MOLECULAR PLANT-MICROBE INTERACTIONS
Vol. 2, No. 1, pp. 11-16, 1989

chvA Locus May Be Involved in Export of Neutral Cyclic β-1,2-Linked D-Glucan from *Agrobacterium tumefaciens*

Kevin P. O'Connell and Jo Handelsman

Department of Plant Pathology, University of Wisconsin, 1630 Linden Drive, Madison 53706 U.S.A.
Received 7 July 1988. Accepted 22 November 1988.

Extracellular and intracellular neutral β-1,2-linked D-glucan content was determined in avirulent, attachment-deficient mutants of *Agrobacterium tumefaciens* that map in the *chvA* locus. *chvA* mutants contained approximately the same amount of intracellular glucan as cells of the virulent control strain A759, but released into the culture medium only 2% of the glucan released by strain A759. Introduction of a cosmid carrying the wild-type *chv* region restored attachment and virulence and restored extracellular glucan production to *chvA* mutant A2505. Exogenous glucan did not enhance or inhibit attachment or tumorigenesis of the virulent control strain or the *chvA* or *chvB* mutants. Our results suggest that the *chvA* locus is involved in the export of glucan from the cell and that export may be required for tumorigenesis.

Additional keywords: chromosomal virulence genes, crown gall, extracellular polysaccharide, nuclear magnetic resonance.

Agrobacterium tumefaciens causes crown gall, a neoplastic disease of dicotyledonous plants. The bacterium transfers a piece of a large plasmid, the Ti plasmid, to the host genome during infection. This process is dependent on genes carried on the Ti plasmid and on a number of chromosomal loci (Douglas *et al.* 1982; Cangelosi *et al.* 1987; Thomashow *et al.* 1987). Two linked chromosomal loci, *chvA* and *chvB*, have been implicated in virulence and in the attachment of *A. tumefaciens* to plant cells. Attachment is regarded as one of the early steps in the transformation of susceptible plant tissue. Mutations in *chvB* are pleiotropic: *chvB* mutants are avirulent on a variety of hosts, defective in attachment to plant cells, and lack flagella (Douglas *et al.* 1982). In addition, Puvanesarajah *et al.* (1985) have reported that *chvB* mutants fail to synthesize a neutral polysaccharide, cyclic β-1,2-linked D-glucan. Although the presence of the glucan in the supernatants of *A. tumefaciens* was established more than four decades ago (McIntire *et al.* 1942) and its production and structure have been well studied (Hodgson *et al.* 1945; Putnam *et al.* 1950; Gorin *et al.* 1961; Gorin and Mazurek 1973; Gorin 1981), only recently has the glucan been implicated in tumorigenesis by *A. tumefaciens*.

In this paper, we demonstrate that *chvA* mutants are deficient in the production of recoverable neutral glucan and that they almost entirely lack extracellular neutral glucan. Furthermore, we present data that suggest that the neutral glucan is not involved directly in attachment. The possible roles of glucan in tumorigenesis are discussed.

MATERIALS AND METHODS

Bacterial strains and culture media. The strains used in this study are listed in Table 1. Strain A759 (Bradley *et al.* 1984) is a virulent, nonmotile derivative of strain A723 (Garfinkel and Nester 1980). Strains of *A. tumefaciens* were grown for carbohydrate analysis in AB broth (Chilton *et al.* 1974) at 28° C with vigorous shaking. Strains of *Escherichia coli* were grown in Luria-Bertani (LB) broth (Maniatis *et al.* 1982) at 37° C. Antibiotics were added at the following concentrations: kanamycin (Km), 25 μg/ml; carbenicillin (Cb), 100 μg/ml; and tetracycline (Tc), 10 μg/ml.

Preparation of extracellular neutral glucan. Glucan was isolated according to the method of Puvanesarajah *et al.* (1985), with the following modifications. Two-day-old cultures were centrifuged at 15,300 $\times$ *g* for 15 min, and the pellets were stored at −20° C. The culture supernatants were lyophilized, resuspended in distilled water, and dialyzed exhaustively against deionized water in dialysis tubing with a molecular weight exclusion limit of 1,000 Da. The material retained by the tubing was lyophilized, resuspended in distilled water, and then poured while stirring into two volumes of ethanol. The mixture was placed at −20° C for 24 hr, and the precipitated material was removed by centrifugation at 15,300 $\times$ *g* for 15 min and discarded. Most of the ethanol was removed from the supernatant under vacuum at approximately 40° C, and the remaining solution was lyophilized. The lyophilized material was resuspended in distilled water and passed through a DEAE-cellulose column (2.6 $\times$ 18.5 cm), which was eluted with distilled water until no hexose-containing material could be detected in the effluent by the phenol-sulphuric acid method

Table 1. Origin of bacterial strains

Strain	Genotype or phenotype[a]	Reference
A. tumefaciens		
A759	C58 chromosome, pTiB$_6$806, Vir$^+$, Fla$^-$	Garfinkel and Nester (1980); Bradley *et al.* (1984)
A2505	*chvA*::Tn*5*, Km^r	Douglas *et al.* (1985)
ME66	*chvA*::(Tn*3*::HoHo1), Cb^r	Douglas *et al.* (1985)
ME45	*chvA*::(Tn*3*::HoHo1), Cb^r	Douglas *et al.* (1985)
A1038	*chvB*::Tn*5*	Douglas *et al.* (1982)
A2505(pCD523)	Vir$^+$	This study
E. coli		
HB101	*thr leu thi recA hsdR hsdM pro* Str^r	Boyer and Roulland-Dussoix (1969)
DH1	F$^-$ *recA1 endA1 gyrA96 thi1 hsdR17 supE44*	Hanahan (1983)

[a] Cb^r = carbenicillin resistant; Km^r = kanamycin resistant; Str^r = streptomycin resistant; Vir$^-$ = avirulent; Vir$^+$ = virulent; Fla$^-$ = lacks flagella.

© 1989 The American Phytopathological Society

(Ashwell 1966). The eluted material was pooled, lyophilized, and weighed.

Preparation of intracellular neutral glucan. The cell pellets that remained after removal of the culture supernatants were washed three times in phosphate-buffered saline (PBS) (per liter, 8.76 g of NaCl, 5.22 g of K_2HPO_4, 1.36 g of KH_2PO_4, pH 7.3) and extracted with phenol according to the method of Johnson and Perry (1975). The phenol phase was discarded, and the aqueous phase was dialyzed exhaustively as described above. After dialysis, the extracts were lyophilized, resuspended in 0.1% sodium dodecyl sulfate (SDS) in distilled water, and 10 mg was applied to a Biogel A-5m column (2.6 × 65 cm) and eluted with the SDS solution. The eluted material was recovered in 5-ml fractions, which were assayed for hexoses. Fractions of the large hexose-containing double peak were pooled and applied to a Dowex Ag10x column (0.5 × 20 cm), and the column was eluted with distilled water until hexoses were no longer detected in the eluted material. The material eluted from the Dowex column was lyophilized and weighed; this material represented the neutral, low-molecular weight polysaccharide fraction.

NMR spectroscopy. ^{1}H- and ^{13}C-NMR spectra were obtained with a Bruker 400MHz spectrometer. Samples were dissolved in 0.5 ml of D_2O (Wilmad Co., Buena, NJ) at a concentration of 12 mg/ml. The ^{1}H-NMR spectra were the time average of at least 16 scans, performed at 70° C with sodium trimethylsilylpropionate-2,2,3,3-d_4 (Wilmad Co.) as the internal reference. ^{13}C-NMR spectra were the time average of at least 20,000 scans, performed at 25° C. Each sample was subjected to both ^{1}H-NMR and ^{13}C-NMR spectroscopy.

Attachment assay. Attachment assays were performed as described by Douglas *et al.* (1982), with the following modifications. Cultures of bacterial cells were grown to early stationary phase, and 50 μl of each culture was added to 0.5 ml of AB broth with 2 μCi of ^{14}C-leucine. The subcultures were grown overnight with shaking at 28° C, and the bacteria were washed twice in Murashige and Skoog (MS) salts (GIBCO, Grand Island, NY) in 10 mM MES buffer, pH 6.0. Zinnia mesophyll cells were prepared by gently grinding zinnia leaves (cv. Polar Bear; Olds Seed Co., Madison, WI) in buffered MS salts with a mortar and pestle. The zinnia cells were washed once in the buffered MS salts, pelleted by gentle centrifugation in a hand-cranked tabletop centrifuge, and resuspended to an A_{600} of approximately 1.0. In assay mixtures to which glucan was added, glucan was first dissolved in buffered MS salts and then added to zinnia cells. The zinnia cell mixtures, with and without glucan, were then incubated with shaking for 1 hr at room temperature.

Bacteria were added to the zinnia cells in a 125-ml flask at a ratio of approximately 4:1 and shaken gently for 2 hr. The flasks were shaken vigorously enough to keep the zinnia cells evenly suspended. One ml of the mixture was passed through a Nitex cloth filter (25-μm pore size) and flushed with 20 ml of buffered MS salts. The Nitex filters were then placed in Aquasol scintillation fluid (New England Nuclear Research Products, Boston, MA), and counted in a Packard Tri-Carb scintillation counter.

Transfer of pCD523 by triparental mating. Cosmid pCD523 was transferred conjugally from strain DH1 of *E. coli* to strain A2505 of *A. tumefaciens* by the filter mating method (Ditta *et al.* 1980), with strain HB101(pRK2013) of *E. coli* as the helper strain.

Tumorigenesis assays. Strains were tested for virulence by inoculating *Kalanchoe daigremontiana* leaves with bacteria from a single colony (Garfinkel and Nester 1980). The effects of glucan on tumorigenesis were measured on Jerusalem artichoke disks (*Helianthus tuberosus* L.) by the method of Tanimoto *et al.* (1979). Following excision, the disks were placed either in distilled water or in a solution of glucan (1 mg/ml in distilled water) for 45 min, and then immersed in 5 ml of the culture to be tested. The disks were placed on solid medium (MS salts, 20 g/L of glucose, 7.5 g/L of agar) in covered petri plates for 2 days and were then transferred to the same medium with the addition of 100 μg/ml of vancomycin, 100 μg/ml of ampicillin, and 10 μg/ml of rifampicin. The disks were incubated at room temperature in total darkness for 10 days, at which time the number of disks with tumors from each treatment was determined.

RESULTS

Extracellular neutral glucan from *chv* mutants. We detected significantly less neutral extracellular polysaccharide (EPS) in the culture supernatants of both *chvA* and *chvB* mutants than in culture supernatants of virulent control strain A759. The mutants A2505 (*chvA*) and A1038 (*chvB*) produced approximately 45-fold less recoverable neutral EPS than did the virulent control strain A759 (Table 2). The ^{1}H-NMR spectrum of the small amount of EPS recovered from mutant A2505 (Fig. 1A) contained peaks that are characteristic of the cyclic glucan from the virulent control (Fig. 2A), whereas the ^{1}H-NMR spectrum of A1038 EPS did not contain such peaks (Fig. 2B). The peak at 4.9 ppm is characteristic of the anomeric proton of neutral glucan (Puvanesarajah *et al.* 1985); however, the other peaks have not been assigned to specific protons. The ^{13}C spectrum of A2505 neutral EPS also revealed the six peaks that are characteristic of the cyclic glucan (Gorin 1981), whereas the ^{13}C spectrum of A1038 neutral EPS did not (data not shown).

Although the neutral EPS fraction of A2505 appears to contain authentic glucan (Fig. 1A), there was 45-fold less material present in this fraction from the mutant than from the virulent control strain A759 (Table 2). Strain A723, the motile parent strain of A2505, produced extracellular glucan in quantities comparable to those produced by strain A759 (data not shown). Furthermore, the supernatants of

Table 2. Recovery of polysaccharides from culture supernatants of strains of *A. tumefaciens* following each step of preparation

	Polysaccharide recovery (mg) after:[a]		
Strain	**Dialysis (retained fraction)**	**Ethanol precipitation (supernatant)**	**DEAE-cellulose chromatography (neutral fraction)**
A759[b]	418	275	168
A1038	235	214	6
ME45	180	16	2
ME66	84	36	4
A2505	90	25	3
A2505(pCD523)[b]	228	160	122

[a] The starting material was 1.2 L of culture supernatant for all of the strains.
[b] Values represent the average of preparations from two cultures.

two *chvA* mutants, ME66 and ME45, that were constructed by marker-exchange mutagenesis (Douglas *et al.* 1985) also contained less neutral EPS than the supernatants of virulent control cultures (Table 2), and the ^{1}H-NMR spectra of the neutral EPS fraction from these mutants contained peaks characteristic of the glucan (data not shown). We have not yet identified the other neutral polysaccharide(s) present in the supernatants of the *chvA* or *chvB* mutants (characterized by the doublet at 4.65 ppm), although we found that the neutral EPS from both A1038 and A2505 is composed almost entirely of glucose (>98%).

Intracellular neutral glucan in *chv* mutants. Crude phenol-water extracts of cells were analyzed by gel filtration chromatography (Fig. 3). The amount of carbohydrate in the large double peaks eluted from the gel filtration column between 130–190 ml of elution buffer was approximately the same in the cell extracts of both *chvA* mutants and the virulent control strain A759, whereas the peak was missing in the extracts of *chvB* mutants. Material from A2505 (*chvA*) extract that eluted between 130–190 ml had a ^{1}H-NMR spectrum (Fig. 1B) that was the same as the neutral glucan (Puvanesarajah *et al.* 1985). This material was derivatized by the method of Jones and Albersheim (1972), analyzed by gas chromatography, and was found to contain only glucose. We obtained identical results with extracts of the other two *chvA* mutants, ME45 and ME66 (data not shown). As Puvanesarajah *et al.* (1985) showed previously, neutral glucan was not found associated with cells or in the culture supernatants of *chvB* mutants.

Restoration of *chvA* mutant A2505. Plasmid pCD523, a pLAFR1 derivative that carries the entire *chv* region (Douglas *et al.* 1985), restored attachment ability (Table 3) and virulence to A2505. Virulence was determined on *K. daigremontiana* leaves and Jerusalem artichoke tuber disks (Table 4). Culture supernatants of the restored strain, A2505(pCD523), contained approximately three-fourths of the amount of neutral EPS as supernatants of the virulent control strain A759 and 40-fold more than recovered from the culture supernatants of *chvA* mutants (Table 2). The ^{1}H-NMR spectrum of the neutral EPS recovered from A2505(pCD523) was identical to that of the neutral glucan obtained from the supernatants of A759 cultures (data not shown). The presence of pCD523 had no detectable effect on the amount of neutral glucan in the cell extracts of A2505 (Fig. 3).

Effects of exogenously applied glucan on tumor formation and attachment. Jerusalem artichokes were treated with either *chv* mutant or virulent strains of *A. tumefaciens* in the presence or absence of neutral glucan. The data in Table 4 show that glucan had no effect on the number of disks that formed tumors after inoculation with either mutant or virulent strains. The mutants A2505 and A1038 did not form tumors on disks that were treated with either distilled water or the glucan solution. Approximately the same proportion of disks inoculated with the virulent

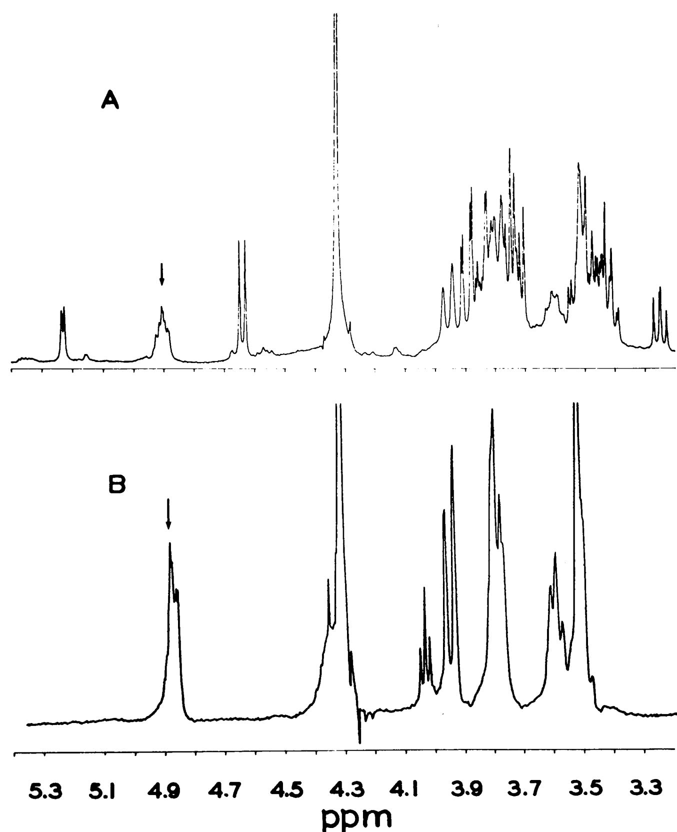

Fig. 1. Proton NMR spectra of neutral polysaccharides from avirulent mutant A2505 (*chvA*) of *A. tumefaciens*. **A**, Extracellular polysaccharides after DEAE-cellulose chromatography. **B**, Intracellular polysaccharides after gel filtration chromatography. The triplet at 4.05 ppm (**B**) is due to residual sodium dodecyl sulfate in the sample. Each sample contained 6 mg of polysaccharides. Arrows indicate the position of the chemical shift of the anomeric proton of neutral β-1,2-linked D-glucan.

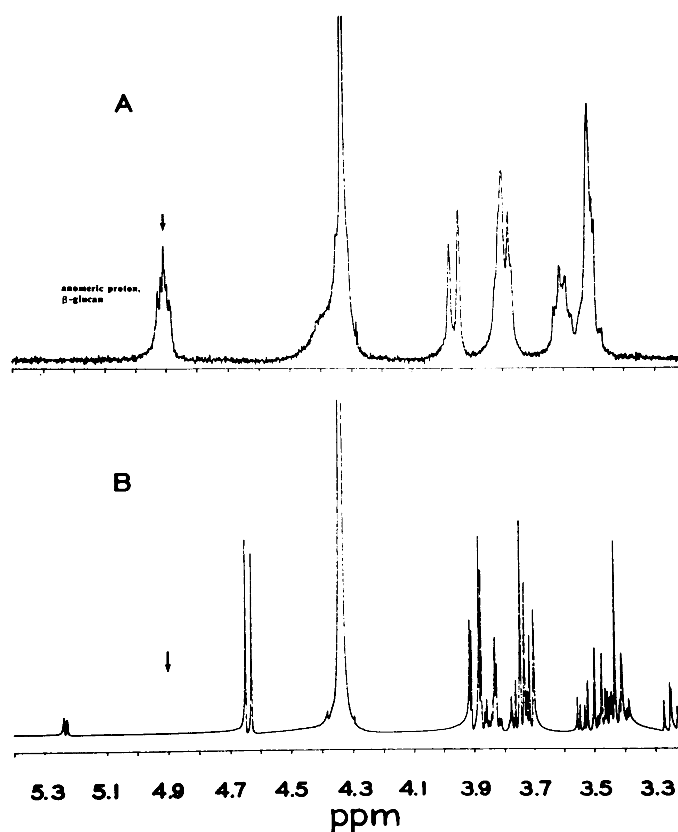

Fig. 2. Proton NMR spectra of neutral extracellular polysaccharides of (**A**) virulent control strain A759 and (**B**) *chvB* mutant A1038 following DEAE-cellulose chromatography. Each sample contained 6 mg of polysaccharides. Arrows indicate the position of the chemical shift of the anomeric proton of neutral β-1,2-linked D-glucan.

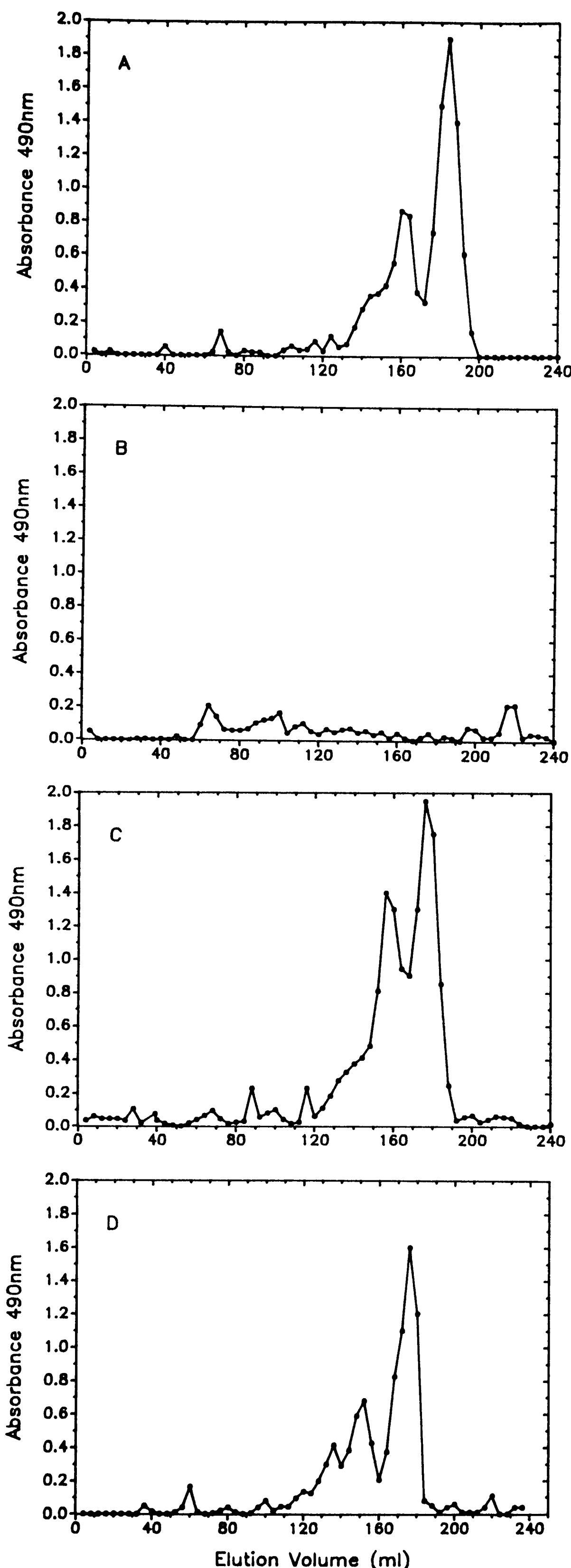

Fig. 3. Gel filtration chromatography on a Biogel A-5m column of phenol-water extracts of cells of *A. tumefaciens*. **A**, Virulent control strain A759; **B**, *chvB* mutant A1038; **C**, *chvA* mutant A2505; **D**, A2505(pCD523) (Vir$^+$). The neutral glucan was eluted from the column between 130–190 ml.

control strain A759 or the restored mutant A2505(pCD523) formed tumors in the presence and absence of glucan.

We examined the effect of glucan on the attachment of strains of *A. tumefaciens* to plant cells in the attachment assay that originally defined the *chv* mutants as attachment deficient (Douglas *et al.* 1982). Pretreatment of zinnia mesophyll cells with 1 mg/ml of glucan had no effect on the attachment of *chv* mutants or virulent strains A759 and A2505(pCD523) to the zinnia cells (Table 3). The data presented in Table 3 are representative of three experiments in which *chv* mutants attached to zinnia cells significantly less than did virulent bacteria, as reported previously (Douglas *et al.* 1982; Thomashow *et al.* 1987; Cangelosi *et al.* 1987). Although variability within experiments is quite low, we have found that the results obtained in separate experiments with this assay are variable. Attachment of virulent strains ranges from 20 to 70% of any given population (O'Connell and Handelsman, unpublished data; M. Thomashow, personal communication). *chv* mutants generally exhibit attachment frequencies that are two- to fivefold lower than virulent strains, and are also variable, whereas *pscA* and *exoC* mutants consistently attach quite poorly. Less than 3% of the cells in cultures of these mutants attach to plant cells (Thomashow *et al.* 1987; Cangelosi *et al.* 1987). We have been unable to determine why the attachment of *chv* mutants and virulent strains is more variable than that of *exoC*/*pscA* mutants.

DISCUSSION

We have shown that cells of *chvA* mutants contain intracellular neutral glucan in amounts equivalent to cells of

Table 3. Attachment of strains of *A. tumefaciens* to zinnia mesophyll cells, following treatment of zinnia cells with Murashige and Skoog salts buffer or buffer containing glucan

Strain	− glucan[y]	+ glucan (1 mg/ml)
A759	31.3 c[z]	33.0 c
A1038	18.1 a	14.9 a
A2505	17.9 a	18.1 a
A2505(pCD523)	27.2 bc	25.6 b

[y] All cell suspensions were exposed to the buffer that was used to suspend the glucan.
[z] Percent of bacteria attached to plant cells. Values represent the mean of three measurements from a single flask of bacteria per zinnia cell suspension. Values followed by the same letter do not differ significantly at $P < 0.05$ by using the Newman-Keuls procedure. Results are representative of three independent experiments.

Table 4. Tumor formation on Jerusalem artichoke disks in the presence and absence of glucan

Strain	Treatment	Disks with tumors (%)[y]
A759	Distilled water	63.3 a[z]
	Glucan solution	79.5 a
A1038	Distilled water	0 b
	Glucan solution	0 b
A2505	Distilled water	0 b
	Glucan solution	0 b
A2505(pCD523)	Distilled water	69.5 a
	Glucan solution	74.2 a

[y] Values represent the mean of three replicates, 10 slices per replicate.
[z] Values followed by the same letter do not differ significantly at $P < 0.05$ by using the Newman-Keuls procedure.

virulent strains but lack extracellular neutral glucan; thus, the *chvA* mutants produce less total recoverable glucan than the virulent strains. This result is of particular interest because other chromosomal virulence loci, *chvB* and *exoC/pscA* (see Marks *et al.* 1987), that have been identified in strain A723 of *A. tumefaciens* also affect glucan production (Puvanesarajah *et al.* 1985; Cangelosi *et al.* 1987; Thomashow *et al.* 1987). Whereas *chvB* is directly involved in glucan synthesis (Zorreguieta and Ugalde 1986; Zorreguieta *et al.* 1988), *chvA* appears to be required for export of the glucan from the cell. In addition to the direct biochemical evidence presented in this paper suggesting a role for *chvA* in glucan export, the sequence of *chvA* suggests an export function because it has extensive similarity to the *hlyB* locus of *E. coli* (G. Cangelosi, personal communication). *hlyB* encodes hemolysin B, a protein thought to be associated with the inner membrane of *E. coli* and required for the export of the hemolysin A protein (Mackman *et al.* 1986). *chvA* also has extensive homology to the *ndvA* gene of *Rhizobium meliloti*, which is required for normal nodule induction on alfalfa. *ndvA* also appears to be essential for the export of glucan from cells of *R. meliloti* and contains extensive homology to *hlyB* (Stanfield *et al.* 1988).

It is interesting to note that all of the mutants that map in virulence loci that affect neutral glucan production or export (*chvA*, *chvB*, *pscA*, and *exoC*) are also defective in attachment to plant cells, suggesting that extracellular neutral glucan is required for attachment and tumorigenesis. This could implicate the glucan directly in attachment, although purified glucan did not significantly inhibit or enhance attachment or tumorigenesis of mutant or virulent strains except in one experiment, in which preincubation of zinnia cells with 1 mg/ml of glucan resulted in partial inhibition of attachment. However, we believe that this is not a biologically relevant concentration because it would require 2,000 times the number of bacteria added to the attachment assay to produce that amount of glucan, and the high concentration of glucan alters the viscosity of the suspension. Specific recognition between plants and bacteria can often be inhibited by much lower concentrations of the appropriate hapten. For example, the specific recognition of clover roots by *R. trifolii* is completely inhibited by 30 mM of the hapten 2-deoxy-glucose (Dazzo *et al.* 1984), whereas we observed partial inhibition of attachment of *A. tumefaciens* to zinnia cells only at 200–400 mM neutral glucan. It is possible that anionic glucans (Miller *et al.* 1987; Batley *et al.* 1987; Hisamatsu *et al.* 1987) have biological activity, but we have not tested the effects of anionic glucans on tumorigenesis or attachment because the isolation methods used in this study eliminate these molecules during purification (Puvanesarajah *et al.* 1985).

The introduction of a cosmid, pCD523, into *chvA* mutant A2505 restored virulence on Jerusalem artichoke and *Kalanchoe* and restored the ability of the mutant to attach to zinnia cells, but it did not fully restore extracellular glucan production. This may have been due to loss of the plasmid during growth of the culture for glucan isolation. If extracellular glucan is indeed required for plant cell transformation, the ability of A2505(pCD523) to cause tumors despite reduced glucan production suggests that some virulent strains are producing more glucan than is absolutely required for tumorigenesis.

One possible physiological role for β-1,2-linked D-glucans in *E. coli* and *A. tumefaciens* is protection against low environmental osmolarity. *A. tumefaciens* produces periplasmic glucans only under conditions of low environmental osmolarity, suggesting that these molecules serve to balance the osmotic strength of solutions inside and outside the inner membrane (Miller *et al.* 1986). Interestingly, *ndvA* mutants of *R. meliloti* grow under conditions of high osmolarity that are inhibitory to *ndvB* mutants and wild-type strains of *R. meliloti* (Dylan *et al.*, unpublished), possibly because a defect in glucan export causes the cells to accumulate glucan, balancing the osmolarity inside *ndvA* mutants with the high osmolarity of the medium. However, in *A. tumefaciens* the osmolarity of the growth medium does not affect extracellular glucan production (Miller *et al.* 1986), suggesting that extracellular glucan has a physiological role distinct from the response to environmental osmoticum. Because extracellular glucan appears to be required for the virulence of *A. tumefaciens*, it may have a nonosmotic function in the transformation process. For example, the glucan may act as a chemical messenger between the bacteria and plant cells or it may be a part of a complex attachment structure.

It is possible that the loss of attachment ability and virulence is a pleiotropic effect of mutations in the *chv* loci. A single transposon insertion in either locus causes several altered phenotypes (avirulence, loss of flagella, defects in attachment and in glucan production), none of which have been causally linked. It is possible that a defect in osmoregulation caused by glucan deficiency affects a wide variety of cell surface structures. Perhaps mutations in the *chv* loci affect, through an osmoregulatory defect, the production of an unidentified cell-surface structure that is essential for tumorigenesis. Understanding the role of the *chv* loci in the physiology of *A. tumefaciens* may be a key step in elucidating the early events of tumorigenesis.

ACKNOWLEDGMENTS

We thank S. Vicen for assistance in preparing the figures and M. Erhardt for operating the NMR spectrophotometers. We are grateful to L. Sequeira for many helpful discussions and to G. Cangelosi and M. Thomashow for sharing unpublished data.

This research was supported by the University of Wisconsin College of Agricultural and Life Sciences through Hatch project 3012.

K. P. O. received support from National Institutes of Health Cell and Molecular Biology Training Grant GM07215-13.

LITERATURE CITED

Ashwell, G. 1966. New colorimetric methods of sugar analysis. Methods Enzymol. 8:85-94.

Batley, M., Redmond, J. W., Djordjevic, S. P., and Rolfe, B. G. 1987. Characterization of glycerophosphorylated cyclic β-1,2-glucans from a fast-growing *Rhizobium* species. Biochim. Biophys. Acta 901:119-126.

Boyer, H. W., and Roulland-Dussoix, D. 1969. A complementation analysis of the restriction and modification of DNA in *Escherichia coli*. J. Mol. Biol. 41:459-472.

Bradley, D. E., Douglas, C. J., and Peschon, J. 1984. Flagella-specific bacteriophages of *Agrobacterium tumefaciens*: Demonstration of virulence of non-motile mutants. Can. J. Microbiol. 30:676-681.

Cangelosi, G. A., Hung, L., Puvanesarajah, V., Stacey, G., Ozga, D. A., Leigh, J. A., and Nester, E. W. 1987. Common loci for *Agrobacterium tumefaciens* and *Rhizobium meliloti* extracellular polysaccharide synthesis and their roles in plant interactions. J. Bacteriol. 169:2086-2091.

Chilton, M.-D., Currier, T. C., Farrand, S. K., Bendich, A. J., Gordon, M. P., and Nester, E. W. 1974. *Agrobacterium* DNA and PS8

bacteriophage DNA not detected in crown gall tumors. Proc. Natl. Acad. Sci. USA 71:3672-3676.
Dazzo, F. B., Truchet, G. L., Sherwood, J. E., Hrabak, E. M., Abe, M., and Pankratz, S. H. 1984. Specific phases of root hair attachment in the *Rhizobium trifolii*-clover symbiosis. Appl. Envir. Microbiol. 48:1140-1150.
Ditta, G., Stanfield, S., Corbin, C., and Helinski, D. R. 1980. Broad host range DNA cloning system for Gram-negative bacteria: Construction of a gene bank of *Rhizobium meliloti*. Proc. Natl. Acad. Sci. USA 77:7347-7351.
Douglas, C. J., Halperin, W., and Nester, E. W. 1982. *Agrobacterium tumefaciens* mutants affected in attachment to plant cells. J. Bacteriol. 152:1265-1275.
Douglas, C. J., Staneloni, R. J., Rubin, R., and Nester, E. W. 1985. Identification and genetic analysis of an *Agrobacterium tumefaciens* chromosomal virulence region. J. Bacteriol. 161:850-860.
Garfinkel, D. J., and Nester, E. W. 1980. *Agrobacterium tumefaciens* mutants affected in crown gall tumorigenesis and octopine catabolism. J. Bacteriol. 144:732-743.
Gorin, P. A. J. 1981. ^{13}C nuclear magnetic resonance spectroscopy of polysaccharides. Adv. Carbohydr. Chem. Biochem. 38:13-104.
Gorin, P. A. J., and Mazurek, M. 1973. Carbon-13 resonance spectroscopic studies on the formation of borate and diphenylborate complexes of polyhydroxy compounds. Can. J. Chem. 51:3277-3286.
Gorin, P. A. J., Spencer, J. F. T., and Westlake, D. W. S. 1961. The structure and resistance to methylation of 1,2-β-glucans from species of *Agrobacterium*. Can. J. Chem. 39:1067-1073.
Hanahan, D. 1983. Studies on the transformation of *Escherichia coli* with plasmids. J. Mol. Biol. 166:557-580.
Hisamatsu, M., Yamada, T., Higashiura, T., and Ikeda, M. 1987. The production of acidic *o*-acylated cyclosophorans [cyclic (1->2)-β-D-glucans] by *Agrobacterium* and *Rhizobium* species. Carbohydr. Res. 163:115-122.
Hodgson, R., Riker, A. J., and Peterson, W. H. 1945. Polysaccharide production by virulent and attenuated crown-gall bacteria. J. Biol. Chem. 158:89-100.
Johnson, K. G., and Perry, M. B. 1975. Improved techniques for the preparation of bacterial lipopolysaccharides. Can. J. Microbiol. 22:29-34.
Jones, T., and Albersheim, P. 1972. A gas chromatographic method for the determination of aldose and uronic acid constituents of plant cell wall polysaccharides. Plant Physiol. 49:926-936.
Mackman, N., Nicaud, J.-M., Gray, L., and Holland, I. B. 1986. Secretion of Haemolysin by *Escherichia coli*. Pages 159-181 in: Current Topics in Microbiology and Immunology, Vol. 125. H. C. Wu and P. C. Tai, eds. Springer-Verlag, New York.
Maniatis, T., Fritsch, E. F., and Sambrook, J. 1982. Page 68 in: Molecular Cloning: A Laboratory Manual. Cold Spring Harbor Laboratory, Cold Spring Harbor, NY.
Marks, J. R., Lynch, T. J., Karlinsey, J. E., and Thomashow, M. E. 1987. *Agrobacterium tumefaciens* virulence locus *pscA* is related to the *Rhizobium meliloti exoC* locus. J. Bacteriol. 169:5835-5837.
McIntire, F. C., Peterson, W. H., and Riker, A. J. 1942. A polysaccharide produced by the crown-gall organism. J. Biol. Chem. 143:491-496.
Miller, K. J., Kennedy, E. P., and Reinhold, V. N. 1986. Osmotic adaptation by gram-negative bacteria: Possible role for periplasmic oligosaccharides. Science 231:48-51.
Miller, K. J., Reinhold, V. N., Weissborn, A. C., and Kennedy, E. P. 1987. Cyclic glucans produced by *Agrobacterium tumefaciens* are substituted with *sn*-1-phosphoglycerol residues. Biochim. Biophys. Acta 901:112-118.
Putnam, E. W., Potter, A. L., Hodgson, R., and Hassid, W. Z. 1950. The structure of crown-gall polysaccharide. J. Am. Chem. Soc. 72:5024-5026.
Puvanesarajah, V., Schell, F. M., Stacey, G., Douglas, C. J., and Nester, E. W. 1985. Role for 2-linked-β-D-glucan in the virulence of *Agrobacterium tumefaciens*. J. Bacteriol. 164:102-106.
Stanfield, S. W., Ielpi, L., O'Brochta, D., Helinski, D. R., and Ditta, G. S. 1988. The *ndvA* gene product of *Rhizobium meliloti* is required for β-(1->2) glucan production and has homology to the ATP-binding export protein *HlyB*. J. Bacteriol. 170:3523-3530.
Tanimoto, E., Douglas, C., and Halperin, W. 1979. Factors affecting crown gall tumorigenesis in tuber slices of Jerusalem artichoke (*Helianthus tuberosus*, L.). Plant Physiol. 63:989-994.
Thomashow, M. F., Karlinsey, J. E., Marks, J. R., and Hurlbert, R. E. 1987. Identification of a new virulence locus in *Agrobacterium tumefaciens* that affects polysaccharide composition and plant cell attachment. J. Bacteriol. 169:3209-3216.
Zorreguieta, A., and Ugalde, R. 1986. Formation in *Rhizobium* and *Agrobacterium* spp. of a 235-kilodalton protein intermediate in β-D(1,2) glucan synthesis. J. Bacteriol. 167:947-951.
Zorreguieta, A., Geremia, R. A., Cavaignac, S., Cangelosi, G. A., Nester, E. W., and Ugalde, R. A. 1988. Identification of the product of an *Agrobacterium tumefaciens* chromosomal virulence gene. Molec. Plant-Microbe Interact. 1:121-127.