Attachment of *Agrobacterium tumefaciens* to Carrot Cells and *Arabidopsis* Wound Sites Is Correlated with the Presence of a Cell-Associated, Acidic Polysaccharide

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An early step in crown gall tumor formation involves the attachment of *Agrobacterium tumefaciens* to host plant cells. *A. tumefaciens* C58::A205 (C58 *attR*) is a Tn*3*HoHo1 insertion mutant that was found to be avirulent on *Bryophyllum daigremontiana* and unable to attach to carrot suspension cells. The mutation mapped to an open reading frame encoding a putative protein of 247 amino acids which has significant homology to transacetylases from many bacteria. Biochemical analysis of polysaccharide extracts from wild-type strain C58 and the C58::A205 mutant showed that the latter was deficient in the production of a cell-associated polysaccharide. Anion-exchange chromatography followed by ^1H nuclear magnetic resonance and gas chromatography-mass spectrometry analyses showed that the polysaccharide produced by strain C58 was an acetylated, acidic polysaccharide and that the polysaccharide preparation contained three sugars: glucose, glucosamine, and an unidentified deoxy-sugar. Application of the polysaccharide preparation from strain C58 to carrot suspension cells prior to inoculation with the bacteria effectively inhibited attachment of the bacteria to the carrot cells, whereas an identical preparation from strain C58::A205 had no inhibitory effect and did not contain the acidic polysaccharide. Similarly, preincubation of *Arabidopsis thaliana* root segments with the polysaccharide prevented attachment of strain C58 to that plant. This indicates that the acidic polysaccharide may play a role in the attachment of *A. tumefaciens* to host soma plant cells.

Infections of wound sites in dicotyledonous plants by *Agrobacterium tumefaciens* result in the formation of crown gall tumors. The mechanism of pathogenesis involves the transfer of a DNA fragment (T DNA) from the bacterial Ti plasmid to the plant host cell (9). An early step in tumor formation is the attachment of the bacteria to the plant cell surface (22), which is required for pathogenesis. All known nonattaching mutants are avirulent (6, 10, 23, 44). Past studies have identified the *att* gene region (more than 20 kb in size) of the bacterial chromosome, which contains genes required for attachment and virulence (23). Transposon insertions in the left side of this region (genes *attA1* through *attH*) resulted in mutants that could be restored to attachment and virulence by the addition of conditioned medium (26), which is produced by incubation of a virulent strain of *A. tumefaciens* with carrot cells in Murashige and Skoog (MS) medium, followed by removal of the bacteria and plant cells (24). This suggested that the left side of the *att* region contains genes that are involved in some molecular signaling event. In contrast, transposon insertions in the right side of the *att* gene region resulted in nonattaching mutants that were unaffected by the presence of conditioned medium. We report here the characterization of one such *att* mutant (*attR*).

Cell surface polysaccharides of *A. tumefaciens* have been proposed to be important in the attachment process; specifically, preparations of lipopolysaccharides (LPS) of virulent strains were reported to inhibit tumor formation on bean leaves, if applied before the bacteria (49–51). LPS are integral components of the outer membrane and comprise the lipid A membrane anchor, a conserved core oligosaccharide, and the O-antigen polysaccharide (48).

In addition to LPS, members of the family *Rhizobiaceae* of plant-associated bacteria, including *A. tumefaciens*, also produce capsular polysaccharides, or K antigens (16, 33). The K antigens of rhizobia are distinguished from LPS by a lack of a lipid anchor and a highly anionic nature and from the extracellular polysaccharides (EPS) by a tight association with the cell proper. Four structurally distinct K antigens have been isolated from rhizobia and characterized (11, 30, 33). All have been found to contain a high proportion of 3-deoxy-D-*manno*-2-octulosonic acid (Kdo) or Kdo variants. In addition, some evidence indicates that the K antigens may have specific functions in the interaction of rhizobia with the host plants (4, 32, 35).

Evidence that shows that the production of an acidic polysaccharide is correlated to the attachment of wild-type strain C58 to cultured carrot cells and *Arabidopsis thaliana* root segments and that this polysaccharide is not produced by the *attR* mutant is presented in this report.

MATERIALS AND METHODS

Bacterial strains, plasmids, and growth conditions. The bacterial strains and plasmids used in this study were previously described (21, 23, 25). Bacteria were grown in Luria broth, nutrient broth, or minimal medium, and viable-cell counts were determined as previously described (21). *A. tumefaciens* was grown at 25°C, and *Escherichia coli* was grown at 37°C. Antibiotics were used at the following concentrations (in milligrams per liter): carbenicillin, 50; tetracycline, 10; chloramphenicol, 100; neomycin, 20 for liquid media and 60 for agar media; and gentamicin, 50. A library of *A. tumefaciens* NT1 DNA cloned as *Sau*3A partial digestion products into the *Bam*HI site of pCP13 in *E. coli* was obtained from S. Farrand (University of Illinois, Urbana).

Generation of *A. tumefaciens* C58::A205 and C58::A610. In order to obtain transposon insertions in cloned DNA in *E. coli*, the Tn*3*HoHo1 system described by Stachel et al. (38) was used. Tn*3*HoHo1 is an artificial transposon which carries the β-galactosidase gene but lacks a transposase. Plasmids containing transposon insertions were introduced into *A. tumefaciens* C58 by conjugation

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using pRK2013 as a mobilizing plasmid for mating, as previously described (39). *A. tumefaciens* C58 strains in which the wild-type DNA was replaced in the chromosome by DNA containing the transposon insertion were isolated by the introduction of plasmid pRK2014, which is incompatible with plasmid pCP13, which carried the cloned DNA. Transconjugants were selected for gentamicin resistance (carried by pRK2014) and carbenicillin resistance (carried by the transposon) and screened for loss of tetracycline resistance (carried by pCP13). Bacterial mutants were characterized with respect to their ability to attach to carrot suspension-culture cells, ability to attach to *A. thaliana* root segments, virulence on *Bryophyllum daigremontiana*, motility, and cellulose synthesis as previously described (15, 21, 25).

DNA sequencing and analysis of sequence data. DNA fragments to be sequenced were subcloned from pCP13 by the protocols described by Maniatis et al. (20) into pBluescript KS⁻ (Stragene). Plasmid DNA was purified as previously described (39), and double-stranded sequencing was carried out at the University of North Carolina Nucleic Acid Sequencing Facility, using a model 373A DNA Sequencer (Applied Biosystems) and a *Taq* DyeDeoxy Terminator Cycle Sequencing kit (Applied Biosystems). In order to sequence regions removed from the ends of the clone, artificial oligonucleotides 20 to 22 bases in length which were identical to a sequence near the end of the region previously sequenced were synthesized by the University of North Carolina Oligonucleotide Synthesis Facility, purified, and used as primers for further sequencing reactions. Unless otherwise indicated, both strands of the DNA were sequenced. DNA sequences were aligned and analyzed by using Genetics Computer Group (GCG) computer analysis programs. BlastX (1, 12) and Blocks (14) were used to search databases for protein amino acid sequences similar to those obtained by translating the DNA sequence. Similar sequences were aligned by using the Pileup program of the GCG. Percent identity and homology were calculated by using the GCG program Bestfit with a gap weight of 3.0 and a gap length weight of 0.10. Probabilities that regions of the predicted protein sequence would be found in the membrane were determined by using the ALOM program for detecting membrane-spanning proteins (19).

Attachment and aggregation assays. Conditioned medium was prepared and tested for its effect on bacterial attachment as previously described (24). Briefly, wild-type *A. tumefaciens* cells were incubated for 6 to 8 h with freshly transferred carrot suspension-culture cells in MS medium. The carrot cells were then removed by filtration through Miracloth (Calbiochem). Conditioned medium was filter sterilized with a 0.2-μm-pore-size filter. Sterile conditioned medium was stored at 4°C.

For studies of bacterial attachment, approximately 10^5 carrot cells/ml were suspended in MS or conditioned medium and incubated for 30 min. Bacteria were then added to a final concentration of 2×10^3 to 5×10^3 cells/ml. Attachment was measured by determining the number of viable free and bound bacteria by plating the mixture after filtration through Miracloth filters, which retained the carrot cells and any bound bacteria. The filtrate was plated directly to determine the number of viable free bacteria. The carrot cells were resuspended in MS medium and ground in a Waring blender before the solution was plated to determine the number of bound bacteria.

Aggregation of the carrot cells was measured visually, as shown below (see Fig. 2). The basis for this phenomenon and the protocol have been described elsewhere (21, 23).

For studies of bacterial attachment to *A. thaliana*, roots were excised from 1- to 3-week-old aseptically grown plants and washed in sterile water. Segments 1 to 5 mm in length were suspended in 0.2 ml of 0.4% sucrose, and bacteria were added to a final concentration of 1×10^4 to 8×10^4 cells/ml. The number of free bacteria was determined by viable-cell counts of the bacteria sampled with a 1- to 100-μl pipette tip with an opening too small to allow the passage of root segments. Attached bacteria were examined under a light microscope as previously described (15).

Polysaccharide preparation and analysis. The cell pellets from 5-liter cultures were extracted by the modified hot phenol-water method (8), and the fractionated phases were dialyzed against H_2O and freeze-dried. During the fractionation step, care was taken to avoid cross-contamination of each phase.

For purification, Sephadex G-150 superfine (Pharmacia, Uppsala, Sweden) size exclusion chromatography was initially attempted (0.2 M NaCl, 1 mM EDTA, 10 mM Tris base, 0.25% deoxycholic acid; pH 9.25); however, the sample formed a gel in the eluent and could not be recovered. DEAE Sephadex (Pharmacia) anion-exchange chromatography (50-ml column) was performed by using a wash with 2 column volumes of H_2O followed by a linear gradient (0 to 1 M) of ammonium formate ($CH_2OO \cdot NH_3$). Any remaining material was removed by a wash with 2 column volumes of 2 M ammonium formate. The eluted fractions were assayed colorimetrically for neutral sugars by the anthrone procedure (52).

Deoxycholic acid-polyacrylamide gel electrophoresis (PAGE) analysis was performed on aliquots of 2 to 5 μg (dry weight) of the polysaccharide preparations, using 18% acrylamide gels. The gels were silver stained to highlight LPS (45) or Alcian blue-silver stained for acidic polysaccharides. These protocols have been described previously and referenced (33).

A second size exclusion chromatography step was performed on Sepharose 4B (1-m by 1.5-cm column; 30 mM triethylamine, 10 mM EDTA; pH 7); the eluted fractions were assayed for neutral sugars, as described above, and for phosphate (37), uronic acids (5), and Kdo by the thiobarbituric acid (TBA) assay (47). The pooled fractions were then dialyzed and freeze-dried.

TABLE 1. Effect of conditioned medium on the attachment of wild-type and mutant *A. tumefaciens* cells to carrot suspension-culture cells

Bacterial strain inoculated	Source of conditioned medium	% Bacterial inoculum attached[a]
C58 (wild type)	None	35 ± 8
C58::A205 (*attR*)	None	5 ± 8
	C58 and carrot cells	9 ± 8
C58::B123 (*attD*)	None	1 ± 1
	C58 and carrot cells	14 ± 3
	C58::A205 and carrot cells	20 ± 5

[a] Attachment was measured after 60-min incubation of *A. tumefaciens* C58 with the carrot cells. Values are means ± standard deviations of a minimum of three experiments.

Carbohydrate composition analysis was performed by gas chromatography-mass spectrometry (GC-MS) of trimethylsilyl methyl glycosides (52) using a 30-m DB1 fused silica column (J&W Scientific, Folsum, Calif.) on a 5890A GC-MSD (Hewlett-Packard, Palo Alto, Calif.). Inositol was used as an internal standard, and retention times were compared to those of authentic standards.

For nuclear magnetic resonance (NMR) spectrometry, the polysaccharide preparation was dissolved in ^2H_2O, and the ^1H NMR analysis was performed on an AM 250 MHz instrument (Brucker, Rheinstetten, Germany). The measurement was made at 316 K. The chemical shifts were referenced to acetone at δ 2.24.

Nucleotide sequence accession number. The GenBank accession number for the *attR* gene reported here is U59485.

RESULTS

Isolation and characterization of the mutant C58::A205. A cosmid clone from a library of *A. tumefaciens* carrying genes (*att*) required for bacterial attachment to host cells and virulence was previously identified (23, 36). Random Tn*3*HoHo1 insertions in this clone resulted in insertions A205 and A610; these insertions were introduced into the bacterial chromosome of the wild-type strain C58 by marker exchange. The resulting mutants (C58::A205 and C58::A610) were found to be avirulent when used to inoculate leaves of *B. daigremontiana* and were unable to attach to carrot suspension-culture cells. Although conditioned medium was able to complement some *att* mutants (*attA1* through *attH* [26]), the addition of conditioned medium failed to restore the ability of C58::A205 or C58::A610 to bind to carrot cells (Table 1).

In order to determine whether the A205 insertion affected steps in the formation of conditioned medium, C58::A205 was used instead of C58 to prepare conditioned medium. Conditioned medium prepared by incubating strain C58::A205 with carrot cells was able to complement C58::B123 (an *attD* mutant), restoring its attachment to carrot cells in a fashion similar to that of conditioned medium prepared by using wild-type strain C58 (Table 1). This result suggests that C58::A205 is blocked at a step in bacterial attachment after the step involving conditioned medium and signaling between the plant and bacterium.

DNA sequence and analysis. The region of the cosmid clone containing the Tn*3*HoHo1 insertion in A205 and A610 was subcloned into pBluescript KS⁻ and sequenced. An open reading frame (ORF) termed *attR*, encoding a putative protein of 247 amino acids, was identified. This ORF either is not part of an operon or is part of an operon consisting of only two genes. The next ORF upstream of *attR* is oriented in the opposite direction, and downstream of *attR* there is a space of 134 bp before the next ORF (*attS*), which encodes a putative protein of 251 amino acids with no significant homology to any entries in the databases. Following *attS*, the next ORF (*attT*) is oriented in the opposite direction to *attR*. The nonattaching phe-

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catb4  ................  ................  ............  .............         M  TNYFDSPFKG   11
 ecat  ................  ................  ............  .............         M  TNYFESPFKG   11
  cat  ................  ................  ............  .............         M  ENYFESPFRG   11
 sata  ................  ................  ............  ...........M  GPNMKMYPI  EG...NKSVQ   18
  syn  ................  ................  ............  ..........MY  GPDPQNKHPM  AG...FAQIC   19
 attr  VASVAWKGYR  HSDHVNLAYS  TRPTSCSVGM  GSKRFTMSLL  DPDAIHPITL  PDGRVYSDTV   60

catb4  KLLSEQVKNP  NIKVGRYSYY  SGYYHGHSFD  DCARYLFPDR  DDVDKLIIGS  FCSIGSGASF   71
 ecat  KLLTEQVKNP  NIKVGRYSYY  SGYYHGHSFD  DCARYLLPDR  DDVDQLIIGS  FCSIGSGARF   71
  cat  ITLDKQVKSP  NLVVGKYSYY  SGYYHGHSFE  DCARYLLPD.  EGADRLVIGS  FCSIGSGAAF   70
 sata  FIKPILEKLE  NVEVGEYSYY  .DSKNGETFD  KQILYHY..P  ILNDKLKIGK  FCSIGPGVTI   75
  syn  FIKNTVSN.P  NIIGDYTYY   DDPEDSENFE  RNVLYHF..P  FVGDRLIIGK  FCALARNVKF   76
 attr  YLKNVVDH.P  RIEVGEYSYF  THSGTPEE.T  AQILAPYLGH  TCRRLLIGK   FVQIAKGCYF  118

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 sata  IMNG.ANHRM  DG.STYPFNL  FGNGWEKHMP  KLDQLPIKGD  TIIGNDVWIG  KDVVIMPGVK  133
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 sata  IGDGAIVAAN  SVVVKDIAPY  MLAGGNPANE  IKQRFDQDTI  NQLLDIKWWN  WPIDIINENI  193
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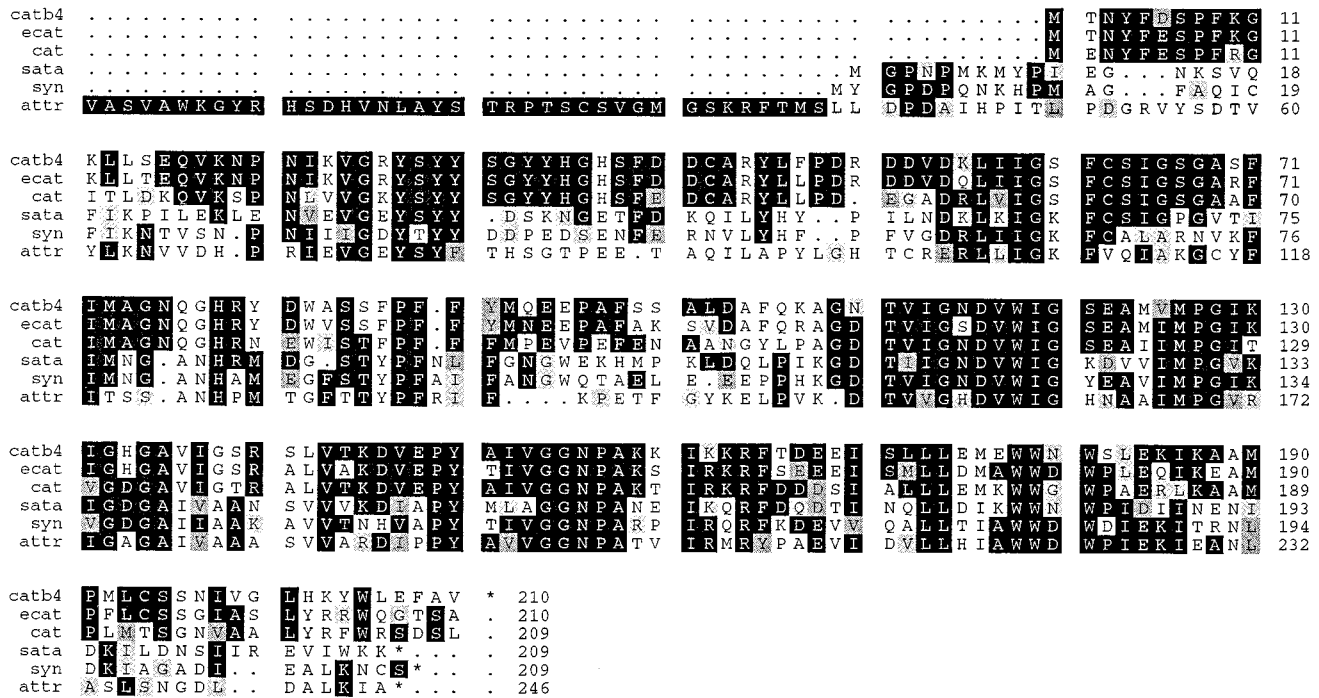
FIG. 1. Homology of the predicted AttR protein to acetyltransferases. Identical (black boxes) and similar (gray boxes) amino acids are indicated. The genes included are listed in the text.

notype of C58::A205 appears to be due to the defect in *attR* only, since the mutant can be complemented in *trans* by a plasmid carrying the complete sequence of *attN-attR* and only the first 150 bp of *attS* or by a plasmid carrying a transacetylase gene only (data not shown).

The predicted AttR protein is hydrophilic, with only one possible membrane-spanning domain located at amino acids 170 to 186. A BlastX search showed that the protein has homology to transacetylases from a *Synechocystis* sp. (68% similarity, 45% identity) (17), *Enterococcus faecium satA* (62% similarity, 40% identity) (29), *A. tumefaciens cat* (62% similarity, 40% identity) (43), *Enterobacter aerogenes* catB4 pBWH301 (61% similarity, 35% identity) (27), *E. coli cat* pNR79 (60% similarity, 38% identity) (27), and many other bacteria (Fig. 1). A lower degree of homology to *nodL* genes from rhizobia (e.g., *R. meliloti nodL* [48% similarity, 29% identity]) was seen (3).

Fractionation and biological analysis of the cell-associated polysaccharides from wild-type *A. tumefaciens* C58 and the *attR* mutant. The facts that the A205 mutation did not affect the production of conditioned medium and could not be complemented by conditioned medium suggested that the defect in C58::A205 might be in the production of the surface molecules involved in the attachment of the bacteria to the plant cell surface. The location of the A205 insertion in an ORF with homology to transacetylases also suggested that this gene might be required for the synthesis of some surface molecule. As there was previous research suggesting that polysaccharides, particularly LPS, might be involved in the attachment of *A. tumefaciens*, we examined the surface polysaccharides of the wild-type strain and the A205 mutant (49–51).

The cell-associated polysaccharides from *A. tumefaciens* C58 and C58::A205 were extracted and fractionated into three pools on the basis of their solubility: the phenol-soluble material (PW/P), the water-soluble component (PW/W), and the

material that forms a cloudy interface between the two phases (PW/Cl). Commonly, the latter phase is partially or wholly combined with the water phase during the fractionation step; however, care was taken here to avoid any cross contamination. Each polysaccharide preparation from C58 was tested for its effect on attachment of *A. tumefaciens* C58 to carrot cells, and it was found that the PW/W preparation, but not the PW/P or PW/Cl pool, from strain C58 inhibited the binding of wild-type bacteria to carrot cells (Table 2). In addition to bacterial attachment, the effect of the polysaccharide preparations on the aggregation of carrot cells caused by the bacteria was noted (Fig. 2 and Table 2): the addition of the PW/W preparation inhibited carrot cell aggregation, whereas the PW/P preparation had no effect. Past work has shown that only virulent strains of *A. tumefaciens*, which are able to attach, bring about the aggregation of the carrot cells (23, 25); nonattaching strains have no effect. This is presumed to be a result of bacterial cell-cell interaction, via cellulose fibrils (21), between the *A. tumefaciens* cells attached to the carrot cells. Therefore, the inhibition of bacterial attachment should also prevent carrot cell aggregation.

From these results, it was evident that wild-type strain C58 produces a molecular factor capable of inhibiting bacterial attachment that was fractionated into the water phase of the hot phenol-water extractions. Therefore, an identical polysaccharide preparation from the nonattaching mutant, C58::A205, was applied in a similar manner to the carrot cell assays (Table 2). This preparation had no effect on bacterial binding to carrot cells and apparently lacked the inhibitory (i.e., attachment) factor, indicating that either the mutant did not produce it or it was abnormally expressed.

A second experiment tested whether the inhibitory factor acted on the carrot cells or on the bacteria. Carrot cells were pretreated with PW/W, washed, and then employed in an at-

TABLE 2. Effect of polysaccharide fractions on attachment of wild-type *A. tumefaciens* C58 to carrot cells and aggregation of carrot cells by the bacteria[a]

Source of polysaccharides[b]	Polysaccharide fraction[c] (concn, µg/ml)	% Attachment[d]	Aggregation of carrot cells[e]
None (control)		100	Yes
C58	PW/P (100)	70 ± 10	Yes
	PW/Cl (100)	81 ± 10	Yes
	PW/W (100)	5 ± 8	No
	PW/W (10)	0 ± 6	No
	PW/W (1)	54 ± 23	No
	PW/W-1 (10)	76 ± 10	Yes
	PW/W-2 (1)	41 ± 8	No
C58::A205	PW/W (100)	70 ± 16	Yes

[a] Carrot cells were incubated with the polysaccharide in MS medium for 30 min prior to the addition of the bacteria.

[b] Polysaccharide fractions were from phenol-water extractions of bacterial pellets and subsequent anion-exchange chromatography (Fig. 3).

[c] PW/P, phenol phase; PW/Cl, cloudy interface; PW/W, water phase; PW/W-1, neutral polysaccharides from PW/W; and PW/W-2, acidic polysaccharides from PW/W.

[d] Expressed relative to the control value (100%). Attachment was measured after 60-min incubation of *A. tumefaciens* C58 with the carrot cells. Values are means ± standard deviations of a minimum of three experiments.

[e] Aggregation of carrot cells was determined after 48-h incubation.

tachment-aggregation assay. The results showed an inhibition of attachment of the wild-type strain and a lack of carrot cell aggregation, indicating that the inhibitory factor acted on the carrot cells (Table 3). The inhibition of attachment by PW/W in this experiment was less than that seen when PW/W was added directly to the attachment reaction mixture. This may be due to loss of some of the PW/W during washing of the carrot cells or to an effect of PW/W on the bacteria as well as the carrot cells. As in the previous assay, the identical polysaccharide preparation from the nonattaching mutant did not have a significant effect.

In order to determine if this was a general phenomenon, the same polysaccharide preparation was also tested on aseptic root segments of *A. thaliana* ecotype Landsberg *erecta*. The results showed that the PW/W, but not the PW/P, polysaccharide prevented attachment of wild-type strain C58 to that plant (Table 4). The difference in the amount of polysaccharide

required for inhibition of attachment to roots and tissue culture cells (approximately 10-fold less) may be due to differences in their surface composition and/or anatomy.

In an initial attempt to fractionate the PW/W preparation by size exclusion chromatography, 20 mg of C58 PW/W was dissolved in 2 ml of deoxycholate-containing eluent; however, prior to application to the column, the solution formed a solid gel, which could not be diluted back into solution. For comparison, 20 mg of the PW/W preparation from the nonattaching mutant C58::A205 was dissolved in 2 ml of the same eluent; the sample dissolved easily and remained in solution. The process was repeated for both preparations, with the same results. This indicated a clear difference in the properties of the two preparations.

The strain C58 PW/W preparation was fractionated by anion-exchange chromatography (Fig. 3). The sample was dissolved in H_2O and applied to a DEAE column; the column was then washed with H_2O, and the bound material was eluted with a gradient of $CH_2OO \cdot NH_3$. This resulted in two subfractions of C58 PW/W: the neutral polysaccharides that eluted in the wash step (PW/W-1) and the acidic polysaccharides (PW/W-2), which eluted with ~0.7 to 0.9 M $CH_2OO \cdot NH_3$. The subfractions were tested in the carrot cell attachment assay, and the results showed that the inhibitory activity was in the acidic fraction, PW/W-2 (Table 2).

Analysis of the C58 PW/W-2 preparation. PAGE analysis of PW/W-2 showed no detectable LPS in this fraction (Fig. 4, lane 3), whereas LPS was clearly detected in both the PW/P and the PW/Cl preparations (Fig. 4, lanes 1 and 2). The initial PW/W preparation contained a trace of LPS, which was removed during purification. The silver stain procedure used here effectively stains LPS but generally does not stain the K antigens or other nonacylated polysaccharides (33, 45). The material that migrates in a ladder pattern in lanes 1 and 2 of Fig. 4 is the smooth LPS, and the high-mobility bands are due to rough LPS. GC-MS analysis of the PW/P preparation confirmed that this fraction contained LPS (data not shown) by identification of the β-OH-fatty acids that are characteristic of *Rhizobium* lipid A (7). This showed that the attachment factor was definitely not LPS. An Alcian blue-silver-stained gel yielded no additional information (data not shown).

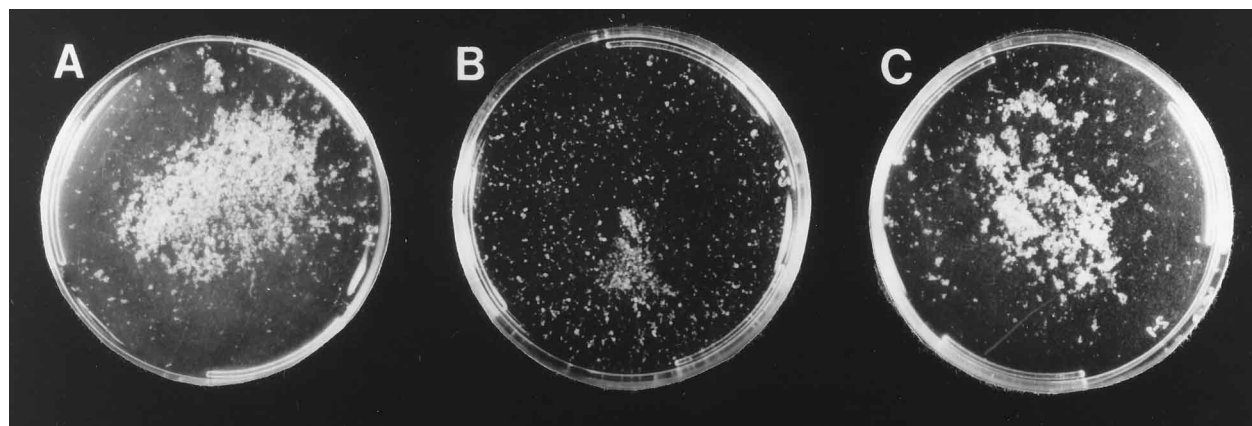GC-MS analysis of the trimethylsilyl methyl glycoside deriv-

FIG. 2. Effect of polysaccharide pools on aggregation of carrot cells incubated with *A. tumefaciens* C58 for 48 h. Past studies had shown that virulent strains of *A. tumefaciens*, which were able to attach, caused aggregation of the carrot cells (see text). (A) Cells with no polysaccharide added. (B) PW/W from strain C58 (100 µg/ml). Note the inhibition of aggregation due to the addition of the PW/W preparation. (C) PW/P from strain C58 (100 µg/ml). All of the petri dishes shown contained approximately 10^5 carrot cells/ml. The addition of an equal amount of the PW/P preparation had little inhibitory effect on carrot cell aggregation. Similar results were obtained with PW/Cl from strain C58 and all three pools from strain C58::A205 (data not shown). Thus, only the strain C58 PW/W preparation was able to effectively inhibit the aggregation of carrot cells.

TABLE 3. Effect of pretreatment of carrot cells with polysaccharide fractions from *A. tumefaciens* on the attachment of C58 to carrot cells and aggregation of carrot cells by the bacteria[a]

Pretreatment of carrot cells[b]	% Attachment[c]	Aggregation of carrot cells[d]
MS medium alone (control)	100	Yes
MS medium plus PW/W (100 µg/ml) from C58 (wild type)	51 ± 8	No
MS medium plus PW/W (100 µg/ml) from C58::A205 (*attR*)	91 ± 10	Yes

[a] Carrot cells were preincubated with the polysaccharide fraction for 30 min in MS medium, collected on Miracloth filters, washed with 2 ml of MS to remove excess polysaccharide, and resuspended in MS medium prior to the addition of the bacteria.
[b] Polysaccharide fractions were from phenol-water extractions of bacterial pellets. PW/W, water phase.
[c] Expressed relative to the control value (100%). Attachment was measured after 60-min incubation of *A. tumefaciens* C58 with the carrot cells. Values are means ± standard deviations of a minimum of three experiments.
[d] Aggregation of carrot cells was determined after 48-h incubation.

atives of the PW/W-2 components showed the presence of three sugars, glucose, glucosamine, and an unidentified sugar, in approximately a 2:1:1 ratio (data not shown). The retention time of the latter sugar derivative suggested that it was a variant of Kdo; i.e., the elution point was between those of Kdo and sialic acid. In addition, the fragmentation ions in the mass spectrum were similar to those of sialic acid (data not shown). Importantly, β-OH-fatty acids, which are indicative of LPS, were not found in PW/W-2.

The PW/W-2 preparation was then analyzed by ^{1}H NMR spectrometry (Fig. 5), which showed that PW/W-2 contained a relatively simple polysaccharide, carrying numerous *O*- and *N*-acetyl substituents (δ 2.0 and δ 1.9, respectively). The resonances at δ 2.3 and the pair of doublets at δ 1.8 are similar to the resonances from the C_3 methylene protons of Kdo or a Kdo-like component, and the resonances at δ 1.4 indicate the presence of a C-linked methyl group (such as a branch or terminal methyl group) in the unidentified sugar.

Importantly, there are no resonances in Fig. 5 that correspond to protein or lipid, showing that the attachment factor is not LPS or a glycoprotein. The NMR analysis also showed that the polysaccharide was not succinylglycan (i.e., acidic EPS), as no pyruvate or succinate signals were present in the spectrum (compare to the NMR spectrum in reference 2). Further analyses of this polysaccharide are ongoing.

TABLE 4. Effect of polysaccharide fractions on the attachment of *A. tumefaciens* C58 to *A. thaliana* root segments[a]

Source of polysaccharide[b]	Polysaccharide fraction[c] (concn, µg/ml)	% Attachment[d]
None (control)		100
C58	PW/P (100)	60 ± 25
	PW/W (100)	2 ± 5
	PW/W (10)	90 ± 20
C58::A205	PW/W (100)	80 ± 20

[a] Root segments were incubated with the polysaccharide in 0.4% sucrose for 15 min prior to the addition of the bacteria.
[b] Polysaccharide fractions were from phenol-water extractions of bacterial pellets.
[c] PW/P, phenol phase; PW/W, water phase.
[d] Expressed relative to the control value (100%). Attachment was measured after 60-min incubation of *A. tumefaciens* C58 with the root segments. Values are means ± standard deviations of a minimum of three experiments.
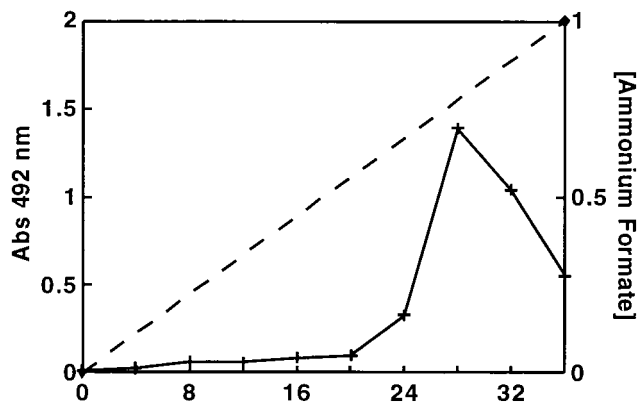

FIG. 3. Anion-exchange chromatography of the PW/W pool from *A. tumefaciens* C58. The column fractions were assayed for hexose. The gradient is 0 to 1 M ammonium formate ($CH_2OO \cdot NH_3$). The column was washed with 2 column volumes of H_2O. The neutral polysaccharides that eluted in the wash constituted pool PW/W-1, and the acidic polysaccharides that eluted in the peak, from fractions 24 through 32, constituted pool PW/W-2. Abs, absorbance.

In order to estimate the size of the active polysaccharide, an aliquot of PW/W-2 was analyzed on a Sepharose 4B size exclusion column (Fig. 6). This yielded only one fraction which eluted in the included volume of the column; previous work with similar samples had shown this to include polysaccharides of ~6 kDa and less. Since the polysaccharide did not pass through dialysis tubing with a 3.5-kDa cutoff, the estimated molecular mass of the polysaccharide that was extracted from cultured cells is between 3 and 7 kDa.

The Sepharose 4B column fractions were assayed for uronic acids, neutral sugars, Kdo or Kdo-like sugars (TBA assay) and phosphate. As shown in Fig. 6, these analyses indicated that the PW/W-2 preparation contained the latter three components but no uronic acid. The TBA-positive response supported the GC and NMR data, which also indicated the pres-
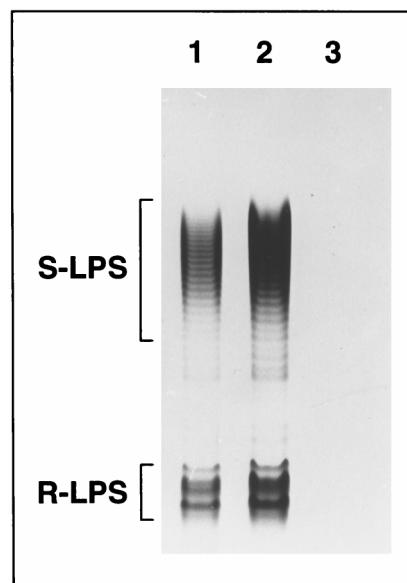

FIG. 4. PAGE analysis of the three preparations isolated from the hot phenol-water extraction of *A. tumefaciens* C58. Lane 1, PW/P; lane 2, PW/Cl; lane 3, PW/W-2. S-LPS and R-LPS, smooth and rough LPS, respectively.
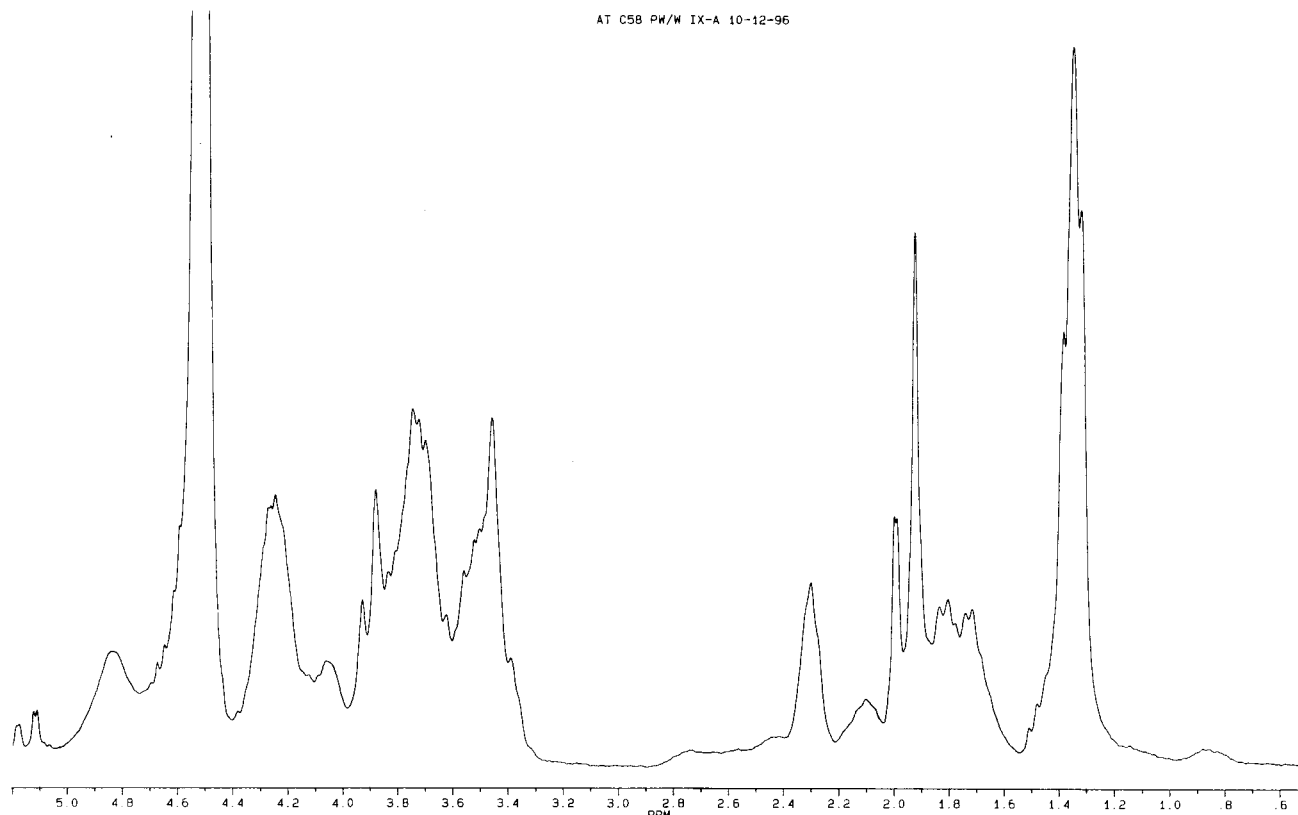
AT C58 PW/W IX-A 10-12-96

FIG. 5. ¹H NMR of the pooled PW/W-2 preparation from *A. tumefaciens* C58. The large signal at 4.5 ppm is from residual water. See the text for details.

ence of a Kdo-like component in the PW/W-2 preparation. The fact that there was only one peak in the column profile showed that the PW/W-2 preparation did not contain any high-molecular-weight contaminants, and subsequent NMR analysis yielded a spectrum that was identical to that shown in Fig. 5 (not shown).
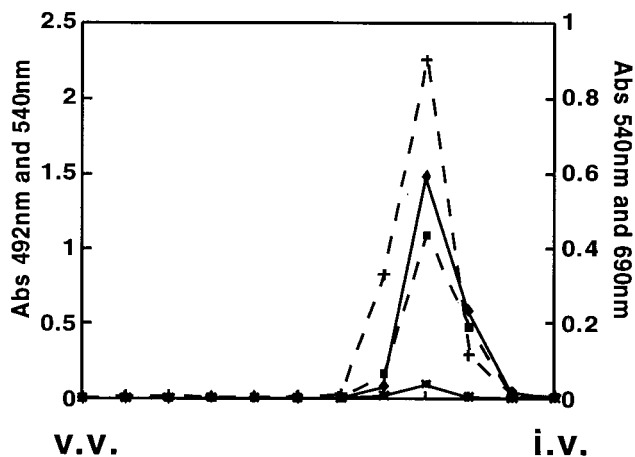

FIG. 6. Sepharose 4B size exclusion chromatography. The eluted fractions were assayed for neutral sugars (crosses; absorbance [Abs] at 492 nm), phosphate (squares; absorbance at 690 nm), uronic acids (asterisks; absorbance at 540 nm), and Kdo (diamonds; absorbance at 540 nm). v.v., void volume; i.v., included volume.

Analysis of the PW/W preparation from *A. tumefaciens* C58::A205 (*attR*), the nonattaching mutant, showed that it did not produce the polysaccharide in any form, i.e., with or without acetate (data not shown). The C58::A205 PW/W preparation contained only glucans (probably cyclic β-glucans) and some LPS.

DISCUSSION

The *A. tumefaciens* *attR* mutant (C58::A205) described in this report appears to lack the surface component(s) involved in the attachment of the pathogen to host cells, which is prerequisite to virulence; the *attR* mutant was avirulent and non-attaching. Unlike some other nonattaching mutants, the *attR* mutant was not complemented by conditioned medium (24), and, in fact, C58::A205 was capable of generating effective conditioned medium when incubated with carrot cells. This indicates that the putative bacterial transport system encoded by *attA1* through *attH* (26), which is necessary for the generation of conditioned medium, is not affected by the *attR* mutation in strain C58::A205. Conversely, the fact that conditioned medium had no effect on the *attR* mutant suggests that it is defective in the production of some structural component.

The DNA sequence analyses of *attR* showed that the insertion in C58::A205 was in an ORF with homology to transacetylases. Biochemical analysis of wild-type strain C58 and the *attR* mutant showed that the mutant was unable to produce an acetylated, acidic polysaccharide. In addition, the purified polysaccharide from the parent strain was capable of inhibiting the attachment of *A. tumefaciens* to carrot cells and *A. thaliana*

root segments. The LPS-containing fractions had no effect in the attachment assays.

Previous research showed that many other sugars and polysaccharides, including glucose, galactose, sucrose, methylglucose, LPS, pectin, and arabinogalactans, had no effect on bacterial attachment (13). Divalent cations, chelating agents, and changes in ionic and osmotic strength between 0.001 and 0.50 M also had no effect on the attachment of biotype 1 *A. tumefaciens* to carrot cells. In fact, the only substances which have been found to inhibit bacterial attachment are those which affect the viability of the plant and/or bacterial cells and the protein vitronectin and antivitronectin antibodies (13, 42, 46). None of the polysaccharide fractions tested here appeared to affect cell viability, as judged by viable-cell counts for bacteria and the continuation of cytoplasmic streaming for carrot cells. These results indicate that the acidic polysaccharide may be involved in the attachment of the microbe to the surface of the host plant cell.

A. tumefaciens is closely related to *Rhizobium meliloti* and *Rhizobium fredii*, and these bacteria have been shown to produce acidic capsular polysaccharides that are structurally analogous to the group II K antigens of *E. coli* (16, 31). Several K antigens from *R. fredii* and *R. meliloti* have been characterized (11, 30, 33, 34), and each has been found to comprise small repeating units of hexose and Kdo, or other 1-carboxy-2-keto-3-deoxy variants, such as sialic acid and 2-keto-3,5,7,9-tetradeoxy-5,7-diaminononulosonic acid. The hexose components found thus far include glucose, galactose, mannose, 2-*O*-methylmannose, and glucosamine. In addition, many of the K antigens carry noncarbohydrate substituents, such as acetate and β-hydroxybutyrate.

Although it has not been firmly established if the acidic polysaccharide isolated from *A. tumefaciens* C58 is also some form of a K antigen, the data presented here indicate that this is the case. (i) There is no lipid moiety on the polysaccharide, distinguishing it from LPS. (ii) Unlike EPS, the polysaccharide was isolated from the cell pellet of cultured cells, and it does not carry the characteristic succinate and pyruvate substituents of succinylglycan (EPS). (iii) It is a highly anionic polysaccharide that comprises simple repeats, composed of hexose and an acidic sugar, which may be a variant of Kdo. (iv) As this bacterial product may mediate a cell-cell attachment, it is presumably on the cell surface, as is a capsular polysaccharide.

The facts that the polysaccharide identified in this study is highly anionic and that it is extracted from cell pellets with hot phenol-water may explain some past observations. Earlier reports suggested that the LPS of *A. tumefaciens* was required for attachment, as pretreatment with a hot phenol-water extract inhibited tumor formation on the host plant (49–51); however, past work and the results presented here show that non-LPS, acidic polysaccharides, such as that identified in this report, are also extracted by that method (31, 33–35).

In contrast to the LPS studies, our results do not offer any clear insight into the possible role and importance of rhicadhesin in attachment (40, 41). It may be that both components are necessary but that the *attR* (i.e., polysaccharide) mutation alone is sufficient for disruption of the process.

A clear relationship between K-antigen expression, infectivity, and the *rkp* gene region of *R. meliloti* AK631 (18, 28) has been established. A mutation in any of the four cistrons of this region (*rkpABCDEF*, *rkpGH*, *rkpI*, and *rkpJ*) results in the inability to properly express the K antigens and an aborted infection of alfalfa. This is also true of mutations in the unlinked *rkpZ* gene (35). The genome of *A. tumefaciens* C58 has been probed for *rkpABCDEF*, and the results showed that it was present (30). Future work should establish if there is a link

between that gene region, attachment of *A. tumefaciens* to host cells, and the polysaccharide identified in this report.

The identity of the molecule(s) on the host cell surface to which the bacteria bind is unknown; however, present evidence suggests that it may be a protein which is sufficiently related to human vitronectin for antibodies to human vitronectin to cross-react with it (46). Preliminary results suggest that the cross-reacting antibodies recognize the side of the protein that is involved in binding of vitronectin to heparin, an acidic polysaccharide (24a); therefore, it is not surprising that the bacterial surface component that may participate in an interaction with a vitronectin-like protein on the plant surface is an acidic polysaccharide.

Further investigations are necessary to structurally characterize this acidic polysaccharide and correlate the sequence homologies of the *att* genes with the biological deficiencies of the mutants. The results of this study, however, show that an attachment step, required for the virulence of *A. tumefaciens* on *B. daigremontiana*, is correlated to the production of this acetylated, acidic polysaccharide and that expression of this polysaccharide is disrupted by a mutation in *attR*, which shows significant homology to bacterial transacetylases.

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