. tumefaciens* attachment in transformation have been implicated by these studies, a direct measurement of bacterial attachment to plant surfaces is not possible using this approach. It has been shown in direct binding studies that both avirulent and virulent strains of *A. tumefaciens* bind to either freshly isolated or suspension-cultured dicot or monocot cells and various characteristics of this attachment have been examined (10, 11, 20–22, 25, 33). Such studies have used several approaches to quantify the bound bacteria. These include measurement of agglutination of plant cells by virulent *A. tumefaciens* (11), viable plating of unbound bacteria (20–22), and measurement of bound radiolabeled bacteria (10, 25, 33). Use of radiolabeled bacteria provides a straightforward quantitation of binding if it is shown that plant cell associated radioactivity is not due to entrapment of flocs of agglutinated bacteria, a criterion previously unaddressed in studies of *A. tumefaciens* binding. We have used radiolabeled bacteria in a binding assay modified so that bacterial binding to plant cells and radioactivity due to agglutinated bacteria can be measured separately in the same assay. A corrected value for bound bacteria can then be obtained.

In none of the previous direct binding studies has the specificity of *A. tumefaciens* binding to plant cells been demonstrated. Since attachment appears to be the initial and also a required event in the transformation process, we have examined the binding of *A. tumefaciens* to suspension cultured cells using a quantitative direct binding assay as the first step in our studies of the cellular basis of this plant-pathogen interaction. Using this assay, we report here a characterization of the initial attachment of *A. tumefaciens* to tomato suspension-cultured cells and show that bacterial binding is saturable and that a pectin-enriched fraction

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from the wall of these plant cells is able to inhibit bacterial attachment.

MATERIALS AND METHODS

Materials. Cellulase (Cellulysin) from *Trichoderma viride*, Macerase Cellulase from *Rhizopus sp.*, and pronase protease were obtained from Calbiochem-Behring. Pectolyase, pectinesterase, soybean trypsin inhibitor type 1-S, trypsin (crystallized from porcine pancreas), galacturonic acid, CDTA,[2] citrus pectin (containing by weight 77% partially methoxylated pectin), PGA, and sodium salt, were purchased from Sigma Chemical Company.

Tissue Culture. Callus cultures of LA 1221 red cherry tomato VFNT were provided by Dr. Bruce Thomas, Arco Plant Cell Research Institute, Dublin, CA. LA 1221 callus was grown in the dark at 26°C with monthly sub-culture on 2D/IP medium, (MS salts [20] modified to contain 2.5 mM KH_2PO_4) 0.087 M sucrose, 0.55 mM myo-inositol, 2.9 μM thiamine-HCl, 3.9 μM nicotinic acid, 2.4 μM pyridoxine-HCl, 4.9 μM 6-(γ,γ-dimethylallylamino)purine riboside, 10 μM dichlorophenoxyacetic acid, and 0.9% agar. Suspension cultures were initiated from callus in 2D/IP medium and were grown in the dark at 26°C with shaking at 120 rpm on a Queue rotary shaker. The cells were subcultured weekly at a 1 to 10 ratio (1 volume cells in conditioned medium, *i.e.* medium from previous week's growth) to 9 parts fresh medium. Suspension cultures were screened monthly for mycoplasma contamination by the Dapi stain method (29).

Growth and Labeling of Bacteria. *A. tumefaciens* stock strains were maintained on minimal medium (per liter: 5 g glucose, 1 g NH_4Cl, 2 g KH_2PO_4, 0.2 g $MgSO_4$, 1 g glutamic acid, 1 μg biotin, 5 μg H_2BO_3, 10 μg $CaCO_3$, 10 μg $CuSO_4$, 300 μg $FeSO_4$, 1 μg KI, 15 μg $MnSO_4$, 10 μg Na_2MoO_4, 5 μg $ZnSO_4 \cdot 7H_2O$, 14 g agar) at 4°C and were transferred once a month. The following strains were used: A6 (containing pTiA6 plasmid), A136 (plasmid free), and DS1-1 (A136 bacterial chromosome with pTiA6, constructed and provided by Daniela Sciaky, Brookhaven National Laboratory). Liquid cultures were initiated weekly in minimal medium (without agar) from 48- to 72-h streaks and were grown at 26°C in a New Brunswick Rotary Shaking water bath model G76. Stationary phase cultures were diluted 10-fold in fresh minimal medium at 24-h intervals. Visible aggregates occasionally appeared in liquid cultures and were removed by sterile filtration (Nucleopore membrane filters, 8 μm pore size) before use of bacteria. A rough estimation of bacterial cell number was obtained by measuring the O.D. of suspensions at 666 nm (0.001 O.D. units = $1-5 \times 10^7$ bacteria). Accurate counts of viable bacteria were obtained by plating appropriate dilutions of bacteria in 0.15 M NaCl on L medium plates (per liter: 10 g tryptone, 5 g yeast extract, 5 g NaCl, 14 g agar) and counting colonies after 48 h at room temperature. For binding experiments, 0.25 to 1.0 ml aliquots of bacterial cultures in early log growth were labeled for 1 to 1.5 h with 10 to 100 μCi [^{35}S]methionine (>600 Ci/mmol, Amersham-Searle). Depending upon input radioactivity, this protocol resulted in between 0.0006 and 0.06 cpm per bacterium. Radiolabeled bacteria were washed in minimal medium by three 2-min centrifugations in a rheostated Eppendorf centrifuge (model 5414) and used immediately for binding studies. Storage of radiolabeled bacteria in 0.15 M NaCl at room temperature or at 4°C for 24 h was found to substantially reduce binding capacity.

Binding Assay. Aliquots of actively growing suspension cultures of LA 1221 red cherry tomato cells were filtered through 400 μm wire mesh to remove large cell clumps. This resulted in suspensions mainly consisting of small aggregates of between 2 and 40 cells. Unless otherwise specified, cell pellets (0.5–1.0 ml packed cell volume) were washed five times followed by centrifugation (500*g*, 4 min) in 40 ml buffer (20.5 mM NH_4NO_3, 19 mM KNO_3, 2.9 mM $CaCl_2$, 1.5 mM $MgSO_4$, 10 mM Mes (pH 6.0) (MSMES). Cells were resuspended in MSMES at a density of 5×10^4 to 2.5×10^5 cells/ml and 200-μl portions were transferred with a truncated Eppendorf tip to 12 × 75 mm polypropylene tubes (Sarstedt). Assays were initiated by adding 20 μl of [^{35}S] methionine-labeled bacteria in MSMES to the plant cells in suspension. Depending upon the experiment, this resulted in the addition of 1×10^5 to 1.8×10^9 bacteria per assay tube. Assays were incubated for varying times at room temperature and shaken at 150 rpm on a New Brunswick rotary shaker, model G2. Assays were terminated by addition of 2 ml MSMES to each tube immediately prior to filtration. Tube contents were filtered onto squares of 60 μm pore size Nitex (Tetko) (previously soaked in BSA, 1 mg/ml in H_2O) using a Millipore filter manifold. Assay tubes were rinsed with an additional 2 ml MSMES and filters were washed with two 10-ml aliquots of MSMES. Filters were counted in 3 ml Liqiscint (National Diagnostics) in a Beckman Liquid Scintillation counter model LS 7000.

Radioactivity due to clumps of agglutinated bacteria was retained by the Nitex filters, whereas single bacterial cells were not retained. To determine radioactivity due to such agglutination (which occurred depending on experimental conditions, *i.e.* bacterial density or inclusion of sugars in the assay buffer), each assay was also carried out without plant cells. In addition to the radiolabeled bacteria, an appropriate volume of MSMES (usually 100–200 μl) was added to these tubes. For binding studies, filter-associated cpm from assay tubes containing bacteria but not plant cells were considered to be background cpm and were substracted from the values obtained from filters containing bacteria and plant cells. For bacterial agglutination studies, filter-associated cpm from assay tubes containing bacteria alone were used directly. Counts per minute were converted to number of bacteria using viable bacterial counts (see above). The number of bacteria bound per plant cell was calculated as follows:

$$\frac{(\text{cpm/assay})/(\text{cpm/bacterium})}{\text{no. plant cells/assay}}$$

Plant cell number was determined by counting the number of cells in 10 μl of an appropriate dilution on a gridded slide. Due to the presence of aggregates, such cell counts approximated to within 20 to 30% the number of cells per assay as determined by multiple cell counts rather than an absolute number. These cell numbers were consistent from experiment to experiment with respect to cell pellet volume. Binding assays including measurement of bacterial agglutination were done in quadruplet. The standard deviation of the assay was within 15% of the mean value. Due to unavoidable variation in the absolute number of both bacteria and plant cells added in different experiments, data points presented are averages from one experiment. Each experiment reported was done at least three times and yielded the same result as the reported experiment. Where appropriate, data were examined by a series of one way analysis of variance in which each treatment group was compared to the control group.

Preparation of Cell Walls and Cell Wall Fractions. Cell walls were prepared from suspension cultured cells as described by Nevins *et al.* (24) with modifications to reduce cytoplasmic contamination as described by Ring and Selvendran (28) and Selvendran (30). All procedures were done at 4°C. Cell pellets (10–20 ml packed cell volume) were obtained from actively dividing suspensions 6 to 7 d after subcultivation and were washed with 10 to 20 volumes of 0.1 M KH_2PO_4 in a fritted glass filter funnel. Washed cells were resuspended in 100 ml 0.1 M

[2] Abbreviations: CDTA, trans-1,2-diaminocyclohexane-*N,N,N',N'*-tetraacetic acid; PGA, polygalacturonic acid; MS, Murashige and Skoog; MSMES, 20.5 mM NH_4NO_3, 19 mM KNO_3, 29 mM $CaCl_2$, 1.5 mM $MgSO_4$, 10 mM Mes (pH 6.0).

KH_4PO_4 (pH 7) and broken by release from a Parr nitrogen disruption bomb by two 5-min passes at 1000 p.s.i. This resulted in almost 100% disruption of the cells as judged by microscopic examination. The broken cell walls were collected by centrifugation at 17,000*g* for 10 min and resuspended in 1% sodium deoxycholate for 30 min. After pelleting, walls were washed three times by centrifugation in distilled H_2O. Walls were extracted three times with 5 volumes chloroform:methanol (1:1, v/v) and three times with acetone by vacuum filtration in a fritted glass funnel. After air drying, walls were resuspended at 10 mg dry weight/ml in 0.1 M KH_2PO_4 (pH 7) containing 40 μg/ml α-amylase (Calbiochem, 3× crystallized from *Aspergillus oryzae*) and 0.01% thimersol, and incubated on a rotary shaker at 120 rpm, 26°C for 48 h. Walls were collected using a fritted disc funnel and washed with 5 volumes of water, air dried, and stored at room temperature.

Cell walls were treated with a Ca^{2+} chelating agent (1,2-diaminocyclohexanediamine *N,N,N',N'*-tetraacetic acid [CDTA]) which extracts a pectin-enriched fraction without degradation of esterified pectin (13). The residue was treated subsequently with 4.8 N KOH, 0.1% $NaBH_4$ to extract mainly neutral polymers. Briefly, cell walls (10 mg/ml) were resuspended in 0.05 M CDTA, 0.05 M acetate (pH 6.5) using a motor-driven Teflon pestle homogenizer to insure a uniform suspension. Walls were shaken 18 h at 4°C on a rotary shaker and the suspension was filtered through two layers of Whatman GF/C paper. The filtrate was dialyzed extensively against H_2O at 4°C and lyophilized; the residue was resuspended in 80 μl H_2O/mg weight of extracted material and stored at −20°C. The residual cell walls were removed from the filter paper and washed with distilled H_2O two times by centrifugation at 17,000*g* for 10 min. These walls were resuspended in 4.8 N KOH, 0.1% $NaBH_4$ for 18 h at room temperature. After filtration through Whatman GF/C filters, the filtrate was neutralized and then dialyzed extensively against H_2O and lyophilized; the residue was resuspended and stored as above. Before use in assays, cell wall fractions were thawed at 40°C for 30 min and aliquots centrifuged for 4 min in an

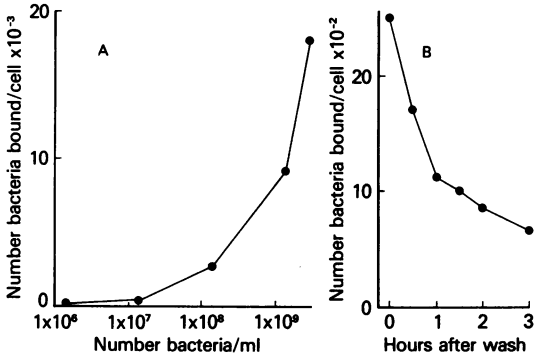

FIG. 1. (A) *A. tumefaciens* adherence to LA 1221 tomato cells as a function of bacterial concentration. Binding assays were performed as described in "Materials and Methods" and contained 2.7×10^3 plant cells and varying numbers of [^{35}S]methionine labeled *A. tumefaciens* (5×10^2 to 5×10^5 cpm) in 200 μl MSMES (pH 6.0). LA 1221 cells were centrifuged out of growth medium and used for binding studies without further washing. Incubation time was 3 h. (B) Effect of time after washing on number of bacteria-bound Erlenmeyer flasks (25 ml) containing 1×10^5 tomato cells and 1×10^9 [^{35}S]methionine-labeled bacteria/ml (2.3×10^5 cpm) were incubated 2 h in MSMES. At this time (time = 0), 200 μl samples were processed to determine the number of bound bacteria as described in "Materials and Methods." Remaining samples were washed two times by centrifugation (500*g*, 4 min) in 5 ml MSMES, resuspended to the original volume in fresh MSMES, and 200 μl samples were processed to determine number of bacteria bound to plant cells at the indicated times.

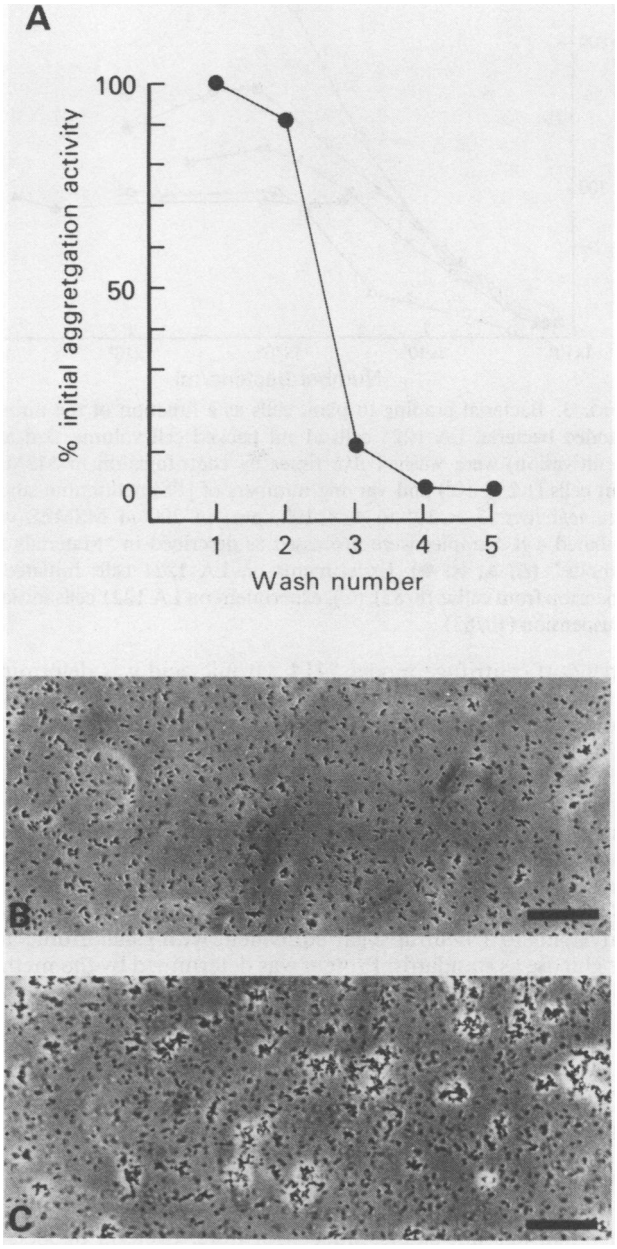

FIG. 2. Agglutination of *A. tumefaciens* by tomato cell washes. (A) Tomato cells (0.5 ml packed cell volume, 4 d after subcultivation) were washed by centrifugation (500*g*, 4 min) five times with 10 ml MSMES and each wash was passed through a 0.45-μm membrane filter before use. Bacterial agglutination assays contained 200 μl of cell wash MSMES or fresh MSMES (control) and 1×10^8 radiolabeled *A. tumefaciens* (1×10^5 cpm). Incubation time was 3.5 h and samples were processed as described in "Materials and Methods." Control radioactivity, due to the minimal bacterial agglutination which occurred in fresh MSMES, was subtracted from the experimental values. Data are presented as the percentage of bacterial agglutination activity in each MSMES cell wash using the activity in the initial cell wash as 100%. (B) Photomicrograph of control. *A. tumefaciens* incubated 45 min in MSMES. (C) Photomicrograph of bacterial agglutination after 45 min in initial cell wash as described above. Note the degree of bacterial agglutination when compared to (B). Bar = 30 μm.

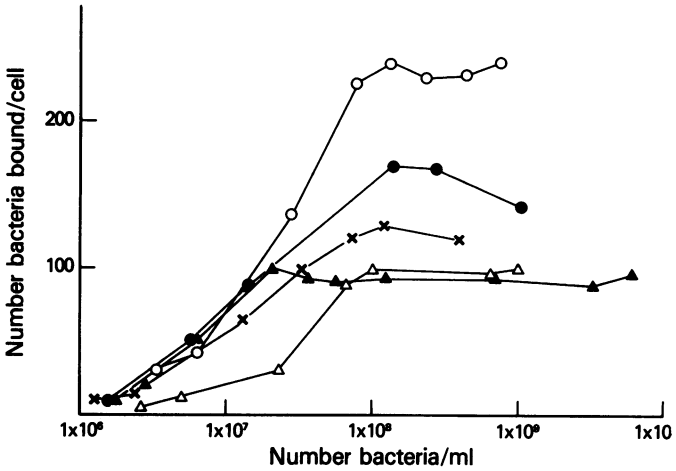

FIG. 3. Bacterial binding to plant cells as a function of the number of added bacteria. LA 1221 cells (1 ml packed cell volume, 3 d after subcultivation) were washed five times by centrifugation in MSMES. Plant cells (1.2×10^4) and varying numbers of [^{35}S]methionine-labeled *A. tumefaciens* (3×10^4 to 4×10^6 cpm) in 200 μl MSMES were incubated 4 h. Samples were processed as described in "Materials and Methods." (△, ▲, ×, ●), Experiments on LA 1221 cells initiated in suspension from callus (8/83); (○), experiment on LA 1221 cells initiated in suspension (10/83).

Eppendorf centrifuge model 5414. Uronic acid was determined according to the method of Blumenkrantz *et al.* (5) with galacturonic acid as a standard. Neutral sugars were estimated by the anthrone assay (9) with glucose as a standard. Although extensive carbohydrate analyses on these fractions were not done, chelating agents have been shown to partially extract pectin (polygalacturonate) -enriched cell wall fractions (1, 13, 14), while alkaline conditions preferentially extract neutral polymers, *e.g.* arabanoxylans, mannans, xyloglucans, and glucomannans (1, 7, 15). The CDTA-extracted fraction contained approximately 4 uronic acid equivalents to 1 neutral sugar equivalent with galacturonic acid and glucose as standards. Protein was determined by the method of Lowry *et al.* (19) with BSA as a standard.

RESULTS

Characterization of *A. tumefaciens* Binding to Suspension-Cultured Cells. Results of initial experiments indicated that binding of *A. tumefaciens* to plant cells was not saturable (Fig. 1A), suggesting that the bacteria were agglutinating at the plant cell wall surface. A high proportion of such bacterial binding or adherence was not stable, since with time, bacteria dissociated from plant cells (Fig. 1B). These initial experiments were carried out on suspension-cultured tomato cells which had been centrifuged out of growth medium but not extensively washed prior to incubation with bacteria. Plant cells grown in suspension secrete cell wall components into the medium (2) and plants also elaborate lectins which agglutinate certain bacteria (31). Therefore, an attempt was made to remove material which caused the observed agglutination of *A. tumefaciens* at the plant cell surface and which possibly masked saturable binding of the bacteria to the plant cells. Tomato cells were washed extensively in a buffered salt solution containing calcium (MSMES). Plant cell-associated material removed in these washes was assayed for its ability to agglutinate *A. tumefaciens*. Agglutinated bacteria were defined as those which remained associated with the Nitex filters after incubation in the absence of plant cells. The bulk of the plant cell-associated *A. tumefaciens* agglutination activity was removed in the first two washes of the tomato cells (Fig. 2A). When *A. tumefaciens* cells were incubated in the cell washes, bacterial aggregates appeared (Fig. 2C), whereas bacteria incubated in fresh buffer did not aggregate (Fig. 2B). Calcium (2.9 mM) was the only component in the buffered salt solution (MSMES) which eluted the plant cell-associated bacterial agglutinating activity (data not shown). In addition, growth medium conditioned by either tomato or tobacco cells contained material that agglutinated *A. tumefaciens* to a much greater extent than fresh, unconditioned medium. To a certain extent, agglutination of *A. tumefaciens* was always observed in media or buffers containing sugars; minimal or no agglutination was observed in MSMES, unless the bacterial density were high, *e.g.* $>1 \times 10^7$/ml. Because material which enhanced bacterial agglutination could be washed from the plant cell surface, tomato suspension-cultured cells were washed five times in MSMES prior to incubating them with the bacteria in the remaining experiments reported here.

Binding of *A. tumefaciens* to extensively salt-washed (MSMES) tomato cells was saturable, resulting in approximately 100 to 300 bacteria bound per plant cell (Fig. 3). At saturating concentrations of either virulent or avirulent isogenic strains of *A. tumefaciens* (strains A136 and DS 1-1), no differences were observed in the extent of binding to tomato cells (data not shown). Experimental variation in the number of bound bacteria was probably due to heterogeneity in plant cell size, growth state, and degree of clumping of plant cells. In eight experiments, however, saturation was always observed at bacterial concentrations above 1×10^7 per ml. Saturable binding of *A. tumefaciens* to tomato suspension cultured cells was maximal at pH 6 (Fig. 4A), and was complete within 1.5 and 2 h (Fig. 4B). With respect to kinetics and pH optimum, these results are consistent with those obtained by Ohyama *et al.* (10) for attachment of *A. tumefaciens* to *Datura* cells. To ascertain the stability of binding, tomato cells were incubated with saturating concentrations of *A. tumefaciens* and, after extensive washing, assayed at subsequent times for adhering bacteria. Saturable binding was stable for up to 3 h after washing of plant cells (Fig. 4C).

Specificity of *A. tumefaciens* binding was assessed in two additional ways. First, tomato cells, which previously had been exposed to saturating concentrations of unlabeled *A. tumefaciens*, were tested for their ability to bind radiolabeled bacteria. This treatment reduced the binding of radiolabeled *A. tumefaciens* by 60% to 75% (Table I). Second, the ability of another bacterium to bind to plant cells was tested. Whereas 100 to 300 *A. tumefaciens* bound per tomato cell, only about three *E. coli* bound under identical experimental conditions (data not shown). This low adherence was probably due to a slight degree of nonspecific sticking or entrapment of these bacteria in plant cell clumps. Taken together results of experiments discussed above suggest that the plant cell wall contains a specific, saturable site for binding of *A. tumefaciens*.

Characterization of the Plant Cell *A. tumefaciens* Adherence Site. In preliminary experiments, the biochemical nature of the *A. tumefaciens* binding site was examined by exposing LA 1221 cells to a variety of treatments (Table II). Protease treatment did not affect binding, suggesting that if the binding site is proteinaceous, it must be highly glycosylated or otherwise modified or protected against proteolytic degradation. Of the carbohydrate-modifying enzymes used, only those enzymes which degrade pectin altered the number of bacteria bound to plant cells. Treatment of plant cells with pectin lyase, which cleaves the α-1,4 bond of PGA, and macerase (a mixture of enzymes from culture filtrates of *Rhizopus* containing cellulase and pectinolytic activities), resulted in a 2- to 3-fold increase in the number of bacteria bound per cell. These enzymes may expose binding sites previously unavailable for attachment of *A. tumefaciens*. Analogous results have been shown in a mammalian system in which increased numbers of sperm bind to mouse oocytes after treat-

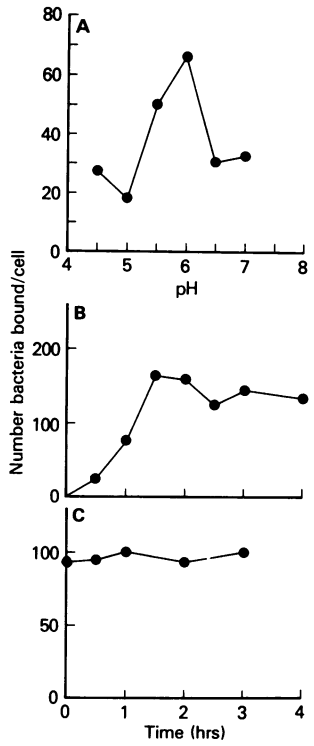

FIG. 4. (A) Effect of pH on binding of *A. tumefaciens* to plant cells. Binding assays contained 6×10^3 tomato cells (washed five times in MSMES) and 2×10^8 bacteria (3.2×10^5 cpm) in 200 μl MS macro salts containing the appropriate buffer. Assays, carried out at pH 4.5, 5.6, and 6.5, were performed in 25 mM Mes and those at 7 and 7.5, in 25 mM Hepes. (B) Time course of *A. tumefaciens* binding. Assays were carried out as described in the legend to Figure 4A except that the buffer was MSMES, pH 6.0. (C) Stability of *A. tumefaciens* binding to MSMES-washed tomato cells. LA 1221 cells washed five times in MSMES. The plant cells (1×10^5) and 8.8×10^8 [^{35}S]methionine-labeled bacteria/ml (5×10^5 cpm) were incubated in MSMES for 3 h. At this time ($t = 0$), 200 μl samples were processed for the number of bound bacteria as described in "Materials and Methods." Remaining cells were washed four times by centrifugation (500*g*, 4 min) in MSMES, resuspended to the original volume in fresh MSMES, and 200 μl samples processed at the indicated times.

Table I. *Binding of ^{35}S-A. tumefaciens to LA 1221 Cells after Prior Incubation with Unlabeled A. tumefaciens*

1.25×10^5 LA 1221 cells/ml MSMES were incubated for the indicated times with 1×10^9 unlabeled log growth phase *A. tumefaciens*. Control LA 1221 cells received no *A. tumefaciens*. Plant cells were then washed on 60 μm Nitex with 30 ml MSMES, resuspended in 20 ml MSMES, centrifuged 500*g* for 4 min, and resuspended to the original cell density in MSMES. ^{35}S-labeled *A. tumefaciens* (1×10^9/ml) (2.5×10^6 cpm) were then added to control and experimental cells and incubation was continued for 3 h. Samples were processed for the number of bacteria bound per cell as described in "Materials and Methods."

Experiment	Prior Incubation with *A. tumefaciens*	No. of Bacteria Bound/Cell
	h	
I	4	22
	0	84
II	3	29
	0	90

Table II. *Binding of A. tumefaciens after Various Treatments of the Plant Cell Surface*

Binding assays containing $1\text{-}3 \times 10^4$ LA 1221 cells and 5×10^7 [^{35}S] methionine-labeled *A. tumefaciens* (4×10^4 cpm) in 200 μl MSMES were incubated for 3 h and processed as described in "Materials and Methods." Prior to treatments, LA 1221 cells were washed five times with MSMES. Control cells received this treatment only.

Treatment of Plant Cells	Binding Relative to Control
1 M NaCl wash	110
1 M KH_2PO_4	109
Pronase (250 μg/ml followed by 1 M KH_2PO_4 wash)[a]	121
Trypin (250 μg/ml followed by 1 M KH_2PO_4 wash)[b]	100
Pectin esterase (0.25 units/ml)[c]	123
Pectinlyase (0.01 units/ml)[c]	377*[d]
Macerase (0.025%)[c]	261*
Cellulase (0.5%)[c]	130

[a] La 1221 cells were washed two times with either 1 M NaCl or 1 M KH_2PO_4 containing 10 mM Mes (pH 6.0) and then two times in MSMES.

[b] LA 1221 cells were incubated 30 min at room temperature with either 250 μg/ml trypsin or pronase (Sigma, protease from *Streptomyces griseus*) in MS macro salts containing 20 mM Hepes (pH 7.2), and then washed as described under footnote a with 1 M KH_2PO_4.

[c] LA 1221 cells were plasmolyzed by incubation for 15 min in MSMES containing 0.4 M sucrose (pH 5.8). The cells were then centrifuged and resuspended in MSMES containing 0.4 M sucrose and the enzymes at the indicated concentrations. After a 30-min incubation at room temperature, cells were centrifuged and washed two times in MSMES (pH 6.0) before the addition of ^{35}S-labeled *A. tumefaciens*. Control cells underwent the same treatment except no enzymes were added.

[d] *, Significantly different from control, $P < 0.005$.

ment of the oocyte with glycosyl transferases (32). Since none of the carbohydrate-degrading enzymes used was homogeneous, however, it was not possible to rule out that the observed effects were due to contaminating enzymes.

A more direct approach to determining the nature of the *A. tumefaciens* binding site was therefore taken. Soluble fractions from plant cell walls were obtained and tested for their ability to compete with intact tomato cells for *A. tumefaciens* binding. Soluble pectin and neutral polymer-enriched fractions were prepared from cell walls isolated from actively growing LA 1221 cells and assayed for both competitive binding activity and effects on bacterial agglutination (Table III). The pectin-enriched soluble cell wall fraction isolated by gentle nondegradative extraction with CDTA ('CDTA-extracted fraction') contained material which inhibited *A. tumefaciens* binding to cells by 85% to 95% and which, in addition, completely inhibited bacterial agglutination. In contrast, a neutral polymer-enriched cell wall fraction only partially inhibited (15–38%) *A. tumefaciens* binding to tomato cells while the fraction actually enhanced bacterial agglutination. Activity inhibiting bacteria-plant cell binding in the neutral polymer-enriched cell wall fraction may be due to the same moiety which is present in higher concentrations in the pectin-enriched wall fraction. This seems likely since the neutral polymer fraction was prepared from cell walls previously extracted with CDTA. CDTA does not extract all of the cell wall pectin (14), and uronic acid was present in the fraction extracted by 4.8 N KOH. Why the neutral polymer fraction enhanced bacterial aggregation is not known at this time. It is possible that the harsh extraction conditions result in hydrolysis of large carbohydrate polymers to cleavage products with specificity for bacterial sites involved in binding.

The CDTA-extracted fraction contained protein in addition

Table III. *Effect of Plant Cell Wall Fractions on A. tumefaciens Binding and Agglutination*

Cell walls were prepared from LA 1221 suspension-cultured cells 6 to 7 d after subcultivation, and soluble pectin and neutral-polymer-enriched fractions were sequentially extracted and prepared as described in "Materials and Methods." Binding and bacterial agglutination assays were carried out as follows: 5×10^6 ^{35}S-labeled bacteria (2.7×10^4 cpm) in 20 μl MSMES were incubated 1 h at room temperature with 20 μl of the desired cell wall fraction or distilled H_2O (control). After this incubation, 4×10^4 LA 1221 cells in 200 μl MSMES were added to binding assays; bacterial agglutination assays received an equal volume of MSMES but no plant cells. Assays were incubated 2.5 h at room temperature on a rotary shaker at 150 rpm and samples were processed for the number of bacteria bound or agglutinated as described in "Material and Methods."

Addition to Assay	No. of Bacteria Bound Cell	% Control	No. of Bacteria Agglutinated ($\times 10^5$)	% Control
H_2O	65	100	0.4	100
CDTA-extracted cell wall fraction	8*[a]	12	0.0*[d]	0
	3*[b]	5	0.0*	0
	3*[c]	5	0.0*	0
Neutral polymer enriched cell wall fraction	55*[a]	85	3.2*	800
	40*[b]	62	2.4*	600
	40*[c]	62	8.1*	2025

[a,b,c] Preparations from three separate cell wall isolations.
[d] *, Significantly different from control $P < 0.0001$.

to acidic and neutral polysaccharides. The ability of this fraction to inhibit *A. tumefaciens* binding to tomato cells decreased upon dilution (Fig. 5) as would be expected if the fraction contained adherence molecules that compete with the plant cell for bacterial binding. The activity was stable to heat. Treatment with pronase and trypsin resulted in partial decrease of the inhibitory activity (Table IV). Partial hydrolysis with TFA substantially decreased the bacterial binding inhibitory activity of the CDTA-extracted fraction (Table IV). We also compared the activities of this fraction with those of pectin and PGA. These substances have been reported to inhibit tumor formation by virulent *A. tumefaciens* on potato tubers and pinto bean leaves presumably by interfering with bacterial binding (26, 27). In our assay, commercial citrus pectin and PGA (0.1–2.0 mg/ml) agglutinated *A. tumefaciens* and substantially (80–100%) inhibited bacterial binding to plant cells. PGA aggregated bacteria to levels 10 times greater than did equivalent concentrations of pectin. Bacterial agglutination caused by galacturonic acid (15 mM) was not as extensive as that observed in the presence of pectin but still exceeded control values by 2- to 3-fold. In addition, bacterial binding to the plant cells was slightly (~30%) inhibited by galacturonic acid. The mode of action of these compounds on *A. tumefaciens* binding and agglutination may therefore be different from that of the binding inhibitory moiety in the CDTA-

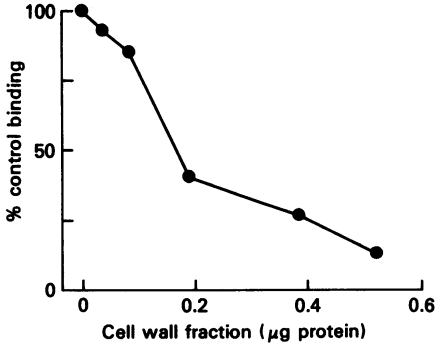

FIG. 5. Dose response of *A. tumefaciens* binding to LA 1221 cells in the presence of CDTA-extracted cell wall fraction. Ten μl containing 1.9×10^6 [^{35}S]methionine-labeled *A. tumefaciens* (2×10^4 cpm) were incubated for 1 h with 10 μl H_2O containing the indicated concentrations of CDTA-extracted cell wall fraction. LA 1221 cells (1×10^4 in 100 μl) were then added and incubated for 2 h with shaking at 150 rpm prior to processing for the number of bound bacteria as described in "Materials and Methods." Control assays contained 10 μl H_2O and no CDTA-extracted material.

Table IV. *Effect of Various Treatments on the Inhibitory Activity of a CDTA-Extracted Plant Cell Wall Fraction*

Aliquots (10 μl) of a CDTA-extracted cell wall fraction, treated as described below were incubated with 10 μl containing 1.9×10^6 ^{35}S-labeled *A. tumefaciens* ($1–2 \times 10^5$ cpm). After 1 h, 1×10^4 LA 1221 cells were added in 100 μl MSMES, and after an additional 2 h with shaking at 150 rpm, assays were processed as described in "Materials and Methods." Experiments were carried out on two different CDTA-extracted cell wall preparations (as described in "Materials and Methods") and reported values are from three separate experiments.

Treatment of CDTA-Extracted Cell Wall Fraction	% Binding Relative to Control		
	Exp. 1	Exp. 2	Exp. 3
None	13	12	16
Trypsin, 500 μg/ml[a]	25*[e]	36*	34*
Pronase, 500 μg/ml[b]	30*	40*	36*
Heat[c]	11	12	20
2 N TFA[d]	78*	60*	

[a] CDTA-extracted cell wall fractions containing 4.8 or 13.6 μg protein were incubated 4 h at 30°C in MS macro salts, 25 mM Hepes (pH 7.2), with 500 μg/ml trypsin, followed by 45 min at 100°C to inactivate the protease. Evaporated volume was replaced by distilled H_2O. Control protease binding assays contained 10 μl of samples which had undergone identical protease treatment except that water replaced the CDTA-extracted fraction.

[b] CDTA-extracted fraction and control, *i.e.* water, treated identically as described for trypsin treatment except that the enzyme was 500 μg/ml protease from *Streptomyces griseus*.

[c] CDTA fraction (65 μl containing 4.8 or 13.6 μg protein) heated 4 h at 100°C. Evaporated volume was replaced with H_2O.

[d] CDTA fraction (65 μl containing 4.8 or 13.6 μg protein) hydrolyzed 4 h at 100°C in 2 N TFA, lyophilized, brought to original volume with water, and pH adjusted to 6.0 with NaOH. Control, *i.e.* 65 μl water, no CDTA fraction, received identical treatment.

[e] *, Significantly different from untreated CDTA-extracted fraction, $P < 0.002$.

extracted cell wall fraction since it completely suppressed bacterial agglutination and most of the binding to plant cells.

DISCUSSION

Using a direct binding assay, we have shown that *A. tumefaciens* adherence to plant cells is saturable. This is the first well characterized direct evidence for a specific *A. tumefaciens* plant cell surface adherence site and corroborates results from earlier bioassay experiments which implied the existence of such a site (16). Direct demonstration of this specific interaction defines a reproducible and identifiable step in the transformation process at a cellular level and provides an assay for purifying and analyzing the components in both the bacterial and plant cells that are involved in this heterologous cell interaction.

If suspension-cultured plant cells were not extensively washed with a buffered salt solution, saturable *A. tumefaciens* binding could not be demonstrated, and large aggregates of bacteria became associated with the plant cell surface. It is possible that *A. tumefaciens* adherence to plant surfaces occurs via several stages and there may be several types of adherence interactions with plant surfaces. For example, one adherence site (represented by the highly agglutinating, plant surface material that is removed by salt washes) appears to be multivalent and possibly nonspecific for bacterial binding. A second adherence moiety is saturable, not easily washed from intact plant cells and appears to contain monovalent binding sites for *A. tumefaciens*, since in its presence even the normal extent of bacterial agglutination is inhibited (Table III). While it is important to point out that certain interactions observed *in vitro* may not play a physiological role in the intact plant, it is conceivable that a 'sticky' nonspecific interaction may serve to keep bacteria at the plant cell surface implementing their subsequent interaction with a saturable binding site.

We have found that a pectin-enriched plant cell wall fraction extracted by CDTA inhibits saturable binding of *A. tumefaciens* to intact plant cells. Pueppke and Benny (26) reported that a pectin-enriched cell wall fraction from potato tubers, commercial citrus pectin, and PGA inhibited tumor formation by virulent strains of *A. tumefaciens* on potato tubers. PGA and citrus pectin also inhibited tumor formation on pinto bean leaves with PGA being about 1000 times more effective than pectin on a weight basis (27). Plant cell walls, which could competitively inhibit tumor formation, lost their inhibitory activity after extraction of pectin with hot H_2O (27). In our assay, galacturonic acid, partially esterified citrus pectin, and PGA agglutinated *A. tumefaciens* and inhibited bacterial binding to plant cells with PGA being most effective on a weight basis. In contrast, the CDTA-extracted cell wall fraction inhibited bacterial agglutination as well as binding to plant cells (Table IV). The biochemical nature of this inhibitory moiety is not yet known but it may be different from the bacterial binding moiety in the pectic and PGA preparations used. This inhibitory factor apparently interacts with *A. tumefaciens* in a manner distinct from these compounds. Pectin and/or PGA in solution may contain many sites (specific and/or nonspecific) for bacterial binding and could inhibit binding and tumor formation by aggregating *A. tumefaciens* thereby blocking or occupying bacterial sites necessary for binding to the plant surface. In support of such a hypothesis, Pueppke and Benny (26) pointed out that heparin was also highly effective in inhibition of tumor formation.

Because the CDTA-extracted tomato cell wall contains a moiety which inhibits bacterial binding and does not enhance bacterial agglutination, we feel it is an advantageous preparation for the further characterization of the putative specific *A. tumefaciens* binding molecule on the plant cell surface. This preparation is heterogeneous and contains protein and acidic and neutral polysaccharides. Although it is not yet possible to ascribe inhibitory activity to any of these (or other) components, our data suggest that the factor may be a carbohydrate moiety attached to a protein, since inhibitory activity is stable to heat and substantially inactivated by conditions which would hydrolyze certain sugar polymers (28). The slight sensitivity to proteases is also consistent with the inhibitory component being a heavily glycosylated glycoprotein. Work is in progress to elucidate the nature of this factor and to determine additional steps in the process of cellular transformation by *A. tumefaciens*.

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