JOURNAL OF BACTERIOLOGY, Nov. 1987, p. 5113–5118
0021-9193/87/115113-06$02.00/0

Vol. 169, No. 11

Dual Control of *Agrobacterium tumefaciens* Ti Plasmid Virulence Genes

TIMOTHY J. CLOSE,[1]† PETER M. ROGOWSKY,[1] CLARENCE I. KADO,[1]* STEPHEN C. WINANS,[2] MARTIN F. YANOFSKY,[2]‡ AND EUGENE W. NESTER[2]

Department of Plant Pathology, University of California, Davis, California, 95616,[1] and Department of Microbiology, University of Washington, Seattle, Washington 98195[2]

Received 12 February 1987/Accepted 15 August 1987

The virulence genes of nopaline (pTiC58) and octopine (pTiA6NC) Ti plasmids are similarly affected by the *Agrobacterium tumefaciens ros* mutation. Of six *vir* region complementation groups (*virA*, *virB*, *virG*, *virC*, *virD*, and *virE*) examined by using fusions to reporter genes, the promoters of only two (*virC* and *virD*) responded to the *ros* mutation. For each promoter that was affected by *ros*, the level of expression of its associated genes was substantially elevated in the mutant. This increase was not influenced by Ti plasmid-encoded factors, and the mutation did not interfere with the induction of pTiC58 *vir* genes by phenolic compounds via the VirA/VirG regulatory control mechanism. The effects of the *ros* mutation and acetosyringone were cumulative for all *vir* promoters examined. The pleiotropic characteristics of the *ros* mutant include the complete absence of the major acidic capsular polysaccharide.

Crown gall, a tumor disease of plants caused by *Agrobacterium tumefaciens* (29), affects a wide range of gymnosperms and dicotyledonous angiosperms (7). There is mounting evidence that a number of monocots can be infected, although the production of tumorous growth is not generally characteristic of infection in monocots other than some members of the orders *Liliales* and *Arales* (7, 13, 18). Plants become susceptible to infection at wound sites after a conditioning response takes place (4). The infection process involves attachment of the bacterium to the plant cell (19) and subsequent transfer of part of the Ti plasmid DNA from the bacterium to host plant cells (reviewed in reference 23).

The Ti plasmid contains two clusters of genes that are directly related to the infection process. One of these clusters is the T-DNA, which is transferred to the plant and contains genes for the biosynthesis of plant growth regulators and modified sugars and amino acids, collectively called opines, that serve as carbon and nitrogen sources for the invading bacteria (reviewed in reference 23). The second cluster is the *vir* region, comprising about 30 kilobase pairs (kb) of DNA, which contains genes that are expressed in the bacterium during the course of infection. The *vir* region contains six complementation groups (*virA*, *virB*, *virG*, *virC*, *virD*, and *virE*) that are common to octopine (31) and nopaline (28) Ti plasmids. Octopine Ti plasmids contain a seventh complementation group, *virF* (17), that is not present in nopaline Ti plasmids (15). The functions of the genes within these groups are currently being ascertained. For instance, VirA and VirG are involved in sensing diffusible phenolic compounds produced by wounded plant tissue (2, 31) and in transmitting a regulatory signal that increases the transcription of the remaining *vir* genes (28, 35). At least two of the *virD* genes are involved in T-DNA processing (1, 32, 37), and other Vir functions seem to influence the infection efficiency and host range of *A. tumefaciens* (14, 15, 36).

Genes present elsewhere than on the Ti plasmid are also involved in the infection process. Mutations in either of two chromosomal genes, *chvA* and *chvB*, result in a marked reduction in the binding of *A. tumefaciens* to plant cells and a concomitant reduction of virulence (9). Exopolysaccharide-deficient mutants of *A. tumefaciens* that can be complemented by the *exoC* locus of *Rhizobium meliloti* (5) are also avirulent and attachment deficient, as are a group of *Agrobacterium* mutants that eliminate the production of one or more periplasmic or outer membrane proteins (20). Mutations of another locus, *ros*, increase the expression of *virC* (formerly *bak*) and *virD* (formerly *hdv*) genes in the absence of contact with plant cells and with no additional requirement for Ti plasmid-encoded functions (6). Mutations in the *ros* locus (chromosomal or cryptic megaplasmid) cause a pleiotropic phenotype that includes a dry, florette colony morphology and an inability to grow at low temperatures (6). We have extended our analysis of the basis of this mutation since it seemed possible that the Ros phenotype is in some way characteristic of a physiological state normally experienced by the bacterium in the course of its interactions with plant cells and in which the mutant is genetically trapped. Here we show the effect of the *ros* mutation on the *vir* genes of nopaline and octopine Ti plasmids by using reporter genes fused to the various well-characterized *vir* promoters (28, 31). Our evidence indicates that *ros* regulation operates on a subset of the *vir* genes and is independent of the VirA/VirG mechanism. The major acidic exopolysaccharide is also completely absent in the *ros* mutant.

MATERIALS AND METHODS

Bacterial strains and plasmids. *Escherichia coli* HB101 (*proA leuB lacY thi hsdS20 recA rpsL20*) was from H. W. Boyer. *A. tumefaciens* LBA4301 (Rec$^-$ Rif^r [rifampin resistant], pTiAch5-free) was from Rob Schilperoort. LBA4301 *ros1* was described previously (6). pTiC58 Tra^c is a transfer-constitutive nopaline Ti plasmid (6) that was introduced into LBA4301 *ros1* by conjugal transfer from *A. tumefaciens* NT1, which was obtained from Mary-Dell Chilton. The

* Corresponding author.
† Present address: CSIRO Division of Plant Industry, Canberra, ACT 2601, Australia.
‡ Present address: Division of Biology, California Institute of Technology, Pasadena, CA 91125.

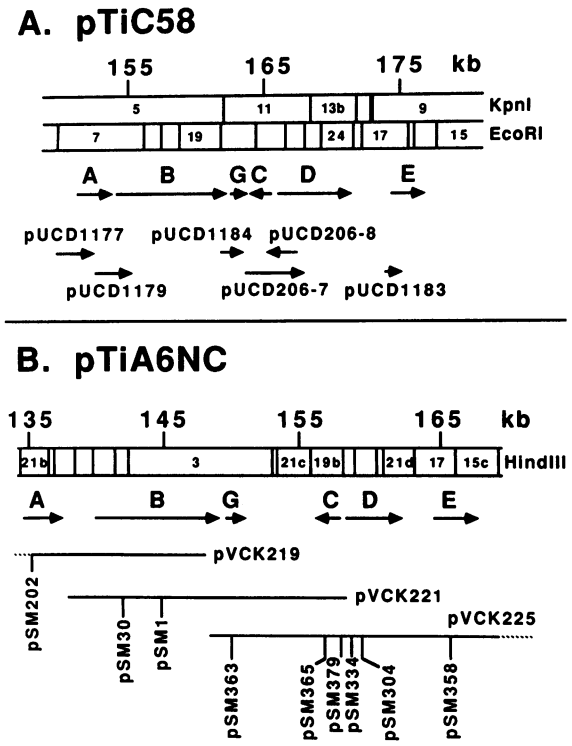

FIG. 1. Maps of Ti plasmid *vir* regions. The positions and directions of transcription of the nopaline (pTiC58) and octopine (pTiA6NC) Ti plasmid *vir* region complementation groups are shown below the physical maps. The coordinates for pTiC58 are based on those of Holsters et al. (16), and the positions of the complementation groups are as described elsewhere (28). pTiC58 *vir* region promoter fragments (pUCD) are also shown and have been described previously (6, 28). The coordinates above the *Hin*dIII map of pTiA6NC are from De Vos et al. (8), and the positions of the complementation groups and the Tn*3*-HoHo1 insertion sites (pSM) in the cosmid fragments (pVCK) covering this region are from Stachel and Nester (31). Octopine plasmids also contain *virF* (not shown) to the right of *virE* (17).

LBA4301 recipient contained a tetracycline resistance (*tet*) gene on a segregatable plasmid. LBA4301 *ros1*(pTiC58 Tra^c) transconjugants were identified as colonies that were tetracycline and rifampin resistant and utilized nopaline as a sole carbon and nitrogen source and also by the additional properties of supersensitivity to agrocin 84 and the typical dry-colony morphology of *ros* mutant strains. The tetracycline resistance plasmid was eliminated from the transconjugant by segregation, and the resulting strain was tested for virulence on several plant hosts.

pTiC58 *vir* region promoter fragments (Fig. 1A) fused to *cat* in the vector pUCD206B (6, 28) were introduced into *A. tumefaciens* by transformation. All of the pTiA6NC *vir-lacZ* fusion plasmids (31) (Fig. 1B) or *R. meliloti* pSym *nod-lacZ* (21) fusion plasmids were introduced by triparental matings from *E. coli* with the helper plasmid pRK2013, obtained from D. Helinski.

Growth conditions. *A. tumefaciens* strains were routinely maintained on medium 523 (6) agar with rifampin (25 μg/ml). Plasmids based on the vector pUCD206B were maintained by the addition of neomycin (25 μg/ml), and plasmids containing pTiA6NC *vir* or pSym *nod* promoters were maintained by adding kanamycin (20 μg/ml). Cultures used to generate the data in Tables 1 and 2 were grown in 523 broth with antibiotics, and those used in Table 3 and Fig. 2 were grown in Murashige and Skoog plant medium (GIBCO Laboratories) supplemented with 12.5 mM potassium phosphate buffer, pH 5.7. Cultures for Table 3 also contained neomycin (20 μg/ml), and those for Fig. 2 contained rifampin (25 μg/ml) and neomycin (10 μg/ml). Induction with acetosyringone is described elsewhere (28).

Enzyme assays. Procedures for determining the specific activity of chloramphenicol acetyltransferase (CAT) and β-galactosidase from crude extracts have been described previously (2, 6). Briefly, log-phase cells were collected by centrifugation, washed once, and sonicated. Cell debris was removed by centrifugation, and the supernatant fluid was assayed for enzyme activity. Bovine serum albumin (Sigma Chemical Co.) was used as a standard for the protein assay of Bradford (3).

T-DNA right-border cleavage assay. The cell pellet from 5 ml of culture, grown as described above, was washed once with H_2O, frozen at −20°C, and then suspended in TEN buffer (10 mM Tris [pH 8.0], 10 mM NaCl, 1 mM EDTA). Pronase (0.1 ml of a 2.5-mg/ml solution) and then *N*-lauroyl sarcosine (0.1 ml of a 5% solution) were added (both were dissolved in TEN buffer). Following a 60-min incubation at 37°C, the viscous lysate was passed 14 times up and down through a disposable 1-ml plastic tip for an adjustable pipette. Phenol and chloroform (0.5 ml of a 1:1 mixture) were added, and the tubes were inverted 80 times. Samples were then centrifuged, and 320 μl of the aqueous phase was transferred to a fresh tube, with care taken to minimize the transfer of material at the solvent interface. The phenol-chloroform extraction was repeated once, this time with 240 μl transferred to a fresh tube. This aqueous solution was extracted two times with 0.5 ml of chloroform by inverting 80 times, centrifuging for 2 min, and then removing the chloroform from the tube after each extraction. The aqueous sample was then centrifuged for 3 min, and 200 μl was transferred to a fresh tube. NaCl (8 μl of a 5 M solution) and then 208 μl of cold (−20°C) isopropanol were added, and the sample was allowed to stand at room temperature for 5 min. Following a 5-min centrifugation at room temperature, the supernatant was removed, and the nucleic acid pellet was washed once with 1 ml of 70% ethanol and then dried inverted. The dried pellet was dissolved in 120 μl of TEN buffer and 1 μl of pancreatic RNase (5 mg/ml in 10 mM Tris [pH 7.5]–15 mM NaCl, heated in a boiling water bath for 15 min). Rehydration of the nucleic acid required 16 h on ice. DNA (6 to 12 μl) was then digested with restriction endonuclease and examined after gel electrophoresis and transfer to Zeta-probe membrane (BioRad Laboratories).

Exopolysaccharide analysis. One milliliter of a fresh overnight culture of each strain grown in medium 523 was used to inoculate 100 ml of a nitrogen-limited minimal medium composed of (per liter): 5 g of glucose, 0.1 g of $(NH_4)_2SO_4$, 0.2 g of $MgSO_4$, 1 g of Na_2HPO_4, 3 g of KH_2PO_4, and 5 ml of Davis City tap water (trace metals). The pH was adjusted to pH 7.4. The cultures were shaken vigorously in 500-ml Erlenmeyer flasks at 28°C for 5 days. Cells were removed by two 20-min centrifugations at 12,000 × *g*. The acidic polysaccharide material in the cleared supernatant fluids was precipitated by the addition of 0.3 volume of 1% HTAB (hexadecyl trimethyl ammonium bromide) and centrifugation for 10 min at 7,000 × *g*. The pellet was dissolved in 10% (wt/vol) NaCl and then precipitated with 3 volumes of 95% ethanol and redissolved in distilled H_2O. The material was reprecipitated twice with 3 volumes of ethanol and dried under a stream of N_2 gas. The quantity of final precipitated

TABLE 1. CAT expression from pTiC58 *vir-cat* fusions in wild-type *A. tumefaciens* and the *ros* mutant

Gene fused to *cat*	Plasmid designation	Avg CAT activity (nmol/min per mg of protein) ± SD[a]			
		Wild type		*ros* mutant	
		−Ti	+Ti	−Ti	+Ti
virA	pUCD1177	63.9 ± 4.2	57.3 ± 4.1	109 ± 14.5	168 ± 2.4
virB	pUCD1179	4.9 ± 0.6	3.8 ± 1.5	3.6 ± 1.5	5.3 ± 0.2
virG	pUCD1184	108 ± 24.2	101 ± 14.1	167 ± 32.6	182 ± 37.3
virC	pUCD206-8	<0.4	<0.4	65.8 ± 13.5	84.2 ± 1.8
virD	pUCD206-7	17.1 ± 7.8	18.8 ± 1.1	148 ± 15.3	164 ± 11.5
virE	pUCD1183	15.1 ± 1.1	15.3 ± 1.2	22.9 ± 1.7	23.5 ± 0.9
None	pUCD206B	2.9 ± 0.3	3.0 ± 0.2	3.3 ± 0.9	2.4 ± 1.2
cat	pSa4	1,187 ± 109	ND[b]	1,503 ± 105	ND

[a] Averages of at least duplicate determinations.
[b] ND, Not determined.

material was determined by an anthrone assay (27) with glucose as the standard, and its hexose composition was analyzed by gas chromatography following complete hydrolysis by trifluoroacetic acid, reduction by $NaBH_4$, and acetylation with acetic anhydride (30). Inositol was used as an internal standard for calibration of the chromatograph against predetermined migration rates for a number of hexoses, including inositol, galactose, and glucose.

RESULTS

***vir* region genes affected by the *ros* mutation.** The *vir* regions of the nopaline Ti plasmid pTiC58 and the octopine Ti plasmid pTiA6NC are shown in Fig. 1. We reported previously that the *bak* (now *virC*) and *hdv* (now *virD*) genes of pTiC58 were expressed at elevated levels in the *A. tumefaciens ros* mutant (6). To examine the effect of *ros* on the remaining pTiC58 *vir* gene promoters, we have now used a set of *cat* fusion plasmids that carry promoters from *virA*, *virB*, *virG*, and *virE*, as well as *virC* and *virD* (28). Each promoter is fused to a promoterless *cat* gene in the vector pUCD206B (6). The expression of *cat* from each promoter was examined in wild-type LBA4301 and the *ros* mutant (Table 1). Each fusion was tested in these strains in the presence and absence of pTiC58 to determine whether the Ti plasmid influences the effect of the *ros* mutation. For this study, all cultures were grown in 523 broth at 30°C and harvested at late log phase (130 Klett turbidity units, green filter). In all cases, including the control plasmid pSa4 (6), whose *cat* gene is regulated by the native *cat* promoter, we observed a slightly higher level of *cat* activity in the *ros* strain than in the wild type. *virC* and *virD* expression was substantially higher in the *ros* mutant than in the wild type. In contrast, none of the other *vir* promoters responded to *ros*, their increase in every case being about the same as the background level for pSa4. The presence or absence of the complete Ti plasmid seemed to have no influence on *vir* gene expression in either strain under these growth conditions.

The homologous loci of the octopine Ti plasmid pTiA6NC were tested in a similar manner. Instead of subcloning promoter active fragments, we used the available transposon Tn*3*-HoHo1 insertions in pTiA6NC cosmid clones (31), which place *lacZ* under the control of the various *vir* promoters (Fig. 1B). Each cosmid was tested in otherwise Ti plasmid-free *A. tumefaciens* LBA4301 and the *ros* strain under conditions identical to those used above for the pTiC58 *vir* promoters (Table 2). In parallel with the results for pTiC58, only *virC* and *virD* fusions responded strongly to the *ros* mutation.

The exact values of the ratios of specific activities in the *ros* mutant strain versus the wild type differ somewhat between pTiC58 and pTiA6NC (132 versus 60.2 and 76.6, respectively, for *virC*, and 8.7 versus 102 and 31.9, respectively, for *virD*). The differences in these ratios may be due to actual differences between pTiC58 and pTiA6NC in the basal levels of *virC* and *virD* expression or may reflect some variability that is inherent in using different enzyme fusions and different fusion sites within any given operon. It is clear that the *ros* mutation resulted in at least a 60-fold increase in *virC* and at least a 9-fold increase in *virD* expression. The *ros* mutation did not affect the expression of *R. meliloti* Sym plasmid promoters for *nodC* and *nodD* (21) (Table 2).

pTiC58 *vir* gene induction with acetosyringone. Since it has been shown for nopaline and octopine Ti plasmids that *virC* and *virD* respond not only to *ros*, but also to phenolic compounds (2) such as acetosyringone (31), we examined the effect of the *ros* mutation on the induction of pTiC58 *vir* gene promoters fused to *cat* (Table 3). The presence of intact Ti plasmid pTiC58 Tra^c, which contains *virA* and *virG*, was used as a means of enabling these strains to respond to acetosyringone. *virA*, *virB*, and *virE*, each of which was induced by acetosyringone but not increased strongly in the *ros* mutant, showed essentially the same ratios of expression with and without inducer whether the fusions were harbored in the wild type or the *ros* mutant. The level of *virC* and *virD* expression still increased in the *ros* mutant in response to

TABLE 2. β-Galactosidase expression from pTiA6NC *vir-lacZ* fusions in wild-type *A. tumefaciens* and the *ros* mutant

Gene fused to *lacZ*	Plasmid designation	β-Galactosidase sp act (nmol/min per mg of protein) ± SD[a]	
		Wild type	*ros* mutant
virA	pSM202	37.4 ± 1.4	40.6 ± 0.3
virB	pSM1	15.0 ± 0.3	11.6 ± 0.8
virB	pSM30	7.2 ± 0.2	4.8 ± 0.2
virG	pSM363	171 ± 17	219 ± 20
virC	pSM365	2.4 ± 0.1	141 ± 18.3
virC	pSM379	1.4 ± 0.1	111 ± 14.9
virD	pSM304	6.4 ± 0.4	658 ± 132
virD	pSM334	2.3 ± 0.4	71.8 ± 6.0
vir E	pSM358	48.9 ± 1.6	47.6 ± 2.5
None		0.0 ± 0.1	0.0 ± 0.1
nodD	pRmM61	360 ± 159	215 ± 24
nodC	pRmM57	6.1 ± 1.1	3.1 ± 0.4

[a] Averages of duplicate determinations ± standard deviation, after subtracting the basal level of activity (3.4 units for the wild type and 2.4 units for the *ros* mutant).

TABLE 3. Effect of *ros* on the VirA/VirG-mediated induction of pTiC58 *vir* genes

Gene fused to *cat*[a]	CAT sp act (nmol/min per mg of protein)[b]			
	Wild type		*ros* mutant	
	Without acetosyringone	With acetosyringone	Without acetosyringone	With acetosyringone
virA	68	223	165	539
virB	5	1,000	5	1,530
virG	134	113	256	209
virC	<0.4	127	37	300
virD	11	550	310	1,040
virE	30	990	51	1,220
None	<0.4	<0.4	<0.4	<0.4

[a] Plasmids are listed in Table 1.
[b] One assay for each sample.

acetosyringone. The VirA/VirG-mediated mechanism of induction is thus not abolished or superseded by the *ros* mutation. In every case, there was no response to acetosyringone when pTiC58 Tra^c was absent from the genetic background (data not shown).

Other evidence from a more direct assay for *virD* expression indicated that *virD* was in fact expressed at the highest levels in the *ros* mutant under acetosyringone-induced conditions (Fig. 2). Using the 14.2-kb *Eco*RI fragment that contained the pTiC58 right border as a probe (Fig. 2A), we observed the smallest amount of double-stranded right-border cleavage in the wild-type strain without acetosyringone induction and the greatest amount in the *ros* strain with acetosyringone induction (Fig. 2B). No detectable border cleavage was observed under the same conditions with a *virD* mutant Ti plasmid (Fig. 2C). That the observed cleavage occurred at the position of the right border was verified by the same assay with *Xba*I- and *Kpn*I-digested DNA (data not shown). In this assay, we used the appearance of double-stranded cleavage products as an indicator of *virD* activity (32), although one could use the appearance of single-stranded T-DNA intermediates for the same purpose (37). The data presented here do not address the issue of which structures are essential to T-DNA processing and transfer. Together, these results indicate that the effects of *ros* and VirA/VirG represent two independent and additive regulatory controls over *virC* and *virD* gene expression.

ros mutant strain lacks the major acidic capsular polysaccharide. As reported earlier, *ros* colonies are nonmucoid. Nonmucoid strains of *A. tumefaciens* have been shown to be deficient in the production of their major acidic capsular polysaccharide and because of this deficiency show decreased fluorescence under UV light when grown on medium containing the optical brightening agent calcofluor (5). We found strong, bright fluorescence for the wild type versus dim, bluish gray fluorescence for *ros* mutant colonies (Fig. 3A). To characterize the capsular material, *A. tumefaciens* strains were grown in carbon-rich, nitrogen-limited medium to stimulate the production of exopolysaccharide (10), which was then processed and characterized as described in Materials and Methods. Supernatant fluids of wild-type cultures were quite viscous, but *ros* cultures looked no more viscous than the starting medium. When HTAB was added (see Materials and Methods), a dense, stringy precipitate immediately formed in the wild-type supernatants, and no visible precipitate formed in the supernatants from the *ros* strains even after overnight incubation (Fig. 3B). The concentration of this material in the culture supernatant was found to be 2.5 to 3.0 g/liter in the wild-type and less than 5 mg/liter in the mutant strains. The hexose composition of this material was approximately 6.7 mol of glucose per mol of galactose. These data are consistent with the idea that the major exopolysaccharide of *A. tumefaciens* contains the same repeating octasaccharide that is the principal component of the major acidic exopolysaccharide of *R. meliloti* (5). Since the *ros* mutant harboring a Ti plasmid is still virulent (Fig. 3C), the production of the major acidic capsular polysaccharide by *A. tumefaciens* must not be required for virulence. Similar conclusions were reached by Cangelosi et al. (5), many of whose calcofluor-dark strains lacked the major acidic exopolysaccharide and were still virulent. Two known effects of the *ros* mutation are thus to abolish acidic exopolysaccharide production by affecting non-Ti-associated genes and to cause constitutive expression of Ti plasmid *virC* and *virD* genes.

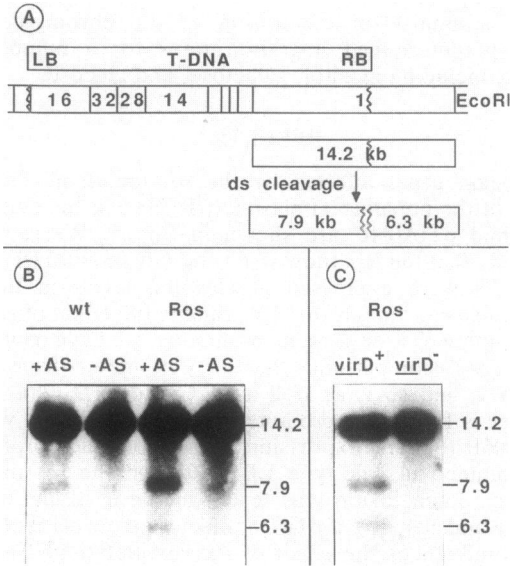

FIG. 2. Conditions for maximum *virD* expression. (A) The pTiC58 T-DNA right border (RB) is contained in *Eco*RI fragment 1 (14.2 kb). One of the events observed after induction of the host strain with acetosyringone is *virD*-dependent, double-stranded (ds) cleavage at T-DNA borders, leading to 7.9-kb and 6.3-kb fragments instead of a single 14.2-kb fragment after *Eco*RI digestion. (B) The wild-type (wt) strain LBA4301 and the *ros* mutant strain were grown in the presence of acetosyringone (AS), and DNA was isolated as described in Materials and Methods. DNA fragments were separated in 0.7% agarose gels in a buffer containing 40 mM Tris (pH 7.9), 5 mM sodium acetate, and 2 mM EDTA. DNA was electrophoretically transferred to a Zeta-Probe membrane in the same buffer following ethidium bromide staining, photography on a UV transilluminator, depurination in 0.25 M HCl, and neutralization in 0.5 M NaOH–1.5 M NaCl. The transferred DNA was cross-linked to the membrane by using UV light and probed with nick-translated ^{32}P-labeled *Eco*RI fragment 1 (0.2 μg, 2 × 10^6 cpm) in 6× SSC (1× SSC is 0.15 M NaCl plus 0.015 M sodium citrate) buffer at 68°C for 8 h. (C) The *ros* mutant strain was similarly examined when containing Ti plasmids with Tn*5* insertions and grown with acetosyringone. The *virD* mutant Ti plasmid is pJK195 (28), and the *virD*-proficient Ti plasmid is pJK503, a *virC* mutant (28). The same cleavage pattern was observed for all Tn*5* insertions except those in *virA*, *virG*, and the first gene of *virD* (T. Steck, personal communication).

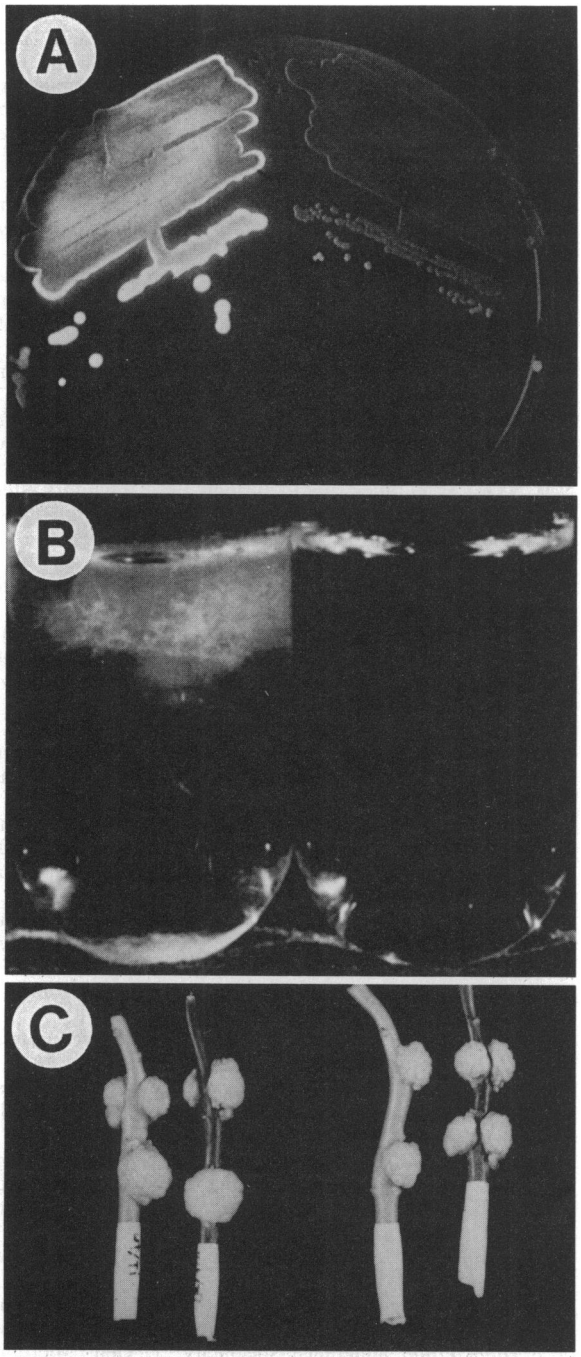

FIG. 3. Properties of the *ros* mutant. (A) Fluorescence of LBA4301 (left) and LBA4301 *ros1* (right) colonies on medium 523 containing 0.05% calcofluor and photographed under UV light. (B) Precipitation of acidic polysaccharide from the supernatant of LBA4301 and LBA4301 *ros1* by the addition of 0.3 volume of HTAB (see Materials and Methods). (C) Tumor formation by LBA4301 and LBA4301 *ros1*, each carrying pTiC58 Tra^c, on *Datura stramonium* stems 30 days after inoculation.

DISCUSSION

The same *vir* genes (*virC* and *virD*) responded to the *ros* mutation in nopaline as well as octopine Ti plasmids, and the VirA/VirG-mediated response to acetosyringone seemed to be unaffected by the *ros* mutation. The production of acidic exopolysaccharide was abolished in the *ros* mutant, but the *ros* strain was still virulent when carrying a Ti plasmid. A model for *ros* regulation must therefore account for decreases as well as increases in the expression of target genes.

Global regulatory networks have been well established for bacteria (11). Among them are examples that can mechanistically account for the type of regulation that we seem to find with *ros*. Direct regulators such as the cyclic AMP receptor protein and the *ntrC* gene product can mediate repression or induction of their target loci (24, 26). Alternatively, as with RecA in the SOS circuit, a modulator can switch the activity of a regulator, such as LexA, so that target genes may be either repressed or induced (33). In the SOS system the target genes may themselves be either activators or inhibitors of cellular functions. As a specific example, the control of capsule synthesis in *E. coli* is mediated by two positive (*rcsA* and *rcsB*) and two negative (*rcsC* and *lon*) regulatory genes, one of which (*lon*) is a component of a global regulatory network (12). In short, when one considers the numerous possibilities in multilayered regulatory networks, the pleiotropic nature of the Ros phenotype does not present a conceptual problem.

Since the *ros* mutant lacked acidic exopolysaccharide regardless of whether a Ti plasmid was present, it is plausible that the effects of the *ros* mutation on *virC* and *virD* would be farther down a regulatory cascade than its effects on exopolysaccharide production. We have isolated a number of exopolysaccharide-deficient mutants of LBA4301 by using Tn*5* mutagenesis, but none resulted in the increase in *virC* and *virD* expression characteristic of the *ros* mutant (data not shown). A deficiency in exopolysaccharide synthesis is therefore not sufficient to increase *virC* and *virD* expression.

Whether the *ros* locus encodes a direct or indirect regulator of its target genes, the ultimate control exerted over the *virC* and *virD* genes via *ros* could be by either a positive or a negative regulator. Two lines of evidence favor negative regulation. (i) As we have shown previously, the *virC* and *virD* genes are expressed at low basal levels in *A. tumefaciens* wild-type strains but are expressed at very elevated levels in *E. coli* (6). It is simpler to suppose that a *virC/D* repressor is not present in *E. coli* than that a suitable positive regulator exists. (ii) DNA can be transcribed efficiently and accurately in vitro from the *virC* and *virD* promoters by RNA polymerase isolated from wild-type *A. tumefaciens* (R. C. Tait and C. I. Kado, unpublished data). RNA polymerase must thus be prevented from initiating *virC* and *virD* transcription in wild-type *A. tumefaciens* by a regulator that the *ros* mutation affects.

Since obvious surface alterations exist in the *ros* mutant, it seems worthwhile to consider the possibility that there is some functional relationship between bacterial surface composition and *virC* and *virD* expression. If the Ros phenotype were to reflect a physiological state that is normally experienced during infection, then it is plausible that the *virC* and *virD* genes (at least some of them, since the first two *virD* genes are known to be involved in T-DNA processing) may be involved in establishing virulence-specific contact. Plant cell wall materials and bacterial surface components that are involved in plant-bacteria adhesion should thus be considered candidates for triggering a "*ros* state." Several candidates already exist in the literature, including pectin-rich plant cell wall fractions that inhibit binding of agrobacteria to plant cells (22), a proteinaceous substance that was reported to induce *virD* (formerly called *virC*) expression (25), and bacterial lipopolysaccharides (34), periplasmic outer mem-

brane proteins (20), and (β-1,2)glucan (5), each of which has been suggested to play a role in binding. Efforts are under way to isolate and characterize the *ros* locus and to establish the mechanism by which the *virC* and *virD* genes are affected by its product(s).

ACKNOWLEDGMENTS

We thank John Labavitch for assistance with the carbohydrate analysis.

This study was supported in part by Public Health Service grants CA-11526, GM-32618, and (to M.F.Y.) GM-07270 from the National Institutes of Health and by USDA-CRGO. S.W. was supported by Damon Runyon-Walter Winchell Cancer Fund Fellowship DRG-800.

LITERATURE CITED

1. **Alt-Morbe, J., B. Rak, and J. Schroder.** 1986. A 3.6 kbp segment from the *vir* region of Ti plasmids contains genes responsible for border sequence-directed production of T region circles in *E. coli*. EMBO J. **5**:1129–1135.
2. **Bolton, G. W., E. W. Nester, and M. P. Gordon.** 1986. Plant phenolic compounds induce expression of the *Agrobacterium tumefaciens* loci needed for virulence. Science **232**:983–985.
3. **Bradford, M. M.** 1976. A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein-dye binding. Anal. Biochem. **72**:248–254.
4. **Braun, A. C.** 1952. Conditioning of the host cell as a factor in the transformation process in crown gall. Growth **16**:65–74.
5. **Cangelosi, G. A., L. Hung, V. Puvanesarajah, G. Stacey, D. A. Ozga, J. A. Leigh, and E. W. Nester.** 1987. Common loci for *Agrobacterium tumefaciens* and *Rhizobium meliloti* exopolysaccharide synthesis and their roles in plant interactions. J. Bacteriol. **169**:2086–2091.
6. **Close, T. J., R. C. Tait, and C. I. Kado.** 1985. Regulation of Ti plasmid virulence genes by a chromosomal locus of *Agrobacterium tumefaciens*. J. Bacteriol. **164**:774–781.
7. **DeCleene, M., and J. Deley.** 1976. The host range of crown gall. Bot. Rev. **42**:389–466.
8. **De Vos, G., M. De Beuckeleer, M. Van Montagu, and J. Schell.** 1981. Restriction endonuclease mapping of the octopine tumor-inducing plasmid pTiAch5 of *Agrobacterium tumefaciens*. Plasmid **6**:249–253.
9. **Douglas, C. J., R. J. Staneloni, R. A. Rubin, and E. W. Nester.** 1985. Identification and genetic analysis of an *Agrobacterium tumefaciens* chromosomal virulence region. J. Bacteriol. **2161**:850–860.
10. **Dudman, W. F.** 1964. Growth and extracellular polysaccharide production by *Rhizobium meliloti* in defined medium. J. Bacteriol. **88**:640–645.
11. **Gottesman, S.** 1984. Bacterial regulation: global regulatory networks. Annu. Rev. Genet. **18**:415–441.
12. **Gottesman, S., P. Trisler, and A. Torres-Cabassa.** 1985. Regulation of capsular polysaccharide synthesis in *Escherichia coli* K-12: characterization of three regulatory genes. J. Bacteriol. **162**:1111–1119.
13. **Grimsley, N., T. Hohn, J. W. Davies, and B. Hohn.** 1987. *Agrobacterium*-mediated delivery of infectious maize streak virus into maize plants. Nature (London) **325**:177–179.
14. **Hille, J., M. Dekker, H. Oude Luttighuis, A. van Kammen, and P. Zabel.** 1986. Detection of T-DNA transfer to plant cells by *A. tumefaciens* virulence mutants using agroinfection. Mol. Gen. Genet. **205**:411–416.
15. **Hirooka, T., and C. I. Kado.** 1986. Location of the right boundary of the virulence region on *Agrobacterium tumefaciens* plasmid pTiC58 and a host-specifying gene next to the boundary. J. Bacteriol. **167**:732–734.
16. **Holsters, M., B. Silva, F. Van Vliet, C. Genetello, M. De Block, P. Dhaese, A. Depicker, D. Inze, G. Engler, R. Villarroel, M. Van Montagu, and J. Schell.** 1980. The functional organization of the nopaline *A. tumefaciens* plasmid pTiC58. Plasmid **3**:212–230.
17. **Hooykaas, P. J. J., M. Hofker, H. den Dulk-Ras, and R. A. Schilperoort.** 1984. A comparison of virulence determinants in an octopine Ti plasmid, a nopaline Ti plasmid, and an Ri plasmid by complementation analysis of *Agrobacterium tumefaciens* mutants. Plasmid **11**:195–205.
18. **Hooykaas-Van Slogteren, G. M. S., P. J. J. Hooykaas, and R. A. Schilperoort.** 1984. Expression of Ti plasmid genes in monocotyledonous plants infected with *Agrobacterium tumefaciens*. Nature (London) **311**:763–764.
19. **Lippincott, B. B., and J. A. Lippincott.** 1969. Bacterial attachment to a specific wound site as an essential stage to tumor induction by *Agrobacterium tumefaciens*. J. Bacteriol. **97**: 620–626.
20. **Matthysse, A. G.** 1987. Characterization of nonattaching mutants of *Agrobacterium tumefaciens*. J. Bacteriol. **169**:313–323.
21. **Mulligan, J. T., and S. R. Long.** 1985. Induction of *Rhizobium meliloti nodC* expression by plant exudate requires *nodD*. Proc. Natl. Acad. Sci. USA **82**:6609–6613.
22. **Neff, N. T., A. N. Binns, and C. Brandt.** 1987. Inhibitory effects of a pectin-enriched tomato cell wall fraction on *A. tumefaciens* binding and tumor formation. Plant Phys. **83**:525–528.
23. **Nester, E. W., M. P. Gordon, R. M. Amasino, and M. F. Yanofsky.** 1984. Crown gall: a molecular and physiological analysis. Annu. Rev. Plant Physiol. **35**:387–413.
24. **Ninfa, A. J., and B. Magasanik.** 1986. Covalent modification of the *glnG* product, NRI, by the *glnL* product, NRII, regulates the transcription of the *glnALG* operon in *Escherichia coli*. Proc. Natl. Acad. Sci. USA **83**:5909–5913.
25. **Okker, R. J. H., H. Spaink, J. Hille, T. A. N. van Brussel, B. Lugtenberg, and R. A. Schilperoort.** 1984. Plant inducible virulence promoter of the *Agrobacterium tumefaciens* Ti plasmid. Nature (London) **312**:564–566.
26. **Railbaud, O., and M. Schwartz.** 1984. Positive control of transcription initiation in bacteria. Annu. Rev. Genet. **18**:178–206.
27. **Roe, J. H.** 1955. The determination of sugar in blood and spinal fluid with anthrone reagent. J. Biol. Chem. **212**:335–343.
28. **Rogowsky, P. M., T. J. Close, J. A. Chimera, J. J. Shaw, and C. I. Kado.** 1987. Regulation of the *vir* genes of *Agrobacterium tumefaciens* plasmid pTiC58. J. Bacteriol. **169**:5101–5112.
29. **Smith, E. F., and C. O. Townsend.** 1907. A plant tumor of bacterial origin. Science **25**:671–673.
30. **Spiro, R. G.** 1972. Study of the carbohydrates of glycoproteins. Methods Enzymol. **28**:3–43.
31. **Stachel, S. E., and E. W. Nester.** 1986. The genetic and transcriptional organization of the *vir* region of the A6 Ti plasmid of *Agrobacterium tumefaciens*. EMBO J. **5**:1445–1454.
32. **Veluthambi, K., R. K. Jayaswal, and S. B. Gelvin.** 1987. Virulence genes *A*, *G*, and *D* mediate the double-stranded border cleavage of T-DNA from the *Agrobacterium* Ti plasmid. Proc. Natl. Acad. Sci. USA **84**:1881–1885.
33. **Walker, G. C.** 1985. Inducible repair systems. Annu. Rev. Biochem. **54**:425–457.
34. **Whatley, M. H., J. B. Margot, J. Schell, B. B. Lippincott, and J. A. Lippincott.** 1978. Plasmid and chromosomal determination of *Agrobacterium* adherence specificity. J. Gen. Microbiol. **107**:395–398.
35. **Winans, S. C., P. R. Ebert, S. E. Stachel, M. P. Gordon, and E. W. Nester.** 1986. A gene essential for *Agrobacterium* virulence is homologous to a family of positive regulatory loci. Proc. Natl. Acad. Sci. USA **83**:8278–8282.
36. **Yanofsky, M., B. Lowe, A. Montoya, R. Rubin, W. Krul, M. Gordon, and E. Nester.** 1985. Molecular and genetic analysis of factors controlling host range in *Agrobacterium tumefaciens*. Mol. Gen. Genet. **201**:237–246.
37. **Yanofsky, M. F., S. G. Porter, C. Young, L. M. Albright, M. P. Gordon, and E. W. Nester.** 1986. The *virD* operon of *Agrobacterium tumefaciens* encodes a site-specific endonuclease. Cell **47**:471–477.