JOURNAL OF BACTERIOLOGY, Feb. 1991, p. 1139–1144
0021-9193/91/031139-06$02.00/0

Vol. 173, No. 3

# Controlled Expression of the Transcriptional Activator Gene *virG* in *Agrobacterium tumefaciens* by Using the *Escherichia coli lac* Promoter

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Received 24 September 1990/Accepted 4 December 1990

**The *Agrobacterium* VirG protein is normally expressed from two promoters in response to multiple stimuli, including plant-released phenolics (at promoter P1) and acidic growth media (at promoter P2). To simplify the analysis of *vir* gene induction, we sought to create *Agrobacterium* strains in which *virG* could be expressed in a controllable fashion. To study the possibility of using the *lac* promoter and repressor, we constructed a plasmid containing the *lac* promoter fused to the *lacZ* structural gene. A derivative of this plasmid containing the *lacI*$^q$ gene was also constructed. The plasmid not containing *lacI*$^q$ expressed high levels of β-galactosidase. The plasmid containing *lacI*$^q$ expressed β-galactosidase at very low levels in the absence of *o*-nitrophenyl-β-D-galactoside (IPTG) and at moderate levels in the presence of IPTG. We also fused the *lac* promoter to a *virG*::*lacZ* translational fusion and found that IPTG elevated expression of this translational fusion to moderate levels, though not to levels as high as from the stronger of the two native *virG* promoters. Finally, the *lac* promoter was used to express the native *virG* gene in strains containing a *virB*::*lacZ* translational fusion. *virB* expression in this strain depended on addition of IPTG as well as the *vir* gene inducer acetosyringone. In a similar strain lacking *lacI*$^q$, *virB* expression was greater than in a strain in which *virG* was expressed from its native promoters. Expression of *virG* from the *lac* promoter did not alter the acidic pH optimum for *vir* gene induction, indicating that the previously observed requirement for acidic media was not due solely to the need to induce P2.**

Models to describe the transcriptional regulation of *Agrobacterium* virulence genes have become steadily more complicated since the phenomenon was first documented just 5 years ago. In 1985, the pioneering work of Stachel and colleagues indicated that the two dozen *Agrobacterium vir* genes required for plant transformation were indeed transcriptionally induced during infection and that *virA* and *virG* were required for this induction (16, 19, 21, 22). Induction depended absolutely on acidic growth media (21). Subsequent studies indicated that VirA and VirG were members of the family of homologous two-component regulatory systems (11, 26). Since that time, it has come to light that *virC* and *virD*, unlike other *vir* genes, are additionally controlled by the chromosomal locus *ros* (6). More recently, it was shown that *vir* gene expression is potentiated by a variety of opines (24). Finally, a variety of monosaccharides and related compounds have been shown to stimulate *vir* gene induction in concert with VirA and a sugar-binding periplasmic protein encoded by the chromosomal *chvE* gene (1, 3).

The transcription of the two regulatory genes *virA* and *virG* is also regulated. *virA* is inducible by acetosyringone in a VirA-, VirG-dependent manner (27). *virG* is expressed from two promoters, one of which (P1) is inducible both by acetosyringone and by phosphate starvation, and the other of which (P2) is inducible by various forms of environmental stress, including acidic media (11a, 22, 23, 25, 27). The second of these promoters resembles the family of *Escherichia coli* heat shock promoters (8). Induction of these promoters during infection results in a several hundred-fold increase in the pool size of VirG. Since the intracellular concentration of VirG (and possibly of VirA as well) limits the efficiency of induction of other *vir* genes (28), these changes in the concentration of VirG are of central importance in the efficiency of *vir* gene expression. These changes also complicate studies of induction. We therefore sought to simplify our analysis of *vir* gene induction by controlling the production of VirG, using the regulatable *lac* promoter.

## MATERIALS AND METHODS

**Strains and plasmids.** *Agrobacterium tumefaciens* A348 is a derivative of A136 containing pTiA6NC (18). A136 is a derivative of strain C58 lacking the nopaline Ti plasmid pTiC58. pSM243, a Tn*3*HoHo1 insertion derivative of cosmid pVK219, contains *virA* and a *virB*::*lacZ* fusion (20). pTZ18R was purchased from U.S. Biochemical. The broad-host-range plasmid pUCD2 was obtained from C. Kado (7). Strain DH5α was purchased from Bethesda Research Laboratories. The *lac* deletion strain MC4100 was obtained from C. Manoil. pMC1403 (4) was obtained from S. Lory. Plasmid pTJS75a (10) was obtained from M. Yanofsky. Plasmid pIC20R (12) was obtained from U. Storb. Plasmid pPC401 (9) was obtained from P. Christie. Plasmid pSW219 was constructed by ligating the large *Bam*HI fragment of plasmid pSM243 (which contains *virA* and a *virB*::*lacZ* translational fusion) to the large *Bam*HI fragment of pUCD2. Antibiotics, 5-bromo-4-chloro-3-indolyl-β-D-galactoside (X-Gal), *o*-nitrophenyl-β-D-galactoside (ONPG), and morpholinoethanesulfonic acid (MES) were purchased from Sigma. Isopropyl-β-D-thiogalactopyranoside (IPTG) was purchased from U.S. Biochemical. Restriction endonucleases and T4 DNA ligase, were purchased from Bethesda Research Laboratories. Acetosyringone was purchased from Aldrich Co.

**Construction of a broad-host-range plasmid containing P*lac* and *lacI*$^q$.** pTJS75a, a 7.5-kb Tc$^r$ plasmid of the IncP incom-

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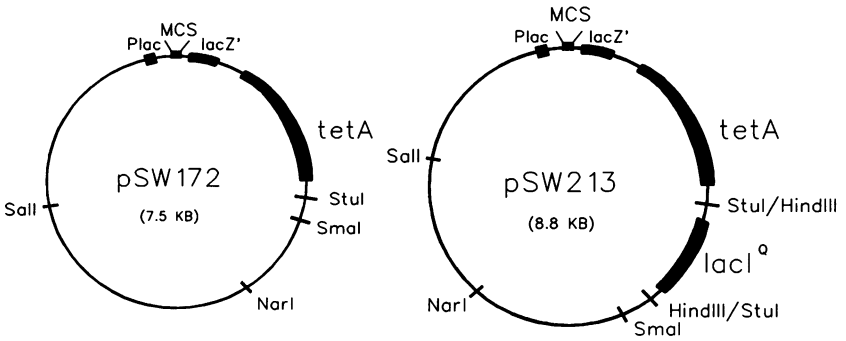


FIG. 1. Physical maps of two broad-host-range plasmids containing the *lac* α complementation group. pSW213 also contains *lacI*$^q$. Restriction sites found at the multiple cloning site (MCS) are *Eco*RI, *Cla*I, *Eco*RV, *Xba*I, *Bgl*II, *Xho*I, *Sac*I, *Nru*I, *Hin*dIII, *Sph*I, *Pst*I, *Sal*I, *Xba*I, *Bam*HI, *Sma*I, *Kpn*I, *Sac*I, and *Eco*RI.

patibility group, replicates in *E. coli* and in *Agrobacterium* spp. (17). This plasmid was subjected to partial digestion with *Hae*II and ligated to *Hae*II fragments of plasmid pIC20R, a plasmid similar to pUC18 but containing 18 restriction sites at the multiple cloning site (12). From this ligation was isolated plasmid pSW172, which is a derivative of pTJS75a containing the multiple cloning site and α complementation group of pIC20R cloned into the *tetR* gene and oriented such that transcription from the *lac* promoter is the same direction as that of the *tetA* promoter (Fig. 1). pSW172 was designed to allow high-level, unregulated expression of the *lac* promoter in *Agrobacterium* strains. To permit controlled expression, a *Hin*dIII fragment containing the *lacI*$^q$ gene from pMMB22 (2) was converted to a blunt-ended form by treatment with the Klenow fragment of DNA polymerase I and introduced into pSW172 digested with *Stu*I, thus giving rise to plasmid pSW213 (Fig. 1).

**Reconstruction of the *lac* operon on broad-host-range plasmids.** To create plasmids that permitted assay of the *lac* promoter activity in *Agrobacterium* strains, we cut plasmids pSW172 and pSW213 with *Bam*HI and introduced *Bam*HI-digested pMC1403. pMC1403 contains the intact *lac* operon beginning at the eighth codon of *lacZ* but does not contain *lacI*, the *lac* promoter, or the *lacZ* translation start site. The resulting plasmids, pCC101 and pCC102, contained the first 27 amino acids of the *lac* α peptide of pSW172 or pSW213 fused in the correct reading frame to the eighth codon of the *lacZ* gene of pMC1403 (Fig. 2).

**Construction of broad-host-range plasmids expressing *virG*.** pSW99 is a derivative of pRK404 cut with *Bam*HI and *Pst*I into which the native *virG* gene was introduced as a *Bgl*II-*Pst*I fragment (Fig. 2). pSW207 and pSW217 were constructed by cutting pSW172 and pSW213, respectively, with *Hin*dIII and treatment with Sequenase and by introducing a *Pvu*II fragment from pPC401. pPC401 is a pTZ18R derivative in which the *lac* promoter strongly transcribes *virG* (9). Both the *lac* promoter and *virG* are on the same *Pvu*II fragment. In both pSW207 and pSW217, the *lac* promoters which express *virG* are oriented in the same direction as the *lac* promoters of the vectors (Fig. 2). To construct a *virG*::*lacZ* translational fusion expressed from the *lac* promoter, pSW217 was cut with *Bam*HI and ligated to *Bam*HI-digested pMC1403, resulting in pCC103 (Fig. 2). pSW174 contains the same *virG*::*lacZ* translational fusion, but expression of this gene fusion is driven from the native *virG* promoters (27).

**Assay of β-galactosidase.** *Agrobacterium* strains were grown in 25 ml of AB medium (5) supplemented with 40 mM MES (pH 7.0) to an optical density of 0.4 at 600 nm, centrifuged, resuspended in 1.0 ml of water containing 15% glycerol, and frozen at −70°C. As needed, cells were thawed, diluted to an optical density of 0.05 at 600 nm into 5.0 ml of induction medium (AB salts, 25 mM phosphate, 40 mM MES [pH 5.25], 0.5% glucose) and cultured at 30°C in culture tubes (20 by 150 mm) in a rotary aerator. After the indicated time intervals, they were assayed for β-galactosidase activity (14).

## RESULTS

To create a broad-host-range plasmid that contained the *lac* promoter and α complementation group, plasmids pSW172 and pSW213 were constructed as described above. Both plasmids contain IncP replication origins, Tc$^r$ determinants, and α complementation groups. pSW213 in addition contains the *lacI*$^q$ gene. DH5α(pSW172) formed blue colonies on solid media containing X-Gal whether or not the gratuitous *lac* inducer IPTG was present, while DH5α

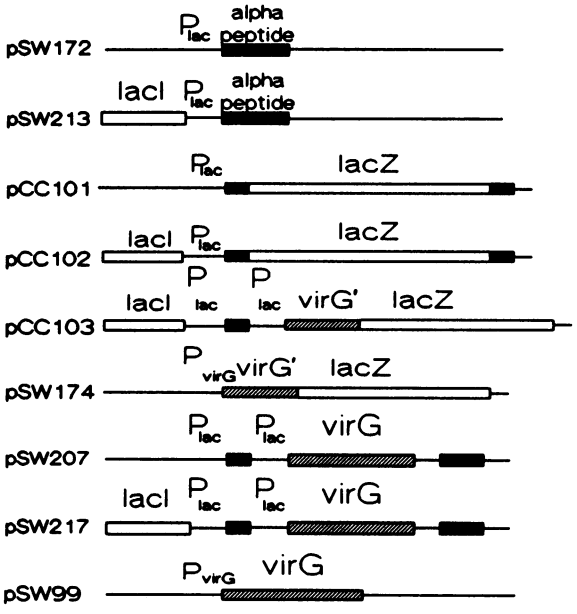


FIG. 2. Schematic representations of plasmids used in this study.

TABLE 1. Activity of the *lac* promoter and *lac* repressor in *E. coli* and *A. tumefaciens*[a]

| Plasmid[b] | Enzyme activity (Miller units) | | | |
|---|---|---|---|---|
| | *E. coli* MC4100 | | *A. tumefaciens* A136 | |
| | 0 mM[c] | 1 mM | 0 mM | 1 mM |
| pCC101 | 2,150 | 2,200 | 2,890 | 3,160 |
| pCC102 | 2 | 1,710 | 3 | 730 |

[a] *E. coli* cells containing the indicated plasmids were cultured in AB medium at 37°C for 3.5 h prior to β-galactosidase assay, while *Agrobacterium* cells were cultured at 30°C for 12 h prior to assay.
[b] pCC101 and pCC102 contain identical P*lac*::*lacZ* translational fusions. pCC102 in addition contains the *lacI*$^q$ gene.
[c] IPTG concentration.

(pSW213) formed white colonies in the absence of IPTG and formed blue colonies only if IPTG was added. This result indicated that the *lac* promoter and the *lacI*$^q$ gene of these plasmids functioned in *E. coli*.

**Measuring *lac* promoter activity in *E. coli* and *Agrobacterium* strains.** Testing of the *lac* promoter of pSW172 or pSW213 depended on the use of a strain that supports α complementation. To test this promoter in other strains of *E. coli* and in *Agrobacterium* strains, the *lac* operon was introduced into these plasmids as described in Materials and Methods, forming plasmids pCC101 and pCC102, respectively. Derivatives of strain MC4100 (which has a *lac* deletion) containing pCC101 or pCC102 were tested for β-galactosidase activity in the presence or absence of IPTG. After culturing logarithmically growing cells for 3.5 h with or without 1 mM IPTG, we obtained the results shown in Table 1. We observed strong expression of the *lac* promoter in pCC101 with or without IPTG, strong repression by *lac* repressor in pCC102, and virtually complete derepression of expression by IPTG. These results confirm that the *lac* promoter and *lacI*$^q$ gene of these plasmids are fully functional in *E. coli* and indicate that these plasmids should be useful to assay quantitatively the activity of these genetic elements in *Agrobacterium* spp.

Accordingly, these plasmids were introduced into *A. tumefaciens* A136 by transformation and assayed for β-galactosidase activity. A136(pCC101) showed approximately 3,000 U of β-galactosidase activity in the presence or absence of IPTG (Table 1). This result indicates that the activity of the *lac* promoter in *Agrobacterium* strains is approximately the same as or slightly higher than in *E. coli*. The fact that IPTG is not required indicates that *Agrobacterium* strains do not contain an endogenous IPTG-sensitive *lac* repressor. Similar assays using A136(pCC102) were undertaken (Table 1). In the absence of IPTG, β-galactosidase was expressed at extremely low levels, indicating that product of the *lacI*$^q$ gene did indeed effectively repress the *lac* promoter. When IPTG was added, β-galactosidase expression was partially but not fully derepressed. We tested derepression of P*lac* by a broad range of IPTG concentrations and found that maximal derepression was achieved if a concentration equal to or greater than 0.1 mM was used (Fig. 3). These data indicate that IPTG is unable to strongly derepress the *lac* promoter in *Agrobacterium* strains even though it does so in an *E. coli* strain containing the same plasmid. We also tried using lactose in place of IPTG as an inducer. Addition of 1 mM lactose to complete AB medium or addition of 0.5% lactose in place of glucose resulted in β-galactosidase levels approximately half as high as with

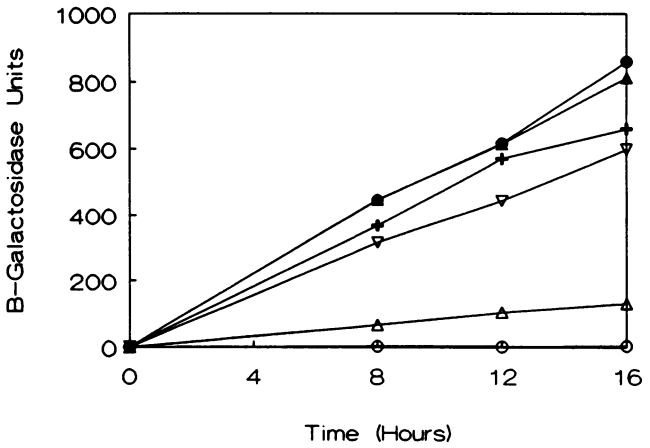


FIG. 3. Expression of P*lac*::*lacZ* in an *Agrobacterium* strain containing *lacI*$^q$. Symbols: ○, 0 mM IPTG; △, 0.01 mM IPTG; ▽, 0.05 mM IPTG; +, 0.1 mM IPTG; ▲, 1 mM IPTG; ●, 10 mM IPTG.

IPTG. Incubation of bacteria in AB medium containing 0.5% glycerol or 0.5% succinate in place of glucose did not affect enzyme levels. Culturing bacteria in acidic broth (pH 5.25) also did not affect enzyme levels.

**Expression of a *virG*::*lacZ* gene fusion from the *lac* promoter.** We wanted to be able to compare directly *virG* expression from the *lac* promoter with that obtained from its native promoters. We therefore created a plasmid (pCC103) in which the *lac* promoter drove the expression of a *virG*::*lacZ* translational fusion and compared it with a plasmid (pSW174) containing the same *virG*::*lacZ* fusion expressed from the native *virG* promoters. A second *lac* promoter in pCC103 may also have contributed to *virG* expression (see Fig. 1). A136(pCC103) expressed virtually no β-galactosidase activity in the absence of IPTG, while it expressed 300 U of β-galactosidase activity in the presence of IPTG, whether cultured at neutral or acidic pH (Table 2). Although a similar construct without *lacI*$^q$ was not constructed, we speculate from the data presented above that such a construct might have expressed 900 to 1,200 U of β-galactosidase activity (since the absence of *lacI*$^q$ resulted in a three- to fourfold increase of expression of *lacZ* expression from pCC102 compared with pCC101). By comparison, strain A348(pSW174) expressed about 70 U of β-galactosidase activity in response to culturing in acidic pH (which induces promoter P2). The same strain expressed 700 U of β-galactosidase in acidic media containing acetosyringone

TABLE 2. Expression of a *virG*::*lacZ* fusion gene from artificial and native promoters[a]

| Plasmid | Description | Inducing treatment | β-Galactosidase (Miller units) |
|---|---|---|---|
| pCC103 | P*lac*::*virG*::*lacZ*, *lacI*$^q$ | None | 2 |
| | | 1 mM IPTG | 300 |
| pSW174 | P*virG*::*virG*::*lacZ* | None | 9 |
| | | pH 5.25 | 70 |
| | | pH 5.25, 100 μM acetosyringone | 700 |

[a] Bacteria were grown in AB medium (pH 7.0) unless otherwise specified. Samples were assayed after 12 h of induction.

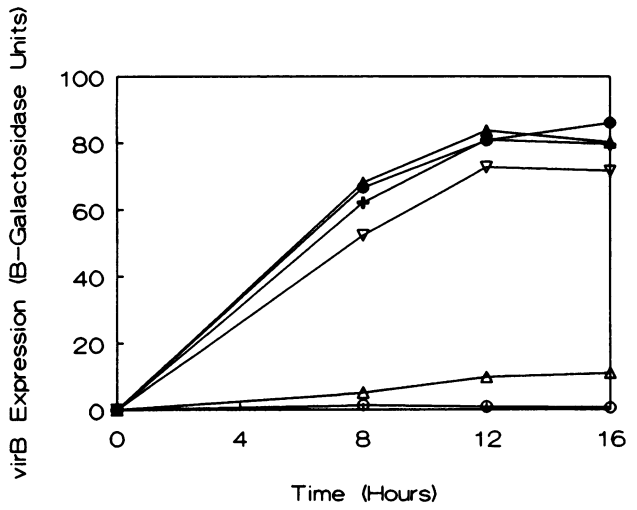


FIG. 4. Expression of a *virB*::*lacZ* fusion in an *Agrobacterium* strain containing P*lac*::*virG* and *lacI*$^q$ in the presence of 100 μM acetosyringone and differing levels of IPTG. Symbols represent the same concentrations of IPTG as in Fig. 3.

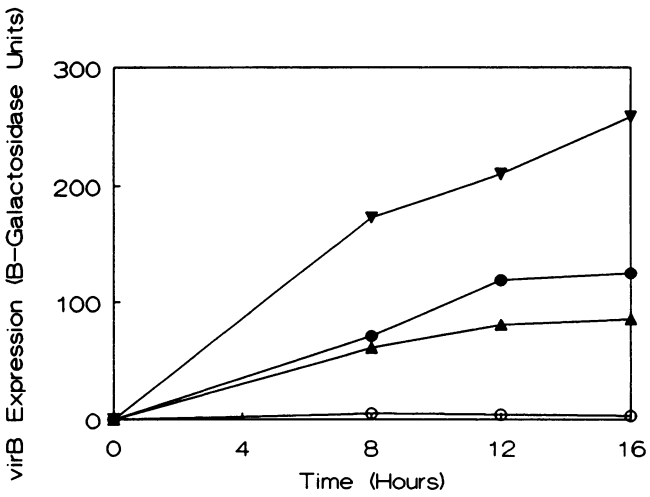


FIG. 5. Expression of a *virB*::*lacZ* fusion by 100 μM acetosyringone in derivatives of A136(pSW219) containing different *virG*-expressing plasmids. pSW99 contains the native *virG* gene (●); pSW207 contains P*lac*::*virG*; (▼); pSW217 contains P*lac*::*virG* and *lacI*$^q$ (▲); pSW213 is a vector control not containing *virG* (○). All incubations were carried out in acidic media containing 1 mM IPTG.

(which induces P1 and P2). Therefore, the *lac* promoter of a plasmid containing *lacI*$^q$ expresses a *virG*::*lacZ* gene fusion during IPTG induction somewhat less strongly than do the native *vir* promoters. Indirect evidence (comparing pCC101 with pCC102; see above) suggests that the same *lac* promoter in the absence of the *lacI*$^q$ probably would have expressed a *virG*::*lacZ* fusion slightly more strongly than did the native *virG* promoters. This speculation is supported by data presented below.

**Expression of native *virG* in *Agrobacterium* cells using the *lac* promoter.** Finally, we used the *lac* promoter to express the native *virG* gene in *Agrobacterium* strains. We used a derivative of strain A136 containing pSW219, which contains *virA* and a *virB*::*lacZ* fusion, and pSW217, which contains P*lac*::*virG* and *lacI*$^q$. Strain A136(pSW219)(pSW217) was tested for the efficiency of induction of the *virB*::*lacZ* fusion carried by pSW219. Acetosyringone (100 μM) was added to all cultures. A136(pSW219)(pSW217) expressed *virB* only in the presence of IPTG, and increasing amounts of IPTG resulted in increased expression of *virB* (Fig. 4).

We used other *virG* constructs to compare *virB* induction in this strain. These *virG*-containing plasmids were pSW217; pSW207, which contains P*lac*::*virG* and does not contain *lacI*$^q$; pSW99, which contains the native *virG* gene; and pSW213, a negative control not containing any *virG* gene. The abilities of different *virG*-expressing plasmids to induce a *virB*::*lacZ* fusion are compared in Fig. 5. Strain A136(pSW219)(pSW217) expressed *virB* slightly less efficiently than did strain A136(pSW219)(pSW99), even when the former strain was incubated in the presence of 1 mM IPTG. In contrast, a strain containing pSW207 expressed *virB* about twofold more efficiently than did a strain containing pSW99. The differences observed are representative of a large number of trials over a range of pHs and acetosyringone concentrations. Expression of *virB* in all strains tested was strictly dependent on inclusion of acetosyringone (data for pSW207 are shown in Fig. 6).

We also tested the following hypothesis. It is known that induction of all *vir* genes occurs only in acidic growth media. It is also known that *virG* promoter P2 is induced by environmental stresses which include acidic media. It has been hypothesized (27) that the underlying reason for this acidic pH optimum is that an acidic medium is required to induce P2. If this were true, then the normal low pH optimum would be lost in a strain containing a plasmid such as pSW207, because *virG* expression would no longer be dependent on acidic pH. We therefore tested induction of *virB* of strain A136(pSW219)(pSW207) in nonacidic media. Under these conditions, absolutely no induction was observed (data not shown). This result indicates that the low pH optimum cannot be explained solely in terms of a requirement for expression of promoter P2.

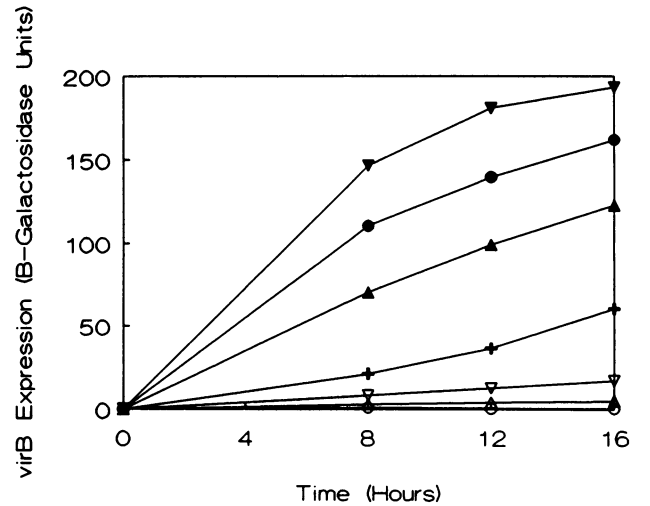


FIG. 6. Expression of *virB* by different levels of acetosyringone in strain A136(pSW219)(pSW207). Symbols: ○, 0 μM AS; △, 5 μM AS; ▽, 10 μM AS; +, 20 μM AS; ▲, 50 μM AS; ●, 100 μM AS; ▼, 200 μM AS.

## DISCUSSION

The *lac* and *tac* promoters in combination with the *lac* repressor and the inducer IPTG provide a time-honored means to control gene expression in *E. coli* but have been used relatively little in other microorganisms, although their use in *Pseudomonas putida* has been reported (2), and the *tac* promoter has been used to express *virG* in an unregulated fashion (15). We have explored the possibilities and limitations of these tools in *Agrobacterium* strains. We here have reported that a plasmid (pCC101) which expresses β-galactosidase in an uncontrolled fashion confers enzyme levels that are similar in *Agrobacterium* strains and *E. coli*. The *lac* promoter is therefore expressed in *Agrobacterium* strains at the same or slightly greater levels than in *E. coli*. This interpretation is subject to the caveats that we do not know whether plasmid copy number, message half-life, translation efficiency, or protein stability is the same in the two organisms. We have also shown that the *lac* repressor protein is an effective repressor of the *lac* promoter in *Agrobacterium* strains and that IPTG derepresses the promoter partially but not fully. The reasons for the lack of full induction are unknown. We conclude that this system is useful for controlled gene expression in situations in which strong gene expression is not required. Strong overproduction is also possible by using plasmids that do not contain $lacI^q$, although in that case, one sacrifices the ability to control gene expression. It is possible that using the wild-type allele of *lacI* in place of $lacI^q$ might also permit more complete derepression of the *lac* promoter.

We were able to compare the strengths of the *lac* and *virG* promoters by using each to express a *virG*::*lacZ* translational fusion. In the presence of *lac* repressor and IPTG, the *lac* promoter was about half strong as the *virG* promoters (Table 2). Although direct measurements were not made, indirect data suggests that in the absence of *lac* repressor, the *lac* promoter is about two- to threefold stronger than the *virG* promoters. Therefore, the level of expression of the native *virG* promoters is extraordinarily strong compared with those of other regulatory genes. The reasons for this strong expression are not known.

We were also able to alter the pool size of the native VirG protein and measure consequent changes in the expression of a representative *vir* promoter (*virB*). Addition of acetosyringone and increasing amounts of IPTG to a strain containing P*lac*::*virG* and $lacI^q$ resulted in increased expression of *virB*. In a strain that does not contain $lacI^q$, expression of *virG* from the *lac* promoter resulted in a moderate and reproducible enhancement of *virB* transcription. This result indicates that the intracellular concentration of VirG produced by the native *virG* promoters is rate limiting for induction even when expressed on a multicopy plasmid. It is possible that still higher levels of VirG protein would result in still higher levels of *vir* gene expression. We believe that the transcription of other *vir* genes may also be increased and that virulence itself may also be stimulated. Experiments to test this hypothesis are currently in progress. This overexpression of *vir* genes could be exploited to increase the utility of *Agrobacterium* strains in creating transgenic plants.

Finally, while *virG* is normally expressed only in acidic or otherwise stressful environments, these constructs allowed us to express VirG protein in nonacidic and nonstressful conditions. We found that an acidic medium is a requirement for *vir* regulon induction even in a strain containing an induced P*lac*::*virG* fusion. It appears that we must look elsewhere to understand the reasons for the acidic pH optimum for *vir* gene induction. Recently, it was reported that a deletion of the periplasmic domain of VirA made this acidic optimum considerably less severe, suggesting that the wild-type VirA protein may have an acidic optimum (13).

## ACKNOWLEDGMENTS

We thank N. Mantis and M. Sessler for helpful discussions. This work was supported by NIH grant 1 R29 GM2893-01. This work was also supported by a grant from the Cornell Biotechnology Program, which is sponsored by the New York State Science and Technology Foundation, a consortium of industries, the U.S. Army Research Office, and the National Science Foundation.

## REFERENCES

1. **Ankenbauer, R. G., and E. W. Nester.** 1990. Sugar-mediated induction of *Agrobacterium tumefaciens* virulence genes: structural specificity and activities of monosaccharides. J. Bacteriol. **172**:6442–6446.
2. **Bagdasarian, M. M., E. Amann, R. Lurz, B. Ruckert, and M. Bagdasarian.** 1983. Activity of the hybrid *trp-lac* (*tac*) promoter of *Escherichia coli* in *Pseudomonas putida*. Construction of broad-host-range, controlled-expression vectors. Gene **26**:273–282.
3. **Cangelosi, G. A., R. G. Ankenbauer, and E. W. Nester.** 1990. Sugars induce the *Agrobacterium* virulence genes through a periplasmic binding protein and a transmembrane signal protein. Proc. Natl. Acad. Sci. USA **87**:6708–6712.
4. **Casadaban, M. J., J. Chou, and S. N. Cohen.** 1980. In vitro gene fusions that join an enzymatically active β-galactosidase segment to amino terminal fragments of exogenous proteins: *E. coli* plasmid vectors for the detection and cloning of translational initiation signals. J. Bacteriol. **143**:971–980.
5. **Chilton, M.-D., T. Currier, S. Farrand, A. J. Benddich, M. P. Gordon, and E. W. Nester.** 1974. *Agrobacterium tumefaciens* DNA and PS8 bacteriophage DNA not detected in crown gall tumors. Proc. Natl. Acad. Sci. USA **71**:3672–3676.
6. **Close, T. J., P. M. Rogowsky, C. I. Kado, S. C. Winans, M. F. Yanofsky, and E. W. Nester.** 1987. Dual control of the *Agrobacterium tumefaciens* Ti plasmid virulence genes. J. Bacteriol. **169**:5113–5118.
7. **Close, T. J., D. Zaitlin, and C. I. Kado.** 1984. Design and development of amplifiable broad-host-range cloning vectors: analysis of the *vir* region of *Agrobacterium tumefaciens* plasmid pTiC58. Plasmid **12**:111–118.
8. **Cowing, D. W., J. C. A. Bardwell, E. A. Craig, C. Woolford, R. W. Hendrix, and C. A. Gross.** 1985. Consensus sequence for *Escherichia coli* heat shock promoters. Proc. Natl. Acad. Sci. USA **82**:2697–2683.
9. **Jin, S., T. Roitsch, P. J. Christie, and E. W. Nester.** 1990. The regulatory VirG protein specifically binds to a *cis*-acting regulatory sequence involved in transcriptional activation of *Agrobacterium tumefaciens* virulence genes. J. Bacteriol. **172**:531–537.
10. **Klee, H. J., M. F. Yanofsky, and E. W. Nester.** 1985. Vectors for transformation of higher plants. Bio/Technology **3**:637–642.
11. **Leroux, B., M. F. Yanofsky, S. C. Winans, J. E. Ward, S. F. Ziegler, and E. W. Nester.** 1987. Characterization of the *virA* locus of *Agrobacterium tumefaciens*: a transcriptional regulator and host range determinant. EMBO J. **6**:849–856.

11a. **Mantis, N., M. Sessler, and S. Winans.** Unpublished data.

12. **Marsh, J. L., M. Erfle, and E. J. Wykes.** 1984. The pIC plasmid and phage vectors with versatile cloning sites for recombinant selection by insertional inactivation. Gene **32**:481–485.
13. **Melchers, L. S., T. J. G. Regensburg-Tuink, R. B. Bourret, N. J. A. Sedee, R. A. Schilperoort, and P. J. J. Hooykaas.** 1989. Membrane topology and functional analysis of the sensory protein VirA of *Agrobacterium tumefaciens*. EMBO J. **8**:1919–1925.
14. **Miller, J. H.** 1972. Experiments in molecular genetics. Cold Spring Harbor Laboratory, Cold Spring Harbor, N.Y.
15. **Pazour, G. J., and A. Das.** 1990. *virG*, an *Agrobacterium*

*tumefaciens* transcriptional activator, initiates translation at a UUG codon and is a sequence-specific DNA-binding protein. J. Bacteriol. **172**:1600–1608.

16. **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.
17. **Schmidhauser, T. J., and D. R. Helinski.** 1985. Regions of broad-host-range plasmid RK2 involved in replication and stable maintenance in nine species of gram-negative bacteria. J. Bacteriol. **164**:446–455.
18. **Sciaky, D., A. L. Montoya, and M.-D. Chilton.** 1978. Fingerprints of *Agrobacterium* Ti plasmids. Plasmid **1**:238–253.
19. **Stachel, S. E., E. Messens, M. Van Montagu, and P. Zambryski.** 1985. Identification of the signal molecules produced by wounded plant cells that activate T-DNA transfer in *Agrobacterium tumefaciens*. Nature (London) **318**:624–629.
20. **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.
21. **Stachel, S. E., E. W. Nester, and P. C. Zambryski.** 1986. A plant cell factor induces *Agrobacterium tumefaciens vir* gene expression. Proc. Natl. Acad. Sci. USA **83**:379–383.
22. **Stachel, S. E., and P. C. Zambryski.** 1986. *virA* and *virG* control the plant-induced activation of the T-DNA transfer process of *A. tumefaciens*. Cell **46**:325–333.
23. **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.
24. **Veluthambi, K., M. Krishnan, J. H. Gould, R. H. Smith, and S. B. Gelvin.** 1989. Opines stimulate induction of the *vir* genes of the *Agrobacterium tumefaciens* Ti plasmid. J. Bacteriol. **171**: 3696–3703.
25. **Winans, S. C.** 1990. Transcriptional induction of an *Agrobacterium* regulatory gene at tandem promoters by plant-released phenolics, phosphate starvation, and acidic growth media. J. Bacteriol. **172**:2433–2438.
26. **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.
27. **Winans, S. C., R. A. Kerstetter, and E. W. Nester.** 1988. Transcriptional regulation of the *virA* and *virG* genes of *Agrobacterium tumefaciens*. J. Bacteriol. **170**:4047–4054.
28. **Yanofsky, M. F., S. G. Porter, C. Young, L. A. Albright, M. P. Gordon, and E. W. Nester.** 1986. The *virD* operon of *Agrobacterium tumefaciens* encodes a site-specific endonuclease. Cell **47**:471–477.