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Characterization of Three *Agrobacterium tumefaciens* Avirulent Mutants with Chromosomal Mutations That Affect Induction of *vir* Genes

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Three *Agrobacterium tumefaciens* mutants with chromosomal mutations that affect bacterial virulence were isolated by transposon mutagenesis. Two of the mutants were avirulent on all hosts tested. The third mutant, Ivr-211, was a host range mutant which was avirulent on *Bryophyllum diagremontiana*, *Nicotiana tabacum*, *N. debneyi*, *N. glauca*, and *Daucus carota* but was virulent on *Zinnia elegans* and *Lycopersicon esculentum* (tomato). That the mutant phenotype was due to the transposon insertion was determined by cloning the DNA containing the transposon insertion and using the cloned DNA to replace the wild-type DNA in the parent bacterial strain by marker exchange. The transposon insertions in the three mutants mapped at three widely separated locations on the bacterial chromosome. The effects of the mutations on various steps in tumor formation were examined. All three mutants showed no alteration in binding to carrot cells. However, none of the mutants showed any induction of *vir* genes by acetosyringone under conditions in which the parent strain showed *vir* gene induction. When the mutant bacteria were examined for changes in surface components, it was found that all three of the mutants showed a similar alteration in lipopolysaccharide (LPS). LPS from the mutants was larger in size and more heavily saccharide substituted than LPS from the parent strain. Two of the mutants showed no detectable alteration in outer membrane and periplasmic space proteins. The third mutant, Ivr-225, was missing a 79-kDa surface peptide. The reason(s) for the failure of *vir* gene induction in these mutants and its relationship, if any, to the observed alteration in LPS are unknown.

Infection of susceptible dicotyledonous plants by *Agrobacterium tumefaciens* results in crown gall tumor formation. The mechanism by which the bacterium infects the plant is not fully understood. An early step in tumor formation involves the attachment of the bacterium to host plant cells (12). Once attached to the plant, the bacterium begins a process that results in the movement of tumor-inducing (Ti) plasmid DNA sequences from the bacterium into the plant host genome (2, 28). Depicker et al. have identified the DNA sequences transferred and termed them T-DNA (5). Without successful transfer of the T-DNA from the bacterium to the plant, tumor formation cannot occur. This paper examines the properties of three avirulent Tn*5* insertion mutants of *A. tumefaciens*, Ivr-211, Ivr-223, and Ivr-225. These Tn*5* insertions are contained within the chromosome of the bacterium; thus, the insertions have no direct affect on the *vir* region of the Ti plasmid which is responsible for T-DNA transfer. The only previously identified chromosomal genes required for virulence are *chvA* and *chvB* (6), *att* (16), *pscA* (27) (also called *exoC* [1]), *chvD* (32), and *chvE* (9). Mutations in each of the first four genes listed result in the inability of the bacteria to attach to host cells and the consequent loss of bacterial virulence. Mutations in *chvD* result in reduced virulence on *Bryophyllum diagremontiana* (visible tumors were still formed) and reduced induction of *virG* by acetosyringone (32). Transposon insertions in *chvE* result in a restricted host range and attenuated virulence. *vir* gene induction by acetosyringone is greatly reduced or absent in *chvE* mutants. However, induction of *virG* by a combination of low pH and low phosphate concentration is unimpaired in *chvE* mutants as compared with wild type. DNA sequence analysis suggests that *chvE* may encode a periplasmic space protein involved in chemotaxis or sugar uptake (9). The mechanism by which this protein affects acetosyringone induction of *vir* genes is not understood. The three mutants described in the present study appear to be unaltered in their ability to bind to plant cells and to be reduced in *vir* gene induction. These mutations appear to be distinct from *chvE* mutations.

MATERIALS AND METHODS

Bacterial strains. *A. tumefaciens* strains were maintained on minimal media plus 0.5 mg of biotin (3, 18) per liter. *Escherichia coli* strains were maintained on Luria agar (20). Antibiotic concentrations were as follows: carbenicillin, 100 mg/liter; neomycin, 60 mg/liter; and tetracycline, 5 mg/liter.

Transposon Tn*5* was introduced into *A. tumefaciens* A6 as described previously (15). The transposon mutants were selected by growth on minimal medium with 60 mg of neomycin per liter. They were screened for virulence on leaves of *B. diagremontiana*. Avirulent mutants were retained and screened for the location of Tn*5* in the bacterial chromosome or plasmids as described below. Mutants in which Tn*5* was located in the chromosome were then screened for their ability to bind to carrot cells as described below. Eleven avirulent chromosomal mutants which showed normal binding to plant cells were obtained from the screening of about 3,000 Tn*5* mutants. Three of these mutants were randomly selected for study. The properties of

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TABLE 1. Bacterial strains and plasmids

Strain or plasmid	Characteristics	Source or reference
Bacteria		
A. tumefaciens		
A6	Wild type, virulent, Neo^s, prototrophic	Matthysse
Ivr-211, Ivr-223, Ivr-225	Tn*5* insertion mutants, avirulent, Neo^r, prototrophic	This paper
E. coli		
JM-83	*thi lac proAB*	13
Plasmids		
pBR325	Tet^r Cm^r Amp^r	13
pTTQ9	Amp^r, polylinker between *ptac* and *rrnB* terminator, *lacI*^{q}	Amersham Corp.
pSM30	Tn*3*HoHoI insertion in *virB* in pVCK221	26
pSM358	Tn*3*HoHoI insertion in *virE* in pVCK225	26
pSW174	*lacZ* fusion with *virG* promoter, Amp^r Kan^r Neo^s	32

these three mutants are described here. The bacterial strains and plasmids used in this study are listed in Table 1.

Plants and plant cells. *B. daigremontiana*, *Nicotiana tabacum* cv. Coker 319, *N. debneyi*, *N. glauca*, *Lycopersicon esculentum* (tomato) cv. Rutgers and cv. Homestead, and *Zinnia elegans* cv. Enchantress Rose were greenhouse grown; *Daucus carota* plants used in virulence assays were purchased fresh with tops from a local market. *D. carota* used for attachment and aggregation experiments was a suspension cell line (from W. Boss, North Carolina State University) grown at room temperature in Murashige and Skoog medium (21).

Bacterial attachment to plant cells. Attachment of bacteria to carrot cells was measured by the procedure of Matthysse et al. (17). Carrot cell cultures were used at 2×10^5 to 75×10^5 cells per ml in Murashige and Skoog medium; suspensions of bacteria were added to the carrot cultures to a final concentration of approximately 10^3 bacteria per ml. The number of bacteria attached to plant cells was determined after various incubation times by filtering an aliquot of the carrot cell bacterial suspension mix through Miracloth (Calbiochem), which allowed the passage of free bacteria but not carrot cells; the number of bacteria in the filtrate and the number bound to carrot cells were determined by viable cell counts. Measurements were made at 0, 30, and 120 min. For one mutant, Ivr-223, time points were taken every 2 to 3 min for the first 20 min to study the initial kinetics of attachment. Aggregation was determined by visual inspection of the carrot cell bacteria mixture after 1 to 3 days (17).

Virulence assays. Virulence assays on *B. diagremontiana* were done by making puncture wounds on young leaves with a sterile toothpick that had been wiped across a stock plate of the appropriate *A. tumefaciens* strain. Strain A6 was used as a virulent control on each plant. Plants were scored for the presence or absence of tumors at each wound site weekly for 6 weeks. *N. tabacum*, *N. debneyi*, *N. glauca*, *L. esculentum*, and *Z. elegans* were used for virulence testing by wounding the stem with a sterile scalpel, followed by toothpick inoculation with the appropriate strain. Wounds were made on the stem near the base and upward to the growing tip of the plant.

Virulence assays on carrot root disks used the procedure of Klein and Tenenbaum (10).

DNA isolation, hybridization, and cloning. To determine the location of the Tn*5* insertion in the mutant strains, the hybridization of radioactive Tn*5* DNA to bacterial plasmid and chromosomal DNAs separated by lysing the bacteria in the well of an agarose gel followed by electrophoresis was carried out as described previously (14, 15). Tn*5* hybridized only to the chromosomal, and not the plasmid, DNA from each of the three mutants.

DNA was extracted from *A. tumefaciens* as described previously (15). The cloning of the *Eco*RI fragments containing the Tn*5* insertion (Tn*5* has no *Eco*RI sites) from mutants Ivr-211 and Ivr-225 into the *Eco*RI site of vector pTTQ9 was done by the protocols of Maniatis et al. (13). Positive selection for the desired clone after transformation of *E. coli* JM83 was by selection for neomycin resistance, a gene marker on Tn*5*. In the case of mutant Ivr-223, vector pBR325 was used in place of pTTQ9. The protocol was as above.

The cloned *A. tumefaciens* DNA containing the Tn*5* insertion was marker exchanged into the parent A6 strain by the procedures of Ruvkun and Ausubel (23) and Matthysse (15).

Characterization of bacterial mutants. Mapping of the location on the chromosome of the Tn*5* insertions in each of the three mutants was done by the R′ mapping procedure of Robertson et al. (22).

Extraction of bacterial outer membrane and periplasmic space proteins was done by the method of Sonoki and Kado (25). The proteins removed were examined by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) by the method of Laemmli (11) and the silver stain method of Merril et al. (19). A running gel of 11% acrylamide and a stacking gel of 3% acrylamide were used.

Lipopolysaccharide (LPS) extracts were prepared as described previously (16) and examined by SDS-PAGE by the method of Tsai and Frasch (29). A running gel of 15% acrylamide and a stacking gel of 3% acrylamide were used. LPS concentrations were determined by measuring the concentration of ketodeoxyoctonate as described previously (16).

vir gene induction was measured by using a Tn*3*HoHo1 transposon which has a promoterless *lacZ* gene inserted into *virB*(pSM30) or *virE*(pSM358) or *virG*(pSW174) and introduced into the parent A6 strain and the mutants Ivr-211, Ivr-223, and Ivr-225 on a wide-host-range plasmid (26, 32). In this arrangement, activation of the *vir* gene produces β-galactosidase. β-Galactosidase assays were performed by the protocol of Stachel and Nester (26).

Crystal violet dye penetration assays were performed by the protocol of Gustafsson et al. (7) to determine whether the enlarged LPS on mutants Ivr-211, Ivr-223, and Ivr-225 prevented the uptake of the compound as compared with the

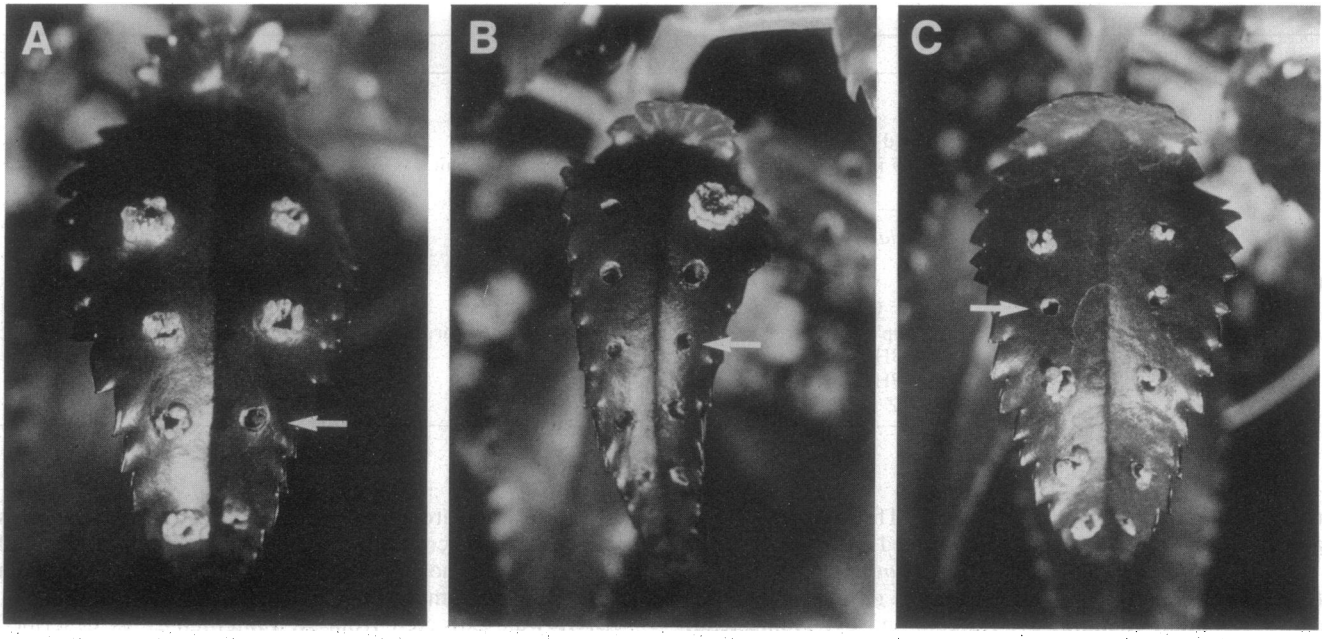

FIG. 1. Virulence of mutant *A. tumefaciens* inoculated into leaves of *B. diagremontiana*. (A) Mutant Ivr-211 was inoculated at the arrow. The A6 parent strain was inoculated at the upper left. (B) Mutant Ivr-223 was inoculated at the arrow. The A6 parent strain was inoculated at the upper right. (C) Mutant Ivr-225 was inoculated at the arrow. The A6 parent strain was inoculated at the upper left. All three mutants were avirulent on *B. diagremontiana* leaves. The photograph was taken 1 month after inoculation.

parent A6. The concentrations of crystal violet used were 0.2, 0.3, 0.4, 0.5, 0.6, 0.7, 0.8, 0.9, 1.0, and 2.0 mg/ml. Growth was measured after 24 h by Klett photometry.

Spheroplasts from A6 and the mutants were prepared by the procedure of Sonoki and Kado (25).

RESULTS

Isolation of mutants. Transposon Tn*5* mutants of *A. tumefaciens* isolated as described in Materials and Methods were screened for virulence on *B. diagremontiana*. Avirulent mutants were retained and tested for the location of the Tn*5* insertion as described above. Eleven mutants in which the Tn*5* insertion was in the chromosome were obtained. Three of these mutants were selected randomly for study and are described here.

Bacterial virulence. Mutants Ivr-223 and Ivr-225 were avirulent on the leaves of *B. diagremontiana* and on the stems of *N. tabacum*, *N. debneyi*, *N. glauca*, *Z. elegans*, and *L. esculentum*. Parent strain A6, used as a control, was virulent on *B. diagremontiana*, the *Nicotiana* species, *Z. elegans*, and *L. esculentum* (Fig. 1). Mutant Ivr-223 showed reduced tumor formation on *D. carota* disks inoculated on the apical surface but no tumor formation on carrot disks inoculated on the basal surface. Mutants Ivr-211 and Ivr-225 showed no tumor formation on carrot disks on either surface. The parent strain, A6, showed tumor formation on carrot disks inoculated on either surface (Table 2). Mutant Ivr-211 was avirulent on *B. diagremontiana* leaves, carrot disks, and the *Nicotiana* species. However, it induced tumors on *Z. elegans* and *L. esculentum* stems (Table 2). No evidence of attenuation of virulence of Ivr-211 was seen on

TABLE 2. Characterization of *A. tumefaciens* wild-type and mutant bacteria

Strain	Cellulose production[a]	Virulence in:								
		B. diagremontiana leaves	*N. tabacum* stems	*N. debneyi* stems	*N. glauca* stems	*D. carota* root disks		*Z. elegans* stems	*L. esculentum* (tomato) stems	
						Apical end up	Basal end up		cv. Rutgers	cv. Homestead
Wild-type parent A-6	+	+[b]	+	+	+	+	+	+	+	+
Mutant										
Ivr-211	+	−[c]	−	−	−	−[d]	−	+	+	+
Ivr-223	+	−	−	−	−	+/−[e]	−	−	−	−
Ivr-225	+	−	−	−	−	−	−	−	−	−

[a] As judged by production of material which fluoresced with cellufluor.
[b] +, >90% of the inoculated sites produced tumors within 6 weeks.
[c] −, No tumors were obtained from the inoculation of more than 10 sites on each of three different plants.
[d] −, No tumors were obtained from the inoculation of a minimum of four carrot disks from each of three different plants.
[e] +/−, A few small tumors (about 20% the number seen with A6) were observed consistently on each disk inoculated.

TABLE 3. Coinheritance frequency of chromosomal genes and Tn5 insertions

Tn5 mutant	% Coinheritance of Tn5 and marker (no. of transconjugants carrying both markers/total no. scored)						
	met-6[a]	*trp-13*	*trp-2*	*trp-1*	*leu-8*	*ilv-13*	*trp-22*
Ivr-211	63 (163/260)	13 (36/260)	14 (36/258)	0 (0/334)	1 (2/334)	0 (0/256)	2 (7/256)
Ivr-223	3 (12/367)	0 (0/367)	6 (7/113)	57 (75/131)	44 (57/131)	0.6 (1/158)	0 (0/158)
Ivr-225	2 (4/241)	0 (0/241)	1 (5/529)	4 (10/233)	3 (8/233)	35 (110/313)	44 (139/313)

[a] Chromosomal gene.

Z. elegans or *L. esculentum*; the tumors appeared at the same time as those caused by inoculation of the parent strain. Thus, mutant Ivr-211 appears to be a host range mutant.

Cloning of the Tn5 insertion and marker exchange. To determine whether the Tn5 insertion in each of the mutants was responsible for the mutant phenotype, the *Eco*RI fragment of the bacterial mutant DNA containing the Tn5 insertion was cloned and used to replace the wild-type DNA in the parent A6 strain by marker exchange. All of the properties tested of the mutants resulting from the marker exchange were the same as those of the original mutants. Characteristics tested included virulence, attachment, LPS, and crystal violet uptake. Measurements of *vir* gene induction were done with marker exchange mutants.

Chromosomal mapping. The technique of Robertson et al. (22) for determining the location on the *Agrobacterium* chromosome of Tn5 insertions was used to map the Tn5 insertions in Ivr-211, Ivr-223, and Ivr-225. This technique involves the formation of random R's which are used to measure the coinheritance frequency of *A. tumefaciens* chromosomal markers with known locations and the inserted Tn5.

The site of Tn5 insertion into the chromosome was different for each of the mutants studied. Mutant Ivr-211 had a Tn5 insertion in the region between *trp-13* and *trp-2* close to *met-6*. The insertion in strain Ivr-223 mapped in the region between *trp-1* and *leu-8*. That in strain Ivr-225 mapped between *trp-22* and *ilv-13* (Table 3, Fig. 2).

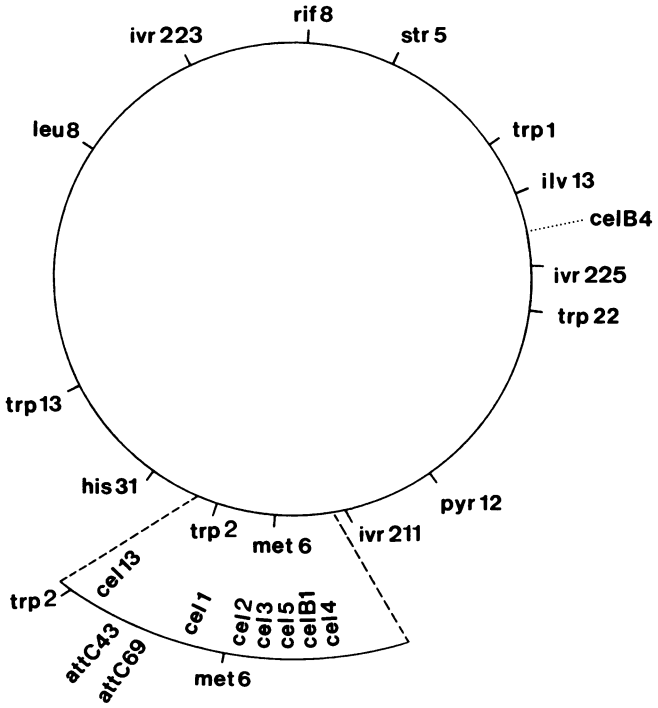

FIG. 2. Chromosomal linkage map of *A. tumefaciens*, based on the maps of Hooykaas et al. (8) and Robertson et al. (22), showing the locations of the mutations in Ivr-211 (near *met-6*), Ivr-223 (between *leu-8* and *rif-8*), and Ivr-225 (near *trp-22*).

Attachment to plant cells. Mutants Ivr-211, Ivr-223, and Ivr-225 showed no significant alterations in attachment to carrot cells when compared with the parent strain over a 2-h time course (Table 4). To be certain that the mutants did not differ from the parent strain in attachment to carrot cells, initial rates of attachment were measured. No difference between the parent and mutant strains was detected. Aggregation of carrot cells by all three mutants was normal when compared with the parent strain (Fig. 3), as was cellulose synthesis (Table 2).

Surface proteins. An alteration in surface proteins has been observed in avirulent chromosomal *att* gene mutants (16). Therefore, the surface proteins of Ivr-211, Ivr-223, and Ivr-225 were examined. SDS-PAGE analysis of outer membrane and periplasmic space proteins from the mutants revealed the loss of one band of approximately 79 kDa in mutant Ivr-225 (Fig. 4). Mutants Ivr-211 and Ivr-223 showed no detectable surface protein changes when compared with the parent strain (data not shown). These results suggest that a factor other than altered surface proteins was responsible for the loss of virulence in Ivr-211 and Ivr-223.

LPS. Changes in LPS composition have been reported to be associated with changes in virulence of *A. tumefaciens* (31) and thus potentially could be responsible for the phenotypic changes seen in these mutants. LPS extracted from the three mutants and the parent strain was run on SDS-polyacrylamide gels, using equal concentrations of ketodeoxyoctonate. The parent LPS was composed of a heavy concentration of core LPS with very little saccharide-substituted LPS. All three mutants showed small quantities of core LPS and heavy concentrations of larger saccharide-substituted LPS (Fig. 5).

***vir* gene induction.** Once *A. tumefaciens* has attached to

TABLE 4. Attachment of *A. tumefaciens* mutants to carrot suspension culture cells

Bacterial strain	% Control attachment after:	
	30 min	120 min
Parent (A6)	100 (control)	100 (control)
Mutants		
Ivr-211	80 ± 5	75 ± 5
Ivr-223	116 ± 10	95 ± 5
Ivr-225	127 ± 10	103 ± 5

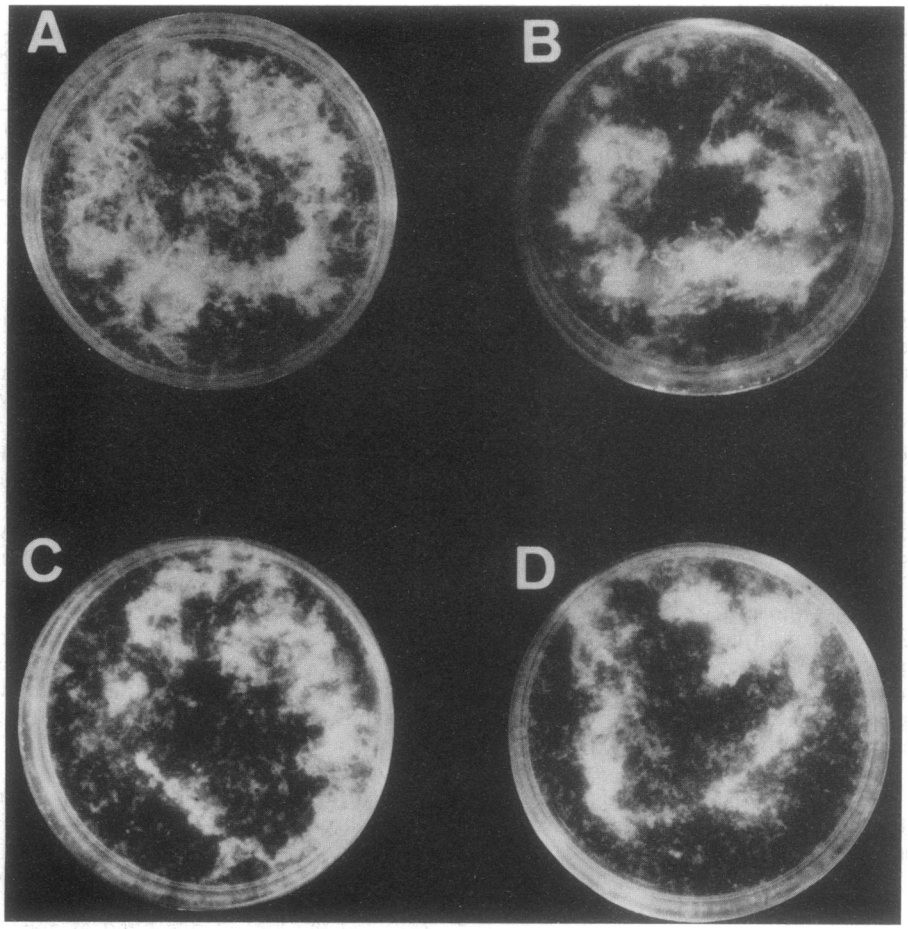

FIG. 3. Aggregation of carrot suspension culture cells by *A. tumefaciens*. Carrot cells in Murashige and Skoog medium were incubated with bacteria for 24 h. The cultures were poured into petri dishes for ease of photography. The bacteria used were (A) wild-type parent strain A6, (B) mutant Ivr-211, (C) mutant Ivr-223, and (D) mutant Ivr-225. The mutants attached to, and aggregated, carrot cells as efficiently as the wild-type bacteria. This observation suggests that bacterial attachment to carrot cells, bacterial cellulose synthesis, and aggregation are not affected in these mutants.

host cells, the next identified step in tumor formation is *vir* gene induction. To measure *vir* gene induction in the mutants and the parent A6 strain, plasmids pSM30 (26), pSM358 (26), and pSW174 (32) which have Tn*3*HoHo1 insertions in *virB*, *virE*, and *virG*, respectively, were introduced into *A. tumefaciens* by conjugation from *E. coli*. Tn*3*HoHo1 has a promoterless β-galactosidase gene at one end and the induction of *virB*, *virE*, or *virG* with these insertions results in the synthesis of β-galactosidase. The parent and the mutant strains showed little *virB*, *virE*, and *virG* activity in the absence of acetosyringone (differences in uninduced enzyme levels in the parent and mutant strains may reflect differences in plasmid copy number). The cause of the low β-galactosidase level in Ivr-225 with each of the plasmids is unknown. In the presence of acetosyringone the parent A6 strain showed about a 5- to 10-fold induction of β-galactosidase in MSSP medium (21, 26). Acetosyringone induction of the *vir* genes in AB medium (32) was much stronger: 30- to 100-fold. In both media the induction of the *virB* and *virE* fusions was similar; *virG* was induced to a lesser extent. Our results are similar to those of Stachel and Nester (26) for these particular insertions in *virB* and *virE*. None of the mutants showed any induction of β-galactosidase in the presence of acetosyringone in either MSSP or AB medium (Table 5). For this reason these mutants were named Ivr (induction of *vir* genes).

As described by Winans et al. (32), *virG* could be induced to a limited extent (about one-fifth the induction seen with acetosyringone) by low-pH, low-phosphate medium in wild-type strain A6. Low-pH, low-phosphate medium failed to induce *virG* to any significant extent in all three Ivr mutants.

One possible explanation for the failure of acetosyringone to induce *vir* gene expression in Ivr-211, Ivr-223, and Ivr-225 is reduced ability of acetosyringone to enter the mutant bacteria. Susceptibility of bacteria to crystal violet has been used as a measure of cell permeability to small hydrophobic molecules (7). Parent strain A6 was sensitive to crystal violet concentrations of 0.4 μg/ml. Mutants Ivr-211 and Ivr-223 were resistant to 0.4 μg/ml and sensitive to concentrations of >0.7 μg/ml. Mutant Ivr-225 was only sensitive to concentrations of >0.9 μg/ml.

DISCUSSION

The known steps required for tumor formation by *A. tumefaciens* are bacterial attachment to host cells, *vir* gene induction, T-DNA transfer, and T-DNA integration into host cell DNA. One group of previously described avirulent

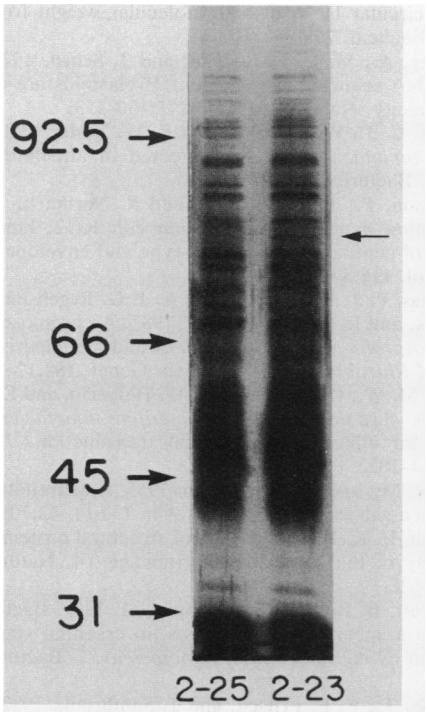

FIG. 4. Protein SDS-PAGE of outer membrane and periplasmic space proteins extracted from mutants Ivr-223 and Ivr-225. The running gel was 11% acrylamide and the stacking gel was 3% acrylamide. The numbers to the left of the gel indicate the position of protein molecular weight standards. Note that Ivr-225 is missing a band at about 79 kDa which is present in Ivr-223. The gel band patterns for Ivr-211 and the parent A6 strain were the same as that for Ivr-223.

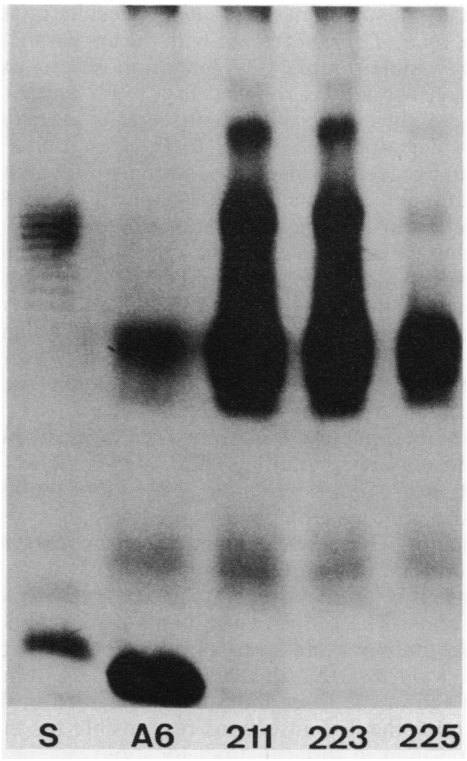

FIG. 5. SDS-PAGE of LPS extracted from the parent *A. tumefaciens* A6 and mutants Ivr-211, Ivr-223, and Ivr-225. Each of the extracts loaded on the gel contained 0.5 μg of ketodeoxyoctonate. A running gel of 15% acrylamide and a stacking gel of 3% acrylamide were used. The standard (S) was 5 μg of *E. coli* O111:B4 LPS purchased from Sigma. Note that the LPS of each of the mutants is larger in average size than that of A6. Similar results were obtained when the LPS of the mutant strains which resulted from the marker exchange of the cloned Tn*5* insertions were examined.

mutations of *A. tumefaciens* which are located on the bacterial chromosome result in inability of the bacteria to bind to plant host cells. The three mutations described in this study do not affect bacterial binding to carrot cells. A second

TABLE 5. Induction of β-galactosidase from insertions in *virB* and *virE* in wild-type and mutant *A. tumefaciens*

Bacterial strain	Plasmid	β-Galactosidase (U)[a] in:					
		MSSP[b]	MSSP with 20 μM acetosyringone	AB[c]			
				pH 7	pH 5.5	pH 5.5, with 50 μM acetosyringone	pH 5.5, low phosphate
Parent (A6)	pSM30(*virB*)	354 ± 40	1,590 ± 150	70 ± 12	75 ± 19	14,920 ± 2,000	155 ± 20
	pSM358(*virE*)	305 ± 20	1,580 ± 100	70 ± 16	116 ± 33	16,065 ± 2,000	284 ± 20
	pSW174(*virG*)	25 ± 5	324 ± 100	25 ± 6	218 ± 150	6,590 ± 2,000	1,394 ± 300
Mutants							
Ivr-211	pSM30(*virB*)	163 ± 40	162 ± 60	12 ± 8	31 ± 15	27 ± 11	76 ± 25
	pSM358(*virE*)	164 ± 20	166 ± 50	15 ± 10	3 ± 3	19 ± 10	100 ± 25
	pSW174(*virG*)	13 ± 7	13 ± 10	1 ± 1	6 ± 6	5 ± 3	8 ± 7
Ivr-223	pSM30(*virB*)	268 ± 30	220 ± 60	565 ± 130	332 ± 140	476 ± 170	371 ± 150
	pSM358(*virE*)	204 ± 80	200 ± 40	480 ± 300	431 ± 210	400 ± 100	570 ± 230
	pSW174(*virG*)	18 ± 2	19 ± 2	3 ± 2	15 ± 3	24 ± 13	35 ± 15
Ivr-225	pSM30(*virB*)	38 ± 30	58 ± 20	2 ± 2	6 ± 3	3 ± 3	1 ± 1
	pSM358(*virE*)	26 ± 9	39 ± 15	97 ± 40	160 ± 150	58 ± 10	161 ± 150
	pSW174(*virG*)	17 ± 7	17 ± 5	2 ± 2	2 ± 2	1 ± 2	7 ± 7

[a] Measured as described by Stachel and Nester (26).
[b] Modified Murashige and Skoog medium (21, 26).
[c] As described by Winans et al. (32).

type of chromosomal mutation, *chvD*, results in attenuated virulence on *B. diagremontiana* with the appearance of small but easily visible tumors (32). Induction of *virG* expression by acetosyringone was reduced in a *chvD* mutant but was not zero. A third type of chromosomal avirulent mutation has been described, *chvE* (9). In these mutants, *vir* gene induction by acetosyringone is strongly reduced (8). The three mutations described in this paper, like *chvE*, reduce the induction of *vir* genes by acetosyringone.

Although *vir* genes in *chvE* mutants cannot be induced by acetosyringone, *virG* could be induced in *chvE* by low-pH, low-phosphate medium (9). *vir* genes in the three Ivr mutants described here could not be induced by acetosyringone or by low-pH, low-phosphate medium. Thus, the Ivr mutants differ from *chvE* mutants in their response to low-pH, low-phosphate medium.

chvE mutations are host range mutations and induce tumors on sunflower, zinnia, and *N. glauca* stems. They are avirulent on *B. diagremontiana* leaves and tomato stems. One of the mutants described here, Ivr-211, is also a host range mutant. However, its host range appears to be different from that of the *chvE* mutants described by Huang et al. (9) since it is virulent on tomato stems and avirulent on *N. glauca* stems. The other two mutants do not appear to be host range mutants and were avirulent on *B. diagremontiana*, *N. tobacum*, *N. debneyi*, *N. glauca*, tomato, and zinnia. Each of the three mutations described in this paper are different; the Tn*5* insertions responsible for the mutant phenotype map in three distinct locations on the bacterial chromosome.

The reason for the failure to induce *virB*, *virE*, and *virG* expression in the Ivr mutants with acetosyringone is unclear. The dye crystal violet has been used to measure the ease of penetration of small hydrophobic molecules into bacteria (7, 33). The MIC of crystal violet was higher for the mutants than for the parent bacteria, but the difference was not very large (at most, a factor of 2). The only surface property which was altered in all three mutants was LPS, which was larger in the mutant than in the parent bacteria. The significance of the altered LPS is unclear. LPS has been proposed to be involved in the binding of *A. tumefaciens* to host cells (31). However, these mutants showed normal binding to host cells. Instead the altered LPS appeared to be associated with these mutations, which also resulted in the inability of the bacteria to induce *vir* genes in response to acetosyringone.

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