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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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 extra- cellular 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 NTI DNA cloned as Sau3A partial digestion products into the BamHI 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 TnHoHo1 system described by Stachel et al. (38) was used. TnHoHo1 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...
using pRK2013 as a mobilizing plasmid for mating, as previously described (39). A. tumefaciens CS8 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, into wild-type CS8 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 B. 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+ (Stratagene). 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 Tac 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 (GCC) computers and analysis programs. BlotX (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 GCC. Percent identity and homology were calculated by using the Pileup program with a gap weight of 0.0 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 filters (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 medium. Conditioned medium and incubated for 30 min. Bacteria were then added to a final concentration of 2 × 10^6 to 5 × 10^7 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.

Conditioned medium was assayed 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 2-week-old plants grown in soil and washed in sterile water. Segments 0.5 to 1 mm in length were suspended in 0.2 ml of 0.4% sucrose, and bacteria were added to a final concentration of 1 × 10^6 to 10^7 cells/ml. The number of free bacteria was determined by viable-cell counts of the bacteria sampled with a 1- to 10-μl aliquot with an opening too small to allow the passage of root segments. Attached bacteria were examined under a light microscope as previously described (15). Poly saccharide 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) was used instead of C58 to prepare conditioned medium. Conditioned medium prepared by using wild-type strain C58 (Table 1) is often described as a source of attachment polysaccharide.

** RESULTS**

Isolation and characterization of the mutant CS8::A205. A cosm id 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 TnHoHo1 insertions in this clone resulted in insertions A205 and A610; these insertions were introduced into the bacterial chromosome of the wild-type strain CS8 by marker exchange. The resulting mutants (CS8::A205 and CS8::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 CS8::A205 or CS8::A610 to bind to carrot cells (Table 1).

In order to determine whether the A205 insertion affected steps in the formation of conditioned medium, CS8::A205 was used instead of CS8 to prepare conditioned medium. Conditioned medium prepared by incubating strain CS8::A205 with carrot cells was able to complement CS8::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 CS8::A205 is blocked at a step in bacterial attachment after the step involving conditioned medium and signaling between the plant and bacterium.

**DNA sequence analysis.** The region of the cosmid clone containing the TnHoHo1 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 location of 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-

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

<table>
<thead>
<tr>
<th>Strain Type</th>
<th>Source of Conditioned Medium</th>
<th>% Bacterial Inoculum Attached</th>
</tr>
</thead>
<tbody>
<tr>
<td>CS8 (wild type)</td>
<td>None</td>
<td>35 ± 8</td>
</tr>
<tr>
<td>CS8::A205 (attR)</td>
<td>None</td>
<td>5 ± 8</td>
</tr>
<tr>
<td>CS8 and carrot cells</td>
<td>9 ± 8</td>
<td></td>
</tr>
<tr>
<td>CS8::B123 (attD)</td>
<td>None</td>
<td>1 ± 1</td>
</tr>
<tr>
<td>CS8 and carrot cells</td>
<td>14 ± 3</td>
<td></td>
</tr>
<tr>
<td>CS8::A205 and carrot cells</td>
<td>20 ± 5</td>
<td></td>
</tr>
</tbody>
</table>
type 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 the bacteria 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

<table>
<thead>
<tr>
<th>Source of polysaccharidesa</th>
<th>Polysaccharide fractionb (conc., μg/ml)</th>
<th>% Attachmentc</th>
<th>Aggregation of carrot cellsd</th>
</tr>
</thead>
<tbody>
<tr>
<td>None (control)</td>
<td></td>
<td>100</td>
<td>Yes</td>
</tr>
<tr>
<td>C58</td>
<td>PW/P (100)</td>
<td>70 ± 10</td>
<td>Yes</td>
</tr>
<tr>
<td></td>
<td>PW/Cl (100)</td>
<td>81 ± 10</td>
<td>Yes</td>
</tr>
<tr>
<td></td>
<td>PW/W (100)</td>
<td>5 ± 8</td>
<td>No</td>
</tr>
<tr>
<td></td>
<td>PW/W (10)</td>
<td>0 ± 6</td>
<td>No</td>
</tr>
<tr>
<td></td>
<td>PW/W (1)</td>
<td>54 ± 23</td>
<td>No</td>
</tr>
<tr>
<td></td>
<td>PW/W-1 (10)</td>
<td>76 ± 10</td>
<td>Yes</td>
</tr>
<tr>
<td></td>
<td>PW/W-2 (1)</td>
<td>41 ± 8</td>
<td>No</td>
</tr>
<tr>
<td></td>
<td>PW/W (100)</td>
<td>70 ± 16</td>
<td>Yes</td>
</tr>
<tr>
<td>C58::A205</td>
<td>PW/P</td>
<td>100</td>
<td>Yes</td>
</tr>
<tr>
<td></td>
<td>PW/Cl</td>
<td>70 ± 10</td>
<td>Yes</td>
</tr>
<tr>
<td></td>
<td>PW/W</td>
<td>81 ± 10</td>
<td>Yes</td>
</tr>
<tr>
<td></td>
<td>PW/W-1</td>
<td>5 ± 8</td>
<td>No</td>
</tr>
<tr>
<td></td>
<td>PW/W-2</td>
<td>0 ± 6</td>
<td>No</td>
</tr>
<tr>
<td></td>
<td>PW/W (1)</td>
<td>54 ± 23</td>
<td>No</td>
</tr>
<tr>
<td></td>
<td>PW/W-1 (10)</td>
<td>76 ± 10</td>
<td>Yes</td>
</tr>
<tr>
<td></td>
<td>PW/W-2 (1)</td>
<td>41 ± 8</td>
<td>No</td>
</tr>
<tr>
<td></td>
<td>PW/W (100)</td>
<td>70 ± 16</td>
<td>Yes</td>
</tr>
</tbody>
</table>

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.

The strain C58 PW/W preparation was fractionated by anion-exchange chromatography (Fig. 3). The sample was dissolved in H2O and applied to a DEAE column; the column was then washed with H2O, and the bound material was eluted with a gradient of CH3OO·NH3. 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).

GC-MS analysis of the trimethylsilyl methyl glycoside derivates 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 H2O and applied to a DEAE column; the column was then washed with H2O, and the bound material was eluted with a gradient of CH3OO·NH3. The results showed that the inhibitory activity was in the acidic fraction, PW/W-2 (Table 2).

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The resonances from the C_3 methylene protons of Kdo or a nances at d-diacetyl substituents (N-relatively simple polysaccharide, carrying numerous spectrometry (Fig. 5), which showed that PW/W-2 contained a 6 means excess polysaccharide, and resuspended in MS medium prior to the addition of the bacteria.

Importantly, there are no resonances in Fig. 5 that corre-

spond 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 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^.

<table>
<thead>
<tr>
<th>Pretreatment of carrot cells^</th>
<th>% Attachment</th>
<th>Aggregation of carrot cells^</th>
</tr>
</thead>
<tbody>
<tr>
<td>MS medium alone (control)</td>
<td>100</td>
<td>Yes</td>
</tr>
<tr>
<td>MS medium plus PW/W (100 µg/ml) from C58 (wild type)</td>
<td>51 ± 8</td>
<td>No</td>
</tr>
<tr>
<td>MS medium plus PW/W (100 µg/ml) from C58::A205 (attR)</td>
<td>91 ± 10</td>
<td>Yes</td>
</tr>
</tbody>
</table>

^ Carrot cells were precultured 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.

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.

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

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-

TABLE 4. Effect of polysaccharide fractions on the attachment of A. tumefaciens C58 to A. thaliana root segments^.

<table>
<thead>
<tr>
<th>Source of polysaccharide^</th>
<th>Polysaccharide fraction^ (conc. µg/ml)</th>
<th>% Attachment^</th>
</tr>
</thead>
<tbody>
<tr>
<td>None (control)</td>
<td></td>
<td>100</td>
</tr>
<tr>
<td>C58</td>
<td>PW/P (100)</td>
<td>60 ± 25</td>
</tr>
<tr>
<td></td>
<td>PW/W (100)</td>
<td>2 ± 5</td>
</tr>
<tr>
<td></td>
<td>PW/W (10)</td>
<td>90 ± 20</td>
</tr>
<tr>
<td>C58::A205</td>
<td>PW/W (100)</td>
<td>80 ± 20</td>
</tr>
</tbody>
</table>

^ Root segments were incubated with the polysaccharide in 0.4% sucrose for 15 min prior to the addition of the bacteria.

^ Polysaccharide fractions were from phenol-water extractions of bacterial pellets.

^ PW/P, phenol phase; PW/W, water phase.

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. 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.
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).

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 transacylases. 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*

**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.

**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.
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, methylgluco-
lose, LPS, pectin, and arabinogalactans, had no effect on bacteri
al 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-tet-
readoxygen-5,7-diaminomonomonosalic acid. The hexose components found thus far include glucose, galactose, mannose, 2-O-meth-
ylmannose, and glucosamine. In addition, many of the K anti-
gens carry noncarbohydrate substituents, such as acetate and β-hydroxybutyrate.

Although it has not been firmly established if the acidic polysaccharide isolated from A. tumefaciens CS8 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 presumab-
ly 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 re-ports 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 rhicad-
hesin 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, infectiv-
ey, 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 (rkpABCD, rkpCH, 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 CS8 has been probed for rkpABCD, 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 poly-
saccharide (24a); therefore, it is not surprising that the bacte-
rial 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 charac-
terize 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 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 transacylases.

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