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Identification of a New Virulence Locus in *Agrobacterium tumefaciens* That Affects Polysaccharide Composition and Plant Cell Attachment

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We have identified a new virulence locus in *Agrobacterium tumefaciens*. Strains carrying Tn*5* inserts at this locus could not incite tumors on *Kalanchoe daigremontiana*, *Nicotiana rustica*, tobacco, or sunflower and had severely attenuated virulence on carrot disks. We termed the locus *pscA*, because the mutants that defined the locus were initially isolated as having an altered polysaccharide composition; they were nonfluorescent on media containing Leucophor or Calcofluor, indicating a defect in the production of cellulose fibrils. Further analysis showed that the *pscA* mutants produced little, if any, of the four species of exopolysaccharide synthesized by the wild-type strain. DNA hybridization analysis and genetic complementation experiments indicated that the *pscA* locus is not encoded by the Ti plasmid and that it is distinct from the previously described chromosomal virulence loci *chvA* and *chvB*. However, like *chvA* and *chvB* mutants, the inability of the *pscA* mutants to form tumors is apparently due to a defect in plant cell attachment. Whereas we could demonstrate binding of the wild-type strain to tobacco suspension cells, attachment of the *pscA* mutants was drastically reduced or completely absent.

Agrobacterium tumefaciens can cause disease on a wide range of plants (for a review, see references 17 and 32). The disease primarily manifests itself in the formation of tumors called crown galls. The basic disease mechanism is novel, in that it involves the exchange of genetic information between bacterium and plant. All virulent *A. tumefaciens* organisms harbor one of a diverse group of plasmids termed Ti (tumor-inducing) plasmids. During infection, a portion of the Ti plasmid, the T-DNA, is transferred to the plant cell, where it becomes integrated into the nuclear genome. Subsequent expression of the T-DNA oncogenes results in the synthesis of an auxin (34, 41, 42) and a cytokinin (1, 4), two classes of phytohormones that control plant cell growth and differentiation. It is the abnormal production of these phytohormones that causes the uncontrolled proliferation of the plant cells and consequent tumor formation.

In addition to the T-DNA sequences, there is a second region of the Ti plasmid, the *vir* region, that encodes genes that are involved in virulence (16, 24). This region is composed of at least seven genetic loci, *virA*, *virB*, *virC*, *virD*, *virE*, *virF*, and *virG* (20, 24, 39). Four of these loci, *virA*, *virB*, *virD*, and *virG*, have essential roles in tumor induction, while the functions of *virC*, *virE*, and *virF* are required on some plants but not others. Although the molecular details remain to be determined, it appears that the *vir* gene products directly process the T-DNA into an intermediate form which is then transferred to the plant (2, 25, 40).

Virulence functions are also encoded by the *Agrobacterium* chromosome (16). Two loci have been described to date, *chvA* and *chvB* (10, 11). Strains carrying mutations at these loci are defective in plant cell attachment and are avirulent. Biochemical analysis of the *chvB* mutants has indicated that they no longer synthesize a low-molecular-weight β-1,2-glucan that is synthesized by the wild-type strain (33). This polysaccharide might have a role in the attachment process, but the nature of that role remains to be determined.

In this paper, we describe a new locus involved in *A. tumefaciens* attachment and virulence. We have termed the locus *pscA* because the mutants that served to define the locus were originally isolated as having an altered polysaccharide composition. Genetic and DNA hybridization analyses indicate that *pscA* is not encoded by the Ti plasmid and that it is distinct from *chvA* and *chvB*.

MATERIALS AND METHODS

Bacterial strains, plasmids, and media. The strains of *A. tumefaciens* and *Escherichia coli* that were used in this study are described in Table 1. *A. tumefaciens* A6.1 was cured of its Ti plasmid as follows. Plasmid pUCD500 (15), which is incompatible with pTiA6, was conjugated into strain A6.1, and transconjugants were selected on media containing kanamycin. After repeated subculturing, individual colonies were tested for the loss of their Ti plasmids by plating them on octopine-BTB medium (21). A colony that could not catabolize octopine was subcultured without antibiotic selection to obtain a strain, A6C.1, that no longer carried pUCD500 (it was identified by its kanamycin-sensitive phenotype). Southern blot analysis was performed to confirm that strain A6C.1 had lost its Ti plasmid.

E. coli was maintained on modified LB medium (8). *A. tumefaciens* was grown on modified LB medium, M9 glucose medium (29), or modified AB minimal medium (43) in which sucrose (1%, final concentration) was substituted for glucose. The antibiotics used in solid media for *A. tumefaciens* were tetracycline (2 μg/ml), rifampin (100 μg/ml), kanamycin (50 μg/ml), and chloramphenicol (10 μg/ml), and the antibiotics used for *E. coli* were tetracycline (25 μg/ml), kanamycin (50 μg/ml), chloramphenicol (30 μg/ml), and ampicillin (100 μg/ml). When antibiotics were

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TABLE 1. Bacteria and plasmids

Bacterial strain or plasmid	Relevant properties[a]	Source or reference
A. tumefaciens		
A6	Wild type	A. Binns
A6.1	Rif^r derivative of A6	This study
A6.1d	A6.1::Tn*5* *pscA* avirulent[b]	This study
A6.1e	A6.1::Tn*5* *pscA* avirulent[b]	This study
A6.1g	A6.1::Tn*5* *pscA* avirulent[b]	This study
A6.1h	A6.1::Tn*5* *pscA* avirulent[b]	This study
A6.1i	A6.1::Tn*5* *pscA* avirulent[b]	This study
A6.1d3	A6.1::Tn*5* *pscA* avirulent[b]	This study
A6.1j	A6.1::Tn*5* avirulent	This study
A6C.1	Ti plasmidless derivative of A6.1	This study
A348	Virulent; C58 chromosomal background	11
E. coli		
HB101	*recA hsdR hsdM pro* Str^r	7
MM294	*hsdR thi endA*	M. Messelson
S17-1	MM294 [RP4-2 (Tc::Mu) (Km::Tn*7*)] Tra (IncP) Tp^r Sm^r	35
SF800	*polA thy* Nal^r	18
Plasmids		
pLAFR1	IncP Tc^r	14
pRK2013	ColE1::pRK2 Tra$^+$ Km^r	9
pBR325	Ap^r Cm^r Tc^r	6
pSUP2021	Tn*5* Mob$^+$ Ap^r Cm^r Km^r	35
pHoHo1	Tn*3*::*lacZ tnpA* Tnp$^+$ Ap^r	38
pSShe	*tnpR tnp* Cm^r	38
pCD523	pLAFR1 containing *chvA* and *chvB* loci from *A. tumefaciens* C58; Tc^r	11
pUCD500	*ori/inc* of pTiC58; Km^r	15
pJ4.0	pLAFR1 containing *pscA* locus of *A. tumefaciens* A6.1j; Tc^r	This study
pJ2.0	pLAFR1 containing *pscA* locus of *A. tumefaciens* A6.1j; Tc^r	This study
pJOYe5.1	pBR325 containing most of *pscA* locus subcloned from pJ4.0	This study

[a] Abbreviations: Ap, ampicillin; Cm, chloramphenicol; Km, kanamycin; Nal, nalidixic acid; Rif, rifampin; Sm, streptomycin; Tc, tetracycline; Tp, trimethoprim.

[b] Avirulent on *K. daigremontiana*, *N. rustica*, tobacco, and sunflower and severely attenuated in virulence on carrot disks.

used in liquid media, their concentrations were decreased by half. Leucophor (1.5 ml/liter) or Calcofluor (0.2 mg/liter) was added to media to test *A. tumefaciens* for an altered polysaccharide composition.

Plant cells and media. Tobacco cells (*Nicotiana tabacum* L. cv. bright yellow 2; obtained from G. An, Washington State University, Pullman, Wash.) were maintained in liquid Linsmaier-Skoog medium (27) supplemented with 0.2 mg of 2,4-dichlorophenoxyacetic acid (2,4-D) per liter. The cultures were grown at 28°C on a gyratory shaker and subcultured each week with a 4% (vol/vol) inoculum.

Restriction endonucleases and chemicals. Restriction endonucleases were obtained from New England BioLabs, Inc., Beverly, Mass., and used according to the supplier. Leucophor BSB Liquid was a gift from Sandoz Chemicals, Charlotte, N.C., and Calcofluor was purchased from Polysciences, Inc., Warrington, Pa. Radiolabeled chemicals were purchased from New England Nuclear Corp., North Billerica, Mass.

Bacterial transformations and conjugations. *A. tumefaciens* was transformed with plasmid DNA as described by Holsters et al. (19). *E. coli* was transformed by the procedures of Mandel and Higa (28). Bacterial conjugations were carried out on Millipore GS filters (Millipore Corp., Bedford, Mass.) incubated on LB agar for 1 to 2 days at 30°C.

DNA isolations. Plasmid DNA was isolated by the procedure of Birnboim and Doly (5). Total DNA was isolated as previously described (8). In some cases, DNA preparations were further purified by using cesium chloride-ethidium bromide density gradients.

Transposon mutagenesis. *A. tumefaciens* A6.1 was mutagenized with Tn*5* by using pSUP2021 (35). This plasmid can be mobilized into *A. tumefaciens* at a high frequency, but it cannot replicate in this host. Thus, *A. tumefaciens* transconjugants that are kanamycin resistant presumably carry Tn*5* inserts. *E. coli* S17-1(pSUP2021) was therefore mated with *A. tumefaciens* A6.1, and the mutagenized recipients were selected on LB agar containing rifampin and kanamycin. Leucophor was included in the media to screen for mutants defective in cellulose production (31). The mutants of interest were plated on media containing chloramphenicol to determine whether other portions of pSUP2021 were present, and in all cases the mutants were chloramphenicol sensitive.

Cosmid pJ4.0 was mutagenized with the *lacZ* fusion transposon pTn*3HoHo1* as previously described (38).

Assay of bacterial attachment to plant cells. Scanning electron microscopy and a bacterial filter assay (10) were used to measure *A. tumefaciens* attachment to plant cells. For scanning electron microscopy, bacterial strains were grown overnight in AB sucrose medium, washed, and added to tobacco suspension cells that had been subcultured for 3 days. Final bacterial and plant cell concentrations were approximately 10^7 and 10^6/ml, respectively. The cell mixture was incubated overnight at 28°C on a gyratory shaker (40 rpm), filtered through Miracloth (Calbiochem-Behring, La Jolla, Calif.), and washed with 100 ml of fresh plant growth

medium to remove unattached bacteria. The samples were fixed in 1% gluteraldehyde in aqueous solutions of 30, 50, 70, and 95% ethanol (10 min each) and 100% (four times for 10 min each), ethanol dried in a Bomar critical point drying apparatus with liquid CO_2, and attached to aluminum stubs (Ted Pella Co., Tustin, Calif.). Finally, the preparations were coated for 9 min with 100% gold (coating thickness was approximately 300 Å [30 nm]) and viewed on an ETEC Autoscan U-1 scanning electron microscope at an accelerating voltage of 20 kV.

For the filter assay, *A. tumefaciens* strains were radiolabeled by growth overnight in AB sucrose medium supplemented with 1 μCi of L-(3-^{14}C)tryptophan (55 mCi/mmol) per ml of medium. The cells were then washed and resuspended to approximately 10^9 cells per ml in fresh AB sucrose medium. Tobacco suspension cells were subcultured and after 3 days were harvested by filtration through Miracloth. The cells were washed three times with 50 ml of fresh plant medium and then resuspended in fresh plant medium at a concentration of approximately 10^6/ml. To begin the assay, the bacteria were added (5×10^6 bacteria per ml, final concentration) to the suspension of plant cells, and the cell suspensions were placed on a gyratory shaker (40 rpm) at 28°C. At various times, 2-ml samples were quickly filtered through Miracloth (free bacteria pass through the filter while plant cells do not); the plant cells and adhering bacteria were resuspended in 20 ml of fresh plant growth medium, and the sample was filtered a second time. The number of bacteria retained on the filters was determined by scintillation counting.

Molecular cloning. Cosmids pJ2.0 and pJ4.0 were isolated from a library that had been constructed from *A. tumefaciens* A6.1j. This mutant, which was derived from a Tn*5* mutagenesis mating, was altered in polysaccharide composition (it formed dark colonies on LB medium supplemented with Leucophor) but was virulent on all plants tested (*N. tabacum*, *Nicotiana rustica*, *Kalanchoe daigremontiana*, sunflower, and carrot). The cosmid library was constructed using standard procedures (29). A partial *Eco*RI digest of total DNA was size fractionated on agarose gels, and fragments of 15 to 30 kilobases (kb) were collected and ligated to *Eco*RI-cut pLAFR1 DNA (14). The ligated DNA was then packaged into bacteriophage and transduced into *E. coli* S17-1. The transductants were plated on LB medium containing tetracycline at a density of approximately 200 colonies per plate to give five independent partial libraries. Cosmids that would complement the nonfluorescent avirulent mutants were identified by mating each mutant with each partial library and then screening the transconjugants on LB medium supplemented with Leucophor, tetracycline, and rifampin. Fluorescent colonies were obtained with two of the libraries. One cosmid from each group, termed pJ2.0 and pJ4.0, was utilized in the experiments presented here.

Plasmid pJOYe5.1 was constructed by cloning the 5.1-kb *Eco*RI fragment of pJ4.0 into the *Eco*RI site of pBR325.

DNA filter hybridizations. DNA fragments were size fractionated on agarose gels and transferred to nitrocellulose (0.45 μm; Schleicher & Schuell, Inc., Keene, N.H.) by the method of Southern (37). DNA hybridization probes were radiolabeled in vitro with [α-^{32}P]dCTP by the nick translation procedure (29). Hybridization and washing conditions have been described previously (8).

Virulence testing. Bacterial strains were grown in liquid LB medium to early stationary phase, washed, and suspended in water. Plants were wounded with a sterile toothpick, and the sites were inoculated with 10 to 50 μl of the bacterial suspensions. Tumor production was scored after approximately 6 weeks.

Preparation and fractionation of EPS. Cultures (100 ml) were grown to early stationary phase (48 h) in AB sucrose medium at 30°C. The cells were removed by centrifugation (40,000 × *g*, 10 min), and the supernatants were dialyzed extensively against water (containing 0.01% phenol) at 7°C to remove the sucrose and other low-molecular-weight compounds. The dialysis tubing used had a molecular mass cutoff of 1,000 daltons. The dialyzed samples were lyophilized and suspended in 2 ml of 1 mM KCl, and total hexose was determined as glucose equivalents by the phenol-sulfuric acid method (3). Samples of the exopolysaccharide (EPS) were fractionated on a column (1.5 cm by 30 cm) of DEAE Bio-Gel A (Bio-Rad Laboratories, Richmond, Calif.); the samples were loaded and eluted, first with 150 ml of 1 mM KCl and then with a 1 to 100 mM gradient of KCl (600 ml). Fractions of 6.5 ml were collected and assayed for total hexose to locate the polysaccharide peaks.

RESULTS

Isolation of *A. tumefaciens* mutants altered in polysaccharide composition and virulence. *A. tumefaciens* A6.1 was mutagenized with Tn*5* by using pSUP2021, and the mutants were screened for an altered polysaccharide composition by plating on LB medium supplemented with either Leucophor or Calcofluor. These compounds are stilbene brighteners which bind to cellulose and other β-linked polysaccharides and fluoresce when irradiated with UV light (22). *A. tumefaciens* plated on media supplemented with either brightener fluoresces a green-blue color. Matthysee et al. (31) have reported that this fluorescence is due primarily to the production of cellulose by the bacteria.

After mutagenesis, nonfluorescent or weakly fluorescent, dark colonies were observed at a frequency of about 5×10^{-3}. (Nonmutagenized cells also produced dark colonies at a relatively high frequency, approximately 5×10^{-4}. The characterization of these spontaneous mutants will be reported elsewhere.) A total of 13 dark mutants from two separate matings were tested for virulence on *K. daigremontiana* leaves, and 6 were found to be avirulent. Mutants A6.1d, A6.1e, A6.1g, A6.1h, and A6.1i came from one mating (a total of nine mutants were tested), and avirulent mutant A6.1d3 came from a second mating (a total of four mutants were tested). Additional testing indicated that all of the mutants affected in virulence on *K. daigremontiana* were avirulent on *N. tabacum*, *N. rustica*, and sunflower and had severely attenuated virulence on carrot disks (after an extended period of time, a few small tumors developed on some of the inoculated disks). The avirulent phenotype of these mutants did not result from simple auxotrophic defects since all of the mutants could grow in liquid M9 glucose minimal medium with generation times approximating that of the wild-type strain. The mutants also grew on glucose minimal plates, but the colonies developed more slowly and attained a smaller final size than those formed by the wild-type strain.

Complementation of virulence mutants. A cosmid library constructed from *A. tumefaciens* A6.1j (a dark Tn*5* mutant that was virulent on all plants tested; see Materials and Methods) was used to screen for cosmids that could complement the dark phenotype of the virulence mutants. Two cosmids were identified, pJ2.0 and pJ4.0, that could restore bright green-blue fluorescence in all of the mutants. These

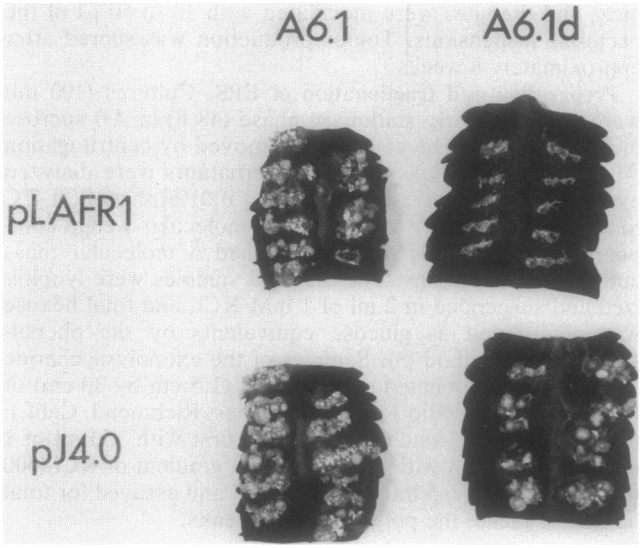

FIG. 1. Complementation of virulence. *A. tumefaciens* A6.1 and A6.1d carrying either pLAFR1 or pJ4.0 were inoculated onto *K. daigremontiana* leaves, and virulence was assessed after approximately 6 weeks.

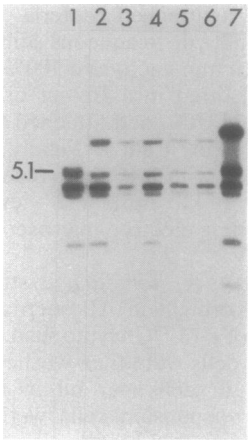

FIG. 2. Physical location of Tn*5* inserts. Samples of total DNA from the wild-type and mutant strains of *A. tumefaciens* were digested with *Eco*RI and subjected to Southern blot analysis with ^{32}P-labeled pJ4.0 as a probe. DNA samples were from *A. tumefaciens* A6.1, A6.1d, A6.1e, A6.1g, A6.1h, and A6.1i (lanes 1 through 6, respectively). Lane 7 contains *Eco*RI-digested pJ4.0 DNA. The largest fragment in this digest is the cloning vehicle, pLAFR1. The position of the 5.1-kb *Eco*RI fragment is indicated (see text).

cosmids were also found to restore virulence in the mutants (Fig. 1). DNA hybridization analysis indicated that both cosmids had *Eco*RI fragments of 1.7, 3.7, and 5.1 kb in common (data not shown). Neither plasmid contained the Tn*5* insert present in strain A6.1j.

Physical location of Tn*5* inserts in virulence mutants. The Tn*5* inserts in the avirulent mutants were presumably located in the region of the genome common to pJ4.0 and pJ2.0, that is, in either the 1.7-, 3.7-, or 5.1-kb *Eco*RI fragments cloned in these cosmids. To determine whether this was true, we isolated total DNAs from the mutants, digested the samples with *Eco*RI, prepared Southern blots, and hybridized them with ^{32}P-labeled pJ4.0. If Tn*5* had inserted into one of the *Eco*RI fragments present in the pJ4.0 region of the genome, that fragment should have been missing and in its place should have been a fragment 5.4 kb larger in size (Tn*5* is 5.4 kb and does not have any *Eco*RI restriction sites). This was the case (Fig. 2). The 5.1-kb *Eco*RI fragment present in the wild-type strain was missing in each of the mutants and in its place was a new fragment of about 10.5 kb. These 10.5-kb fragments hybridized with pSUP2021, the mutagenesis vector containing Tn*5*, and with pJOYe5.1, a plasmid containing the 5.1-kb *Eco*RI fragment from pJ4.0 (data not shown). From these experiments, we concluded that the Tn*5* inserts in each of the mutants was located in the 5.1-kb *Eco*RI fragment from the pJ4.0 region of the genome.

Additional DNA hybridization experiments allowed us to map the sites of the Tn*5* inserts with greater precision (Fig. 3). All of the inserts fell within a 1.5-kb section of DNA. The physical locations of the inserts in A6.1e, A6.1g, and A6.1i were very close, if not identical. Since all of these mutants came from one mutagenesis mating, it was likely that they were siblings. The same situation held for the inserts in strains A6.1d and A6.1h.

Mapping the cosmid pJ4.0 region that complements virulence mutants. Transposon Tn*3HoHo1* was used to generate insertion mutations in cosmid pJ4.0, and then the mutagenized cosmids were tested to see if they could still complement mutants A6.1d, A6.1e, and A6.1d3. The results are summarized in Fig. 3. Cosmids containing Tn*3HoHo1* inserts 3A, 4A, 4C, 5C, and 7A could complement all three mutants (the mutants carrying these plasmids formed bright green-blue colonies on LB Leucophor plates and were virulent on *K. daigremontiana*), while inserts 2D, 5A, and 8A abolished complementation. These data indicated that the section of pJ4.0 that complemented the Tn*5* mutants was the same region of the genome into which the Tn*5*s had inserted. Thus, we concluded that the Tn*5* inserts were the cause of the altered phenotypes of the dark virulence mutants. In addition, the data suggested that the virulence region defined by the Tn*5* inserts consisted of one transcriptional unit that was approximately 3 kb in size, the distance between inserts 4C and 3A. Since the mutants used to define

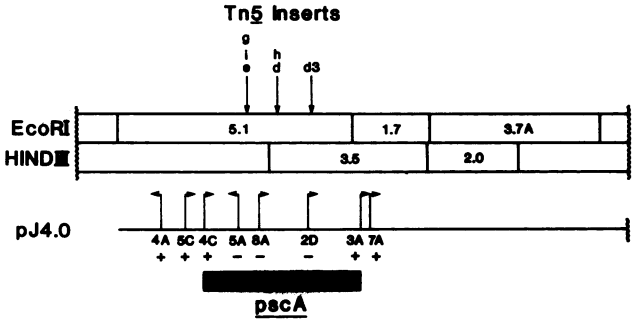

FIG. 3. Map positions of Tn*5* inserts and region of pJ4.0 that complements the nonfluorescent avirulent mutants. The sites of the Tn*5* inserts in *A. tumefaciens* mutants A6.1d, A6.1e, A6.1g, A6.1h, A6.1i, and A6.1d3 are shown (indicated as d, e, etc.), as are the sites of the Tn*3HoHo1* inserts in a number of pJ4.0 derivatives (4A, 5C, etc.). The arrowheads indicate the 5′ to 3′ orientation of the *lacZ* gene in the Tn*3HoHo1* inserts. Also indicated is whether a pJ4.0 derivative containing a particular Tn*3HoHo1* insert could (+) or could not (−) complement the nonfluorescent, avirulent phenotype of the Tn*5* mutants. The numbers within the restriction fragments indicate the approximate sizes of those fragments in kilobases.

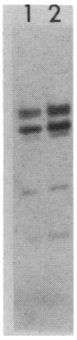

FIG. 4. Detection of pJ4.0 sequences in Ti plasmid-containing and Ti plasmidless strains of *A. tumefaciens*. Total DNA samples isolated from *A. tumefaciens* A6.1 (lane 1) and its Ti plasmid-cured derivative A6C.1 (lane 2) were digested with *Eco*RI and subjected to Southern blot analysis using ^{32}P-labeled pJ4.0 as a probe.

this region were originally isolated as having an altered polysaccharide composition, we termed the locus *pscA*.

Transposon Tn*3HoHo1* can generate both transcriptional and translational fusions to *lacZ* (38). Thus, the Tn*3HoHo1* inserts were also used to determine the direction of transcription of the *pscA* locus. The pJ4.0 derivatives containing Tn*3HoHo1* inserts 2D, 5A, and 8A were conjugated into *A. tumefaciens* A6.1, and the transconjugants were plated on LB medium supplemented with X-Gal (5-bromo-H-chloro-3-indolyl-β-D-galactopyranoside), the chromogenic substrate that is cleaved by β-galactosidase to produce a blue pigment. The transconjugant carrying the cosmid with insert 5A formed dark blue colonies, whereas the transconjugants containing the cosmids with inserts 2D and 8A formed very pale blue colonies. These data indicate that the *pscA* locus is transcribed from right to left (Fig. 3).

***pscA* locus not encoded by Ti plasmid.** To determine whether the *pscA* locus was encoded by the Ti plasmid, we asked whether the DNA sequences cloned in cosmid pJ4.0 were of Ti plasmid origin. To do this, we prepared Southern blots of *Eco*RI-digested total DNA from strains A6.1 and A6C.1 and hybridized them with ^{32}P-labeled pJ4.0. The data show that the pJ4.0 sequences were present in both the Ti plasmid-containing and Ti plasmid-cured strains (Fig. 4). We also could not detect any DNA hybridization between pJ4.0 and blots prepared from *Eco*RI-digested pTiA6 DNA (data not shown). From these data, we conclude that the *pscA* locus is not encoded by the Ti plasmid.

***pscA* locus not related to either *chvA* or *chvB*.** Douglas et al. (11) have previously defined two chromosomal loci of *A. tumefaciens*, *chvA* and *chvB*, that are required for virulence. We conclude that *pscA* is not related to either *chvA* or *chvB* for the following reasons. First, cosmid pCD523, which contains the *chvA* and *chvB* loci, did not complement the phenotypes of our *pscA* mutants. Second, although both pJ4.0 and pJOYe5.1 showed detectable hybridization with total DNA isolated from *A. tumefaciens* A348, the parent strain used by Douglas et al. (11), neither pJ4.0 nor pJOYe5.1 displayed detectable hybridization with pCD523, even when the hybridization was carried out at low stringency (Fig. 5).

Attachment to plant cells affected by *pscA* mutations. One explanation for the avirulent phenotype of the *pscA* mutants was that they might not have been able to bind efficiently to plant cells. To test this hypothesis, we monitored *A. tumefaciens* attachment to plant cells in two ways. First, wild-type and mutant bacteria were incubated with tobacco suspension cells, and after washing, the mixture was examined by scanning electron microscopy (Fig. 6). When the plant cells were incubated with the wild-type strain, bacterial attachment was observed. In contrast, plant cells incubated with mutant A6.1d were essentially free of attached bacteria. Second, we incubated radiolabeled bacteria with tobacco cells and determined attachment by a filtration assay. Again, the results indicated that the wild-type strain could bind to the plant cells, whereas binding by the *pscA* mutants was either drastically reduced or completely absent (Table 2).

Synthesis of EPS affected by *pscA* locus mutations. The *pscA* mutants did not fluoresce on media containing Leucophor or Calcofluor, indicating that the mutants were defective in the production of cellulose. This, however, was probably not the only defect in the *pscA* mutants, since Matthysee (30) had reported that cellulose-negative mutants were virulent. One possibility was that the production of other polysaccharides was also affected in the *pscA* mutants. This was the case. When *A. tumefaciens* A6.1 was grown in AB sucrose minimal medium to early stationary phase, it routinely produced approximately 30 mg of EPS per 100 ml of culture supernatant, whereas the *pscA* mutants produced less than 5% of that value (Table 3). Fractionation of the wild-type EPS on a column of DEAE Bio-Gel A (Fig. 7) separated the polysaccharides into at least four species, one large neutral fraction and three acidic fractions. Fractionation of the EPS from mutant A6.1d indicated that the small amount of material recovered eluted with the neutral fraction (data not shown).

DISCUSSION

By isolating mutants that were altered in polysaccharide composition and then screening for those affected in virulence, we have identified a new genetic locus in *A. tumefaciens* that has a critical role in tumor induction. We have termed the locus *pscA* (polysaccharide composition). Strains carrying mutations at the *pscA* locus do not incite tumors on *K. daigremontiana*, *N. rustica*, tobacco, or sunflower, and they have severely attenuated virulence on carrot disks. The *pscA* locus is not encoded by the Ti plasmid, and it is distinct from the previously described chromosomal virulence loci *chvA* and *chvB* (10, 11). How-

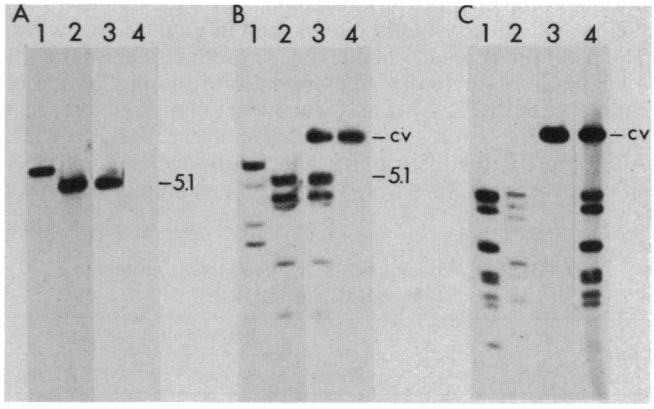

FIG. 5. Nonhomology of *pscA* with either *chvA* or *chvB*. Total DNA isolated from *A. tumefaciens* A348 (lanes 1) or A6.1 (lanes 2) and purified preparations of pJ4.0 (lanes 3) or pCD523 (lanes 4) were digested with *Eco*RI, Southern blots were prepared, and the filters were hybridized with ^{32}P-labeled pJOYe5.1 (A), pJ4.0 (B), or pCD523 (C). The positions of the cloning vehicle (cv) and the 5.1-kb *Eco*RI fragment from pJ4.0 are indicated.

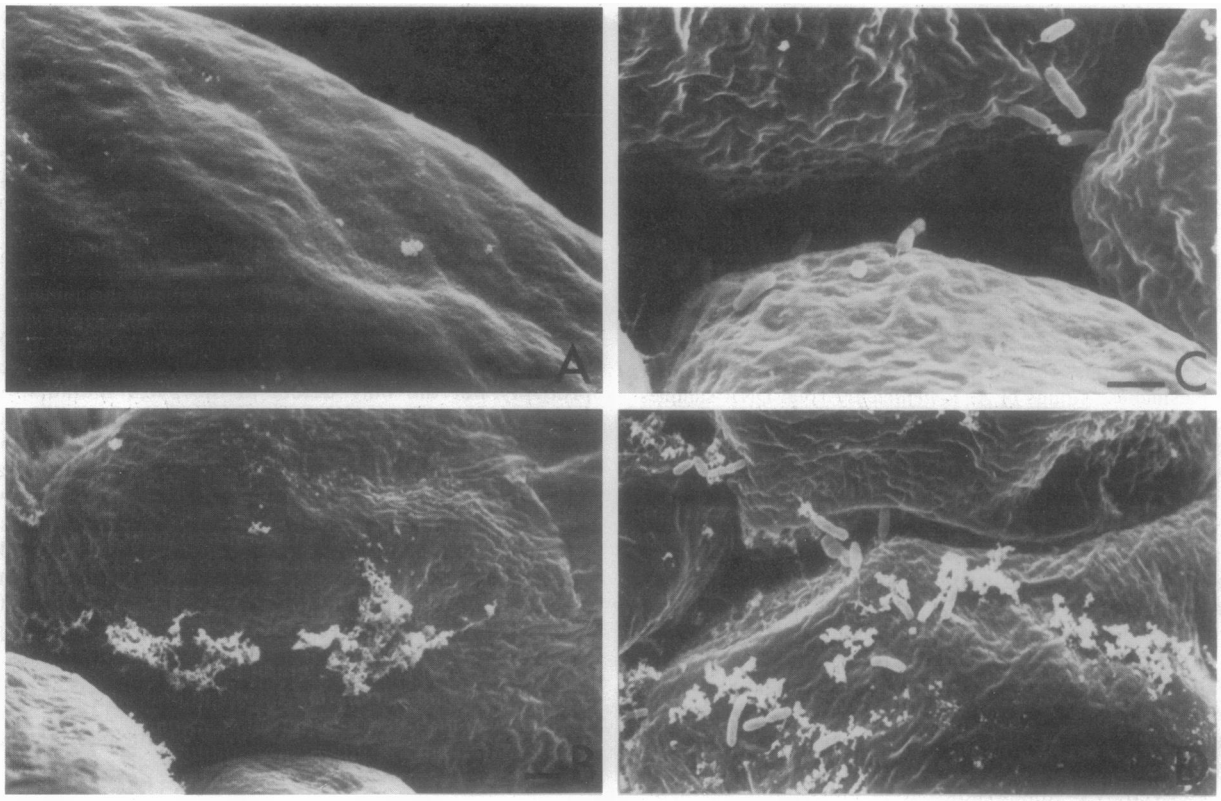

FIG. 6. Attachment of *A. tumefaciens* to plant cells. *A. tumefaciens* A6.1d (A and B) and A6.1 (C and D) were incubated with tobacco suspension cells and viewed by scanning electron microscopy as described in Materials and Methods. Bar, 2 μm.

ever, like *chvA* and *chvB* mutants, the inability of *pscA* mutants to incite tumors appears to result from a defect in plant cell attachment.

The function(s) of the *pscA* gene product(s) and the precise nature of the molecular defects caused by the *pscA* mutations are not yet known. It is clear, however, that the locus has an important role in polysaccharide production. The *pscA* mutants did not produce cellulose fibrils, as judged by the fact that they were nonfluorescent on media containing Leucophor or Calcofluor, and they did not secrete the normal complement of extracellular polysaccharides. Such pleiotropic effects could be explained in a number of ways. The *pscA* locus might encode a gene product(s) required for a synthetic or secretory step common to the production of cellulose and EPS, or it may encode a gene product(s) that regulates the expression of other polysaccharide genes. Alternatively, the locus might affect polysaccharide composition in less direct ways. It may, for example, encode a membrane protein that imparts a general structure required for polysaccharide synthesis. Additional work is needed to distinguish between the various possibilities.

It also remains to be determined whether any of the observed changes in polysaccharide composition are directly responsible for the attachment-defective, avirulent phenotype of the *pscA* mutants. It appears certain that the cellulose-defective phenotype of the mutants is not directly involved. Douglas et al. (10, 11) have shown that the attachment-defective *chvA* and *chvB* mutants produced cellulose fibrils and thus, the presence of cellulose fibrils does not ensure effective attachment. More directly, Matthysee (30) has isolated mutants that were defective in the production of cellulose fibrils and has shown that the mutants were still able to attach to carrot cells, albeit with altered kinetics, and that they could still form tumors on *N. tabacum* and *Bryophyllum daigremontiana*. Cellulose fibrils, therefore, do not appear to be required for virulence. Our results are

TABLE 2. Attachment of *A. tumefaciens* strains to tobacco suspension cells

Strain	% Bacteria attached[a]	
	0 min	120 min
A6.1	0.4 ± 0.2	56 ± 3.0
A6.1d	0.4 ± 0.3	0.8 ± 0.1
A6.1e	ND	0.4 ± 0.1
A6.1d3	ND	0.5 ± 0.2

[a] Bacterial attachment was determined as described in Materials and Methods. Numbers are the mean ± the standard deviation of the mean of three measurements. ND, Not determined.

TABLE 3. Production of EPS by wild-type *A. tumefaciens* and *pscA* mutants[a]

Strain	Amt of EPS[b]	Amt of cell protein[c]	Ratio of EPS to cell protein
A6.1	32.3	31.0	1.04
A6.1d	1.2	35.0	0.03
A6.1e	1.1	31.0	0.04

[a] Cells were grown in AB sucrose minimal medium, and the EPS was prepared and quantified as described in Materials and Methods.

[b] Amount of EPS recovered from a 100-ml culture.

[c] Amount of cell protein recovered from a 100-ml culture. Protein was determined as previously described (23).

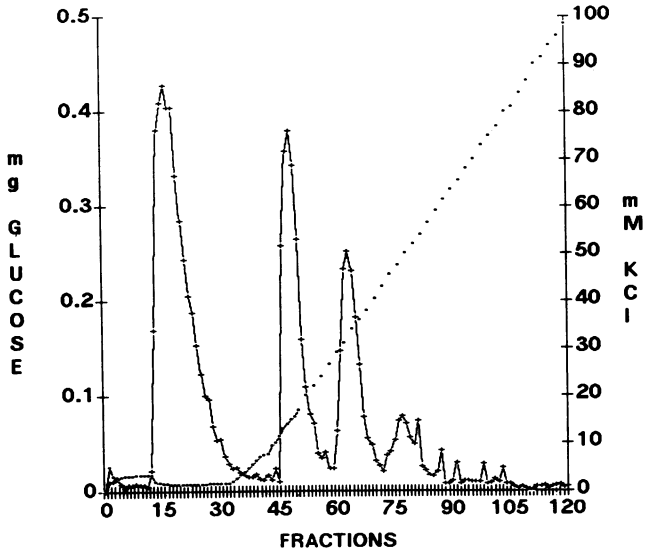

FIG. 7. Fractionation of EPS from wild-type *A. tumefaciens*. EPS was prepared and fractionated on a column of DEAE Bio-Gel A as described in Materials and Methods.

consistent with this conclusion; about half of the Leucophor or Calcofluor nonfluorescent mutants that we isolated appeared to be unaltered in their ability to incite tumors.

Perhaps the inability of the *pscA* mutants to produce a normal complement of EPS is responsible for their attachment-defective, avirulent phenotype. In this regard, it is relevant to note that Puvanesarajah et al. (33) have shown that *chvB* mutants do not synthesize β-1,2-glucan, a polysaccharide that is located both in the periplasmic space and extracellularly in wild-type *A. tumefaciens*. Since no other changes in polysaccharides could be detected in the *chvB* mutants, the investigators suggested that the β-1,2-glucan might have a critical role in attachment and virulence. The general deficiency in EPS production by the *pscA* mutants raises the possibility that they too are defective in the synthesis of this polysaccharide. The nature of the role of glucan in attachment and virulence is not yet clear. Any assigned roles must take into account, however, the fact that *chvA* mutants appear to synthesize this polysaccharide but are still defective in attachment.

Leigh et al. (26) and Finan et al. (13) recently isolated mutants of *Rhizobium meliloti* that were defective in the production of the major acidic EPS, succinoglycan. These *exo* mutants formed aberrant nodules on alfalfa; the nodules were devoid of bacteroids, lacked infection threads, and did not fix nitrogen. Since *A. tumefaciens* also produces succinoglycan (36), it was of interest to determine whether the *pscA* locus was related to any of the *exo* loci of *R. meliloti*. We have found that it is (J. Marks, T. Lynch, J. Karlinsey, and M. Thomashow, manuscript in preparation). Specifically, it is related to the *exoC* locus of *R. meliloti*. Mutations at *exoC*, like those at *pscA*, have pleotropic effects (e.g., they are resistant to nine different bacteriophages). Thus, Leigh et al. (26) have suggested that mutations at *exoC* might affect EPS production in some indirect way, such as affecting general membrane or cell surface structure.

The finding that *pscA* and *exoC* are related is not the first example of an *A. tumefaciens* virulence gene(s) being related to an *R. meliloti* symbiosis gene(s). Dylan et al. (12) have previously shown that *R. meliloti* has two genetic loci, termed *ndvA* and *ndvB* (nodule development), that are functionally and structurally related to *chvA* and *chvB*, respectively. Strains carrying mutations at *ndvA* and *ndvB* form defective nodules that are very similar to those formed by *exo* mutants.

In the previous studies of Garfinkel and Nester (16) and Douglas et al. (11), approximately 23,000 Tn*5*-induced mutants of *A. tumefaciens* were tested for virulence, but the *pscA* locus was not identified. In addition, Matthysee (30) isolated seven Tn*5*-induced mutants of *A. tumefaciens* that were Calcofluor dark, but apparently none of them was equivalent to a *pscA* mutant, as they were all virulent. The question raised then is why *pscA* mutants were not isolated in these studies. It seems unlikely that it was merely due to chance. A more likely possibility involves the initial screening procedures used. In the previous studies, the mutagenized bacteria were first plated on minimal media, whereas in our study, we plated the mutagenized cells on complex media. Although the *pscA* mutants grow with approximately the same doubling time as the wild-type strain in liquid glucose minimal medium, colony development by the mutants was considerably slower than that of the parent strain on solid minimal medium. We do not know if this reflects an actual difference in growth rate on the plates or if it is due to an altered colony morphology caused by the change in polysaccharide composition (one might expect that the colonies formed by the mutants would be less hydrated and thus smaller than those formed by the wild-type strain). Regardless, it might account for the *pscA* mutants being passed over in a screen made on minimal medium.

A final point regards additional virulence loci. We have isolated spontaneous mutants of *A. tumefaciens* A6.1 that do not fluoresce on media containing Leucophor, and we have found that some of them are avirulent or have markedly attenuated virulence on *K. daigremontiana* (H. Malkawi and J. Marks, unpublished results). Genetic complementation studies suggest that the mutations do not map to *chvA*, *chvB*, or *pscA*, and DNA hybridization experiments indicate that the complementing plasmids do not contain Ti plasmid sequences. We, therefore, anticipate that a further characterization of these mutants will define additional virulence loci that are not encoded by the Ti plasmid.

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