Mutation of the *miaA* Gene of *Agrobacterium tumefaciens* Results in Reduced *vir* Gene Expression

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vir regulon expression in *Agrobacterium tumefaciens* involves both chromosome- and Ti-plasmid-encoded gene products. We have isolated and characterized a new chromosomal gene that when mutated results in a 2- to 10-fold reduction in the induced expression of *vir* genes by acetosyringone. This reduced expression occurs in AB minimal medium (pH 5.5) containing either sucrose or glucose and containing phosphate at high or low concentrations. The locus was cloned and used to complement *A. tumefaciens* strains harboring Tn5 insertions in the gene. Sequence analysis of this locus revealed an open reading frame with strong homology to the *miaA* locus of *Escherichia coli* and the *mod5* locus of *Saccharomyces cerevisiae*. These genes encode tRNA: isopentenyltransferase enzymes responsible for the specific modification of the A-37 residue in UNN codon tRNA species. The function of the homologous gene in *A. tumefaciens* was proven by genetic complementation of *E. coli miaA* mutant strains. tRNA undermodification in *A. tumefaciens miaA* mutant strains may reduce *vir* gene expression by causing a reduced translation efficiency. A slight reduction in the virulence of these mutant *Agrobacterium* strains on red potato plants, but not on tobacco, tomato, kalanchoe, or sunflower plants, was observed.

The transfer of T-DNA, transferred DNA, to susceptible plants by the pathogen *Agrobacterium tumefaciens* is governed mainly by the gene products of the *vir* (virulence) regulon that is located on the Ti (tumor-inducing) plasmid (51, 70). The *vir* regulon contains eight operons (53, 59) whose expression is regulated by the two-component regulatory system VirA-VirG (60). The VirA sensor protein responds to plant phenolic and monosaccharide signals by phosphorylating VirG, which then acts as a positive transcriptional activator of itself and other *vir* operons (7, 31, 33, 58). Regulation of *vir* gene expression is more complex, however, and involves a number of chromosomally encoded gene products. *Agrobacterium* strains mutant in *chvE* fail to produce a functional periplasmic glucose-binding protein, show a greatly reduced level of *vir* gene induction, and display an extremely restricted host range. The ChvE protein binds many plant monosaccharides and interacts with the periplasmic domain of VirA (7, 30, 40, 58). The *ros* chromosomal gene encodes a protein that may bind DNA and has a specific repressor effect on the *virC* and *virD* operons (13).

Bacterial pathogenesis entails a large assembly of virulence factors that may not be simultaneously needed or advantageous during all stages of infection. In addition to proteins encoded by the Ti plasmid, there may be chromosomally encoded proteins involved in the infection process of plants by *Agrobacterium* strains. Engstrom et al. (19) reported five such proteins that are induced when *Agrobacterium* strains are grown in the presence of the plant phenolic acetosyringone. The role of these proteins is not known. A number of chromosomal loci affect virulence but not *vir* gene expression per se. These include *chvA* and *chvB*, *att*, and *pscA* (*exoC*) (8, 17, 39, 61). Metts et al. (41) have recently characterized three avirulent *Agrobacterium* strains with chromosomal mutations that severely reduce *vir* gene induction. Eight further mutants isolated by these authors were

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not further characterized. The functions of these recently isolated genes remain to be determined.

Agrobacterium infection is a process in which the bacteria must sense their surroundings and respond in an appropriate manner. That *Agrobacterium* strains are responding to many signals from the plant is indicated by the identification of *picA*, a chromosomal locus that is inducible by pectin-derived polysaccharides from carrot root extracts (54, 55). Work in this laboratory has also shown that certain opines stimulate the induction of *vir* genes by acetosyringone (63). The mechanism of this effect and its role in the infection process remain to be elucidated. Thus, we might envisage that there are other unidentified signals that *Agrobacterium* strains receive and respond to, and some of these may be used to fine-tune *vir* gene expression. The *virG* gene is itself the subject of complex control involving a response to phosphate starvation and acidic pH in addition to plant-released phenolic compounds (62, 66). The *chvD* chromosomal gene encodes a protein apparently involved in both the low pH- and phosphate-starvation-induced expression of *virG* (67). That other cellular components are involved in the expression of *virG* is suggested by evidence that the acidic optimum for *virG* induction is not removed when the native promoter is replaced by a *lacZ* promoter of *Escherichia coli* (9).

Reports on some of the above chromosomal loci and their possible involvement in regulating *vir* gene expression prompted us to identify other chromosomal loci that play a role in the expression of the *vir* regulon. We devised a mutagenesis screen to detect transposon-generated chromosomal mutants with reduced levels of *virB* expression. Such mutants may have reduced virulence; however, the screen allowed for the isolation of genes that play a role in *vir* gene expression and that may have a minor or undetectable role in virulence. In this paper, we describe the isolation of a chromosomal gene that, when mutated, results in a two- to tenfold reduction in the expression of *virB*, *virD*, *virE*, *pinF*, and *virG*. We isolated and sequenced the wild-type gene and

partially complemented the mutant induction phenotype. Sequence analysis of the wild-type gene indicates strong homology to the E. coli miaA locus (12), a gene that encodes a tRNA:isopentenyltransferase. We confirmed the function of the corresponding Agrobacterium gene by complementing an E. coli miaA mutant.

MATERIALS AND METHODS

Bacterial strains, plasmids, phage, and growth conditions. A. tumefaciens and E. coli strains as well as plasmids and phage employed in this study are listed and described in Table 1. A. tumefaciens strains were cultured at 30°C on YEP complete medium or on AB minimal medium containing 0.5% sucrose (36). E. coli strains were cultured on LB, MacConkey-lactose, or eosin-methylene blue (EMB)-lactose plates at 37°C (38). When antibiotics were present in the medium used for A. tumefaciens, concentrations (in milligrams per liter) were as follows: carbenicillin, 100; gentamicin, 100; kanamycin, 100; rifampin, 10; tetracycline, 10. When antibiotics were present in the medium used for E. coli, concentrations (in milligrams per liter) were as follows: ampicillin, 100; kanamycin, 50; nalidixic acid, 30; tetracycline, 10. Plasmids were mated from E. coli to A. tumefaciens by triparental mating (16), using the mobilizing functions of the plasmid pRK2013 (21). Opine-induced conjugal matings were carried out as previously described (22).

Induction media and assay of β-galactosidase activity in A. tumefaciens strains. Agrobacterium strains were grown to mid-exponential phase in YEP medium and diluted to a Klett reading of 50 (red filter no. 66 with spectral range of 640 to 700 nm; approximately 5×10^8 cells per ml) in one of the following media. Initial vir::lacZ induction assays were carried out with AB sucrose minimal medium, pH 5.6, containing 20 mM phosphate buffer. This medium was either autoclaved or filter sterilized as indicated in Table 2. Subsequent induction assays employed AB minimal medium (pH 5.6) containing 1% glucose, 2 mM phosphate buffer, and 30 mM morpholine ethanesulfonic acid (MES) buffer. This latter medium was filter sterilized. Unless otherwise indicated, the concentration of the inducer acetosyringone was 100 μM whenever it was utilized. Bacteria were incubated with shaking at room temperature for 12 to 14 h, after which time β-galactosidase activity was assayed according to the procedure of Miller (42).

Functional complementation test of miaA activity. The presence of a functional miaA gene was assessed in E. coli lacZ(UGA) tester strains (49) (Table 1). The indicator medium employed was either MacConkey plus 0.4% lactose or EMB plus 0.4% lactose. Bacteria were streaked on the relevant plates and incubated at 37°C overnight. In a DEV15lacZ(UGA) background, red or white colony color on MacConkey plus 0.4% lactose medium indicated a miaA+ or miaA strain, respectively. In the DEV15lacZ(UGA)su+9 background, metallic green or pinkish black colony color on EMB plus 0.4% lactose indicated a miaA+ or miaA strain, respectively.

Mutagenesis of A. tumefaciens At44 with Tn5. The suicide plasmid pGS9 (57), containing Tn5, was mobilized into A. tumefaciens At44 (59). Transconjugants were selected and screened for reduced virB expression on AB minimal medium plates (pH 5.6) containing 0.5% sucrose, 20 mM phosphate, 100 μm acetosyringone, 100 μg of kanamycin per ml and 20 μg of the indicator X-Gal (5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside) per ml. On this medium, colonies exhibiting a reduced expression of the virB reporter

fusion were pale blue or white. Analysis of the β-galactosidase activity of these strains was further examined by using a quantitative O-nitrophenyl-β-D-galactopyranoside (ONPG) colorimetric assay.

Cloning of the mutant and wild-type loci from A. tumefaciens. Total DNA isolated from Tn5 mutants of A. tumefaciens At44 was digested with EcoRI, an enzyme that does not cleave within Tn5, thus generating fragments that include the flanking chromosomal regions surrounding the Tn5 insertion site. These fragments were ligated into pUC18 and transformed into E. coli DH5α. Kanamycin- and ampicillin-resistant colonies were selected, and the cloned inserts were analyzed by restriction endonuclease mapping. Plasmids harboring the Tn5-containing fragments thus cloned from At44#37 and At44#38 were called pJG1 and pJG2, respectively.

To recover the DNA fragment carrying the corresponding wild-type allele for these Tn5 insertions, total DNA extracted from A. tumefaciens A136 was digested with EcoRI, and the resulting fragments were separated by agarose gel electrophoresis. DNA fragments 4 to 6 kbp in size were recovered from this gel, ligated into pUC18, and transformed into E. coli DH5α. Colonies harboring plasmids containing wild-type loci were identified by colony hybridization by using the cloned inserts of pJG1 and pJG2 as hybridization probes. The plasmid containing the cloned 5-kbp EcoRI fragment with the expected restriction map for the A. tumefaciens At44#37 wild-type fragment was called pJW100. Southern analysis revealed that the same chromosomal fragment was disrupted in A. tumefaciens At44#38 as was disrupted in A. tumefaciens At44#37.

For complementation studies of the virB::lacZ phenotype, the wild-type EcoRI fragment from pJW100 was cloned into the broad-host-range vector pRK290, generating the plasmid pJG100. pJG100 and the control plasmid pRK290 were moved into the wild-type and mutant strains by triparental mating.

Reconstruction of the miaA mutation of At44#38 in A. tumefaciens A136 by marker exchange. The Tn5 mutation of the miaA mutant At44#38 was reconstructed as follows. The EcoRI fragment containing the Tn5 insertion from pJG2 was cloned into the broad-host-range vector pRK290. The resulting plasmid, pJG38, was mobilized into the wild-type A. tumefaciens strain A136 by triparental mating. Rif' Tet' Kan' transconjugants were selected. The eviction plasmid pPH1JI was mobilized into the resulting strains, and Kan' Gent' colonies were selected. Double-crossover marker exchange recombinants were obtained by screening Kan' Gent' colonies for tetracycline sensitivity. DNA from the reconstructed strain A. tumefaciens At587 was characterized by Southern blot analysis and shown to have the same restriction endonuclease fragment profile as did A. tumefaciens At44#38.

Ti plasmids harboring various vir::lacZ fusions were mated from donor strains (Table 1) into the reconstructed mutant A. tumefaciens At587 by using opine-induced conjugal transfer and selecting for Rif' Carb' Kan' transconjugants that could use octopine as a sole carbon source. The wild-type plasmid pTiR10 was mobilized into At587 in a similar way, except that transconjugants did not exhibit carbenicillin resistance. This strain, which contains a wild-type Ti plasmid in a miaA chromosomal background, was designated At627 and utilized in studies of virulence. To create merodiploid strains, cosmids carrying the relevant vir::lacZ translational fusions were mobilized by triparental mating into A. tumefaciens At627 and Carb' Kan' Rif'

TABLE 1. Bacterial strains, plasmids, and phage

Strain, plasmid, or phage	Relevant genotype	Relevant phenotype[a]	Source or reference
Strains			
E. coli			
DH5α	supE44 ΔlacU169 (φ80lacZΔM15) hsdR17 recA1 endA1 gyrA96 thiL relA1		25
HB101	F⁻ hsdS20 (r_B^- m_B^-) recA13 ara-14 proAZ lacY1 galK2 rpsL20 (Sm^r) xyl-5 mtl-1 supE44 λ⁻		5
BW15723	LR25 creA-lacZ(JC8) DE3(lac) x74 cre(wt) (nonsuppressor strain)		Barry Wanner
SF 800	W3110 thy polA1	Nal^r	26
DEV15	thi relA spoT lacZ(UGA)		49
DEV15miaA	DEV15miaA (originally trpX)		49
DEV15su⁺9	DEV15su⁺9		49
DEV15su⁺9miaA	DEV15su⁺9miaA		49
A. tumefaciens			
A136	Wild-type C58 strain cured of Ti plasmid	Rif^r	43
At109	pTiR10 in C58 chromosomal background	Rif^s	14
At44	A348 (pTiA6mx234) (virB::Tn3-HoHo1)	Rif^r Carb^r	59
At44#37	Tn5 insertion derivative of At44	Rif^r Carb^r Kan^r	This study
At44#38	Tn5 insertion derivative of At44	Rif^r Carb^r Kan^r	This study
A6	Wild-type A6 strain		41
Ivr211	Tn5 insertion derivative of A6, avirulent, prototrophic	Kan^r	41
Ivr221	Tn5 insertion derivative of A6, avirulent, prototrophic	Kan^r	41
Ivr223	Tn5 insertion derivative of A6, avirulent, prototrophic	Kan^r	41
At488	pTiR10 in A136	Rif^r	55
At587	Reconstruction of At44#38 Tn5 insertion in A136	Rif^r Kan^r	This study
At627	pTiR10 in At587	Rif^r Kan^r	This study
At552	pJG100 in At44#37	Rif^r Carb^r Kan^r Tet^r	This study
At553	pJG100 in At44#38	Rif^r Carb^r Kan^r Tet^r	This study
At554	pRK290 in At44#37	Rif^r Carb^r Kan^r Tet^r	This study
At555	pRK290 in At44#38	Rif^r Carb^r Kan^r Tet^r	This study
At556	pRK290 in At44	Rif^r Carb^r Tet^r	This study
At557	pJG100 in At44	Rif^r Carb^r Tet^r	This study
At558	pTiR10(mx30) donor (virB::lacZ)	Carb^r Gent^r	22
At559	pTiR10(mx234) donor (virB::lacZ)	Carb^r Gent^r	22
At560	pTiR10(mx304) donor (virB::lacZ)	Carb^r Gent^r	22
At561	pTiR10(mx358) donor (virE::lacZ)	Carb^r Gent^r	22
At562	pTiR10(mx379) donor (virC::lacZ)	Carb^r Gent^r	22
At338	pTiR10(mx363) donor (virG::lacZ)	Carb^r Gent^r	22
At353	pTiR10(mx219) donor (pinF::lacZ)	Carb^r Gent^r	22
At605	pTiR10(mx30) in At587	Rif^r Carb^r Kan^r	This study
At606	pTiR10(mx234) in At587	Rif^r Carb^r Kan^r	This study
At607	pTiR10(mx304) in At587	Rif^r Carb^r Kan^r	This study
At608	pTiR10(mx358) in At587	Rif^r Carb^r Kan^r	This study
At610	pTiR10(mx379) in At587	Rif^r Carb^r Kan^r	This study
At611	pTiR10(mx363) in At587	Rif^r Carb^r Kan^r	This study
At612	pTiR10(mx219) in At587	Rif^r Carb^r Kan^r	This study
At613	pSM363 (virG::lacZ) in At627	Rif^r Carb^r Kan^r	This study
At614	pSM363 (virG::lacZ) in At488	Rif^r Carb^r Kan^r	This study
At617	pSM30 (virB::lacZ) in At627	Rif^r Carb^r Kan^r	This study
At618	pSM30 (virB::lacZ) in At488	Rif^r Carb^r Kan^r	This study
Plasmids			
pUC18	Cloning vector	Amp^r	68
pUC119	Cloning/sequencing vector	Amp^r	64
pGS9	Tn5 suicide plasmid	Cm^r Kan^r	57
pRK290	Broad-host-range cloning vector	Tet^r	16
pRK2013	Mobilization plasmid	Kan^r	21
pPH1JI	Eviction plasmid	Gent^r	28
pJG1	10.8-kbp EcoRI DNA fragment containing Tn5 insertion from At44#37 cloned in pUC18	Amp^r Kan^r	This study
pJG2	11.3-kbp EcoRI DNA fragment containing Tn5 insertion from At44#38 cloned in pUC18	Amp^r Kan^r	This study
pJG3	pJG1 10.8-kbp EcoRI insert cloned in pRK290	Kan^r Tet^r	This study
pJG4	pJG2 11.3-kbp EcoRI insert cloned in pRK290	Kan^r Tet^r	This study
pJG5, pJG6	1.49-kbp AccI-ClaI DNA subclones of wild-type Agrobacterium miaA locus in pUC119 (orientations A and B, respectively)	Amp^r	This study

TABLE 1—Continued.

Strain, plasmid, or phage	Relevant genotype	Relevant phenotype[a]	Source or reference
pJW100	Wild-type 5-kbp EcoRI DNA fragment containing Agrobacterium miaA locus cloned in pUC18	Amp^r	This study
pJG100	pJW100 5-kbp EcoRI DNA fragment cloned in pRK290	Tet^r	This study
pJG101-127	Tn5 Seq-1 insertion derivatives of pJG100	Kan^r Tet^r	This study
pJG100ΔClaI	ClaI fragment internal to 5-kbp EcoRI insert deleted from pJG100	Tet^r	This study
pJG100ΔHindIII	HindIII fragment internal to 5-kbp EcoRI insert deleted from pJG100	Tet^r	This study
pSM30	pVK221 virB::Tn3-HoHo1	Amp^r Kan^r	59
pSM363	pVK225 virG::Tn3-HoHo1	Amp^r Kan^r	59
pSM379	pVK225 virC::Tn3-HoHo1	Amp^r Kan^r	59
pTiR10	Wild-type Ti plasmid	Onc$^+$, Occ^{+b}	Steve Farrand
Phage λ::Tn5Seq-1	Tn5 Seq-1 b221 cI857 Pam80	Kan^r	46

[a] Antibiotic resistances as follows: Amp^r, ampicillin; Carb^r, carbenicillin; Gent^r, gentamicin; Kan^r, kanamycin; Nal^r, nalidixic acid; Rif^r, rifampin; Tet^r, tetracycline.

[b] Onc$^+$, tumorigenic; Occ$^+$, able to catabolize octopine.

transconjugants were selected. Each of these cosmids was also mated into the wild-type strain A. tumefaciens At488 for use as controls in the relevant vir gene expression studies.

Construction of Tn5Seq-1 derivatives and deletion derivatives of pJG100. Lysates of the phage λ::Tn5Seq-1 were made by using the permissive host E. coli DH5α. pJG100 was transformed into nonsuppressor strain E. coli BW15723, the resulting strain was transfected with phage, and Kan^r Tet^r colonies were selected. To eliminate strains containing transposition events in the bacterial chromosome, Kan^r Tet^r colonies were pooled and mated with E. coli SF800 and Nal^r Kan^r Tet^r transconjugants were selected. These transconjugants contained pJG100::Tn5Seq-1 derivatives. One hundred of these colonies were picked, and plasmid DNA was isolated (38). Restriction endonuclease analysis was used to identify those plasmids containing Tn5Seq-1 insertions within the 5-kbp miaA region. These insertions were mapped with respect to position and orientation within the miaA region (see Fig. 1). Insertions mapping within 200 bp of each other are mapped together.

To construct the pJG100 deletion derivatives pJG100Δ ClaI and pJG100ΔHindIII, pJG104 was digested with ClaI and HindIII, respectively, and religated, and the ligation products were transformed into E. coli DH5α. Tetracycline-resistant colonies were screened for kanamycin sensitivity. Plasmid DNA was isolated from kanamycin-sensitive clones, and restriction endonuclease analysis was used to confirm that the appropriate ClaI or HindIII DNA fragment had been deleted.

The pJG100 deletion derivatives pJG100ΔClaI and pJG 100ΔHindIII and a number of pJG100::Tn5Seq-1 derivatives were individually mated into the original miaA mutants A. tumefaciens At44#37 and At44#38 for use in complementation studies.

DNA sequence analysis of the Agrobacterium miaA locus. A 1.5-kbp AccI-ClaI fragment of pJW100 was cloned into the AccI site of pUC119 in both orientations, generating the plasmids pJG5 and pJG6. A nested set of exonuclease III deletion derivatives of each plasmid was generated with the Erase-a-Base kit (Promega Corp., Madison, Wis.) (27). Both strands of this 1.5-kbp fragment were sequenced according to the dideoxy-chain termination method (56) with Sequenase 2 and the −40 forward primer (U.S. Biochemical Corp., Cleveland, Ohio). Single-stranded DNA templates were prepared for sequencing by using the helper phage M13KO7

(64). Both dGTP and dITP were used to sequence DNA regions with high G+C compositions. Sequence analysis was conducted with the Sequence Analysis Software Package (version 6.0) from the Genetics Computer Group (University of Wisconsin, Madison) (15, 48).

Virulence analysis of A. tumefaciens At627 and At488. The virulence of the mutant miaA strain A. tumefaciens At627 was compared with that of the wild-type strain A. tumefaciens At488 on five different plant hosts: potato, tobacco, tomato, sunflower, and kalanchoe plants. For the red potato disk assay, a modification of the procedure of Rogowsky et al. (53) was employed. Bacteria were grown in YEP medium to a Klett reading of 100 (approximately 10^9 cells per ml), harvested, and resuspended at various concentrations in a solution of MS salts (GIBCO-BRL). Potato disks were immersed in various dilutions of bacteria for 1 h, washed three times in a solution of MS salts, incubated at 25°C on water agar for 2 days, and then transferred to water agar plus 500 μg of carbenicillin per ml. The number of tumors per disk was scored after 12 days. For the tobacco leaf disk assays (29), bacteria were grown to a Klett reading of 100 and harvested, and appropriate dilutions were made with 0.9% NaCl. Leaf disks were immersed for 2 min in the appropriate dilution of bacteria, blotted dry, and placed on MS3 medium containing plant hormones. After 2 days, leaf disks were transferred to MS3 medium without hormones and containing 500 μg of carbenicillin per ml. The number of tumors per disk was determined after 12 days of incubation.

For stem-wound assays, wounds were created on either the leaves of kalanchoe plants or the stems of tomato or sunflower plants by using a sterile toothpick, and the wounds were then inoculated with 10 μl of various concentrations of bacteria. Wounds exhibiting tumors were scored after 21 days for kalanchoe plants and after 14 days for tomato and sunflower stems.

Nucleic acid manipulations. Total DNA was isolated from A. tumefaciens as previously described (23). Restriction endonuclease digestion, agarose gel electrophoresis, and Southern blot analysis were conducted as described previously (38). Recombinant DNA procedures were as described previously (38), using P1 containment conditions as specified by the National Institutes of Health recombinant DNA guidelines.

TABLE 2. *virB::lacZ* induction of *A. tumefaciens* At44, At44#37, and At44#38

Bacterial strain	β-Galactosidase activity (U)[a] in AB minimal medium (pH 5.6)–100 μm acetosyringone[b] with following conditions:					
	0.5% sucrose, 20 mM phosphate		1% glucose, 2 mM phosphate, 30 mM MES, filter sterilized			
	Autoclaved, 12-h induction[c]	Filter sterilized, 12-h induction	6-h induction	12-h induction	15-h induction	18-h induction
At44	445 ± 81	32 ± 10	376 ± 18	647 ± 87	1,072 ± 8	1,096 ± 136
At44#37	49 ± 9	6 ± 4	41 ± 4	149 ± 32	236 ± 11	252 ± 11
At44#38	45 ± 5	1 ± 1	ND[d]	136 ± 45	ND	ND

[a] β-Galactosidase activity expressed in Miller units. Each value is the average activity from three independently induced cultures.
[b] Uninduced levels were 0 to 1 U for each assay point.
[c] Period of culture incubation from time of acetosyringone addition to lysis of cells for enzyme assay.
[d] ND, not determined.

Nucleotide sequence accession number. The nucleotide sequence reported in this paper has been submitted to GenBank (accession number M83532).

RESULTS

Tn5 mutagenesis of *A. tumefaciens* At44 and screening of mutants defective in *virB::lacZ* induction by acetosyringone. We initiated this study to identify new chromosomal loci that play a role in *vir* gene expression. A protocol that would allow a simple visual screen of mutants that exhibited reduced expression of a *virB::lacZ* reporter gene was devised. We mutagenized *A. tumefaciens* At44 by introducing Tn5 on the suicide plasmid pGS9 (57). Approximately 33,000 kanamycin-resistant transconjugants were screened on indicator plates, and 93 pale blue or white colonies were chosen from this initial screen. These putative mutants were assayed for *virB::lacZ* expression by using a quantitative ONPG assay. For these initial induction experiments, sucrose was employed as a carbon source. Seventy-three mutants exhibiting 0 to 50% *virB::lacZ* expression compared with that of wild-type *A. tumefaciens* At44 were retained for further analysis. It was necessary to eliminate those mutants in which Tn5 had been inserted into the Ti plasmid because such mutations were likely to have occurred from the insertion of Tn5 into *virA*, *virG*, or the *lacZ* gene itself. To this end, a modified Eckhardt gel electrophoresis procedure was employed (32) to separate chromosomal DNA from Ti plasmid and cryptic plasmid DNA. DNA blots of these gels were hybridized with a probe from the kanamycin resistance gene of Tn5. Our results indicated that the large majority of these 73 mutants resulted from insertions on the Ti plasmid (data not shown). None were located on the cryptic plasmid, and 13 Tn5 insertions were localized to the chromosome. Each of these latter 13 mutations could play a role in mediating *vir* gene expression, but we wished to exclude those strains carrying insertions in chromosomal loci already known to have a possible role in this process or in virulence. Southern blot analysis indicated that the *chvA*, *chvB*, *chvD*, and *pscA* loci (17, 61, 67) were not disrupted in our mutant strains (data not shown). Additional analysis indicated that the *ivr-211*, *ivr-223*, and *ivr-225* loci (41) were also not disrupted. We found that eight Tn5 insertions had occurred in the *chvE* region of the chromosome, a locus that is now known to mediate *vir* gene induction in response to certain sugars (7, 30, 58). The remaining five mutants had Tn5 insertions in chromosomal regions not previously known to play a role in *vir* gene expression. Two of these mutant strains did not exhibit a reproducibly low induction of *virB* expression and were not pursued further. A third mutant, *A.*

tumefaciens At44#9, exhibited a greatly reduced growth rate that hampered further phenotypic and genetic analyses. The two remaining mutants, *A. tumefaciens* At44#37 and At44#38, exhibited *virB::lacZ* induction levels approximately 10% that of *A. tumefaciens* At44 in AB sucrose induction medium (Table 2).

Characterization of *virB::lacZ* induction in *A. tumefaciens* At44#37 and At44#38. The induction of the *virB::lacZ* fusion gene in *Agrobacterium* strains At44#37 and At44#38 was further characterized, and the results are summarized in Table 2. The initial induction medium utilized sucrose as a carbon source and a relatively high phosphate concentration (20 mM). We observed that both At44#37 and At44#38 exhibited an approximately 5- to 10-fold reduction in *virB::lacZ* expression relative to that of *A. tumefaciens* At44 when assayed in this medium. Although the level of expression relative to that of the wild-type strain remained the same in various experiments, we observed a large variation in the absolute level of induction from experiment to experiment (data not shown). Furthermore, if the AB sucrose medium was filter sterilized rather than autoclaved, very low absolute levels of induction were observed (e.g., 32 versus 447 U, respectively, for At44). In order to avoid low levels of induction and variation in absolute levels of induction among experiments, we utilized the induction medium described by Winans et al. (67). This medium employs glucose as the carbon source as well as a low phosphate concentration (2 mM). MES buffer (30 mM) is utilized to counteract the low buffering capacity due to a low concentration of phosphate. By using these conditions, the absolute levels of induction were increased and were much more reproducible. This increase can be ascribed to the fact that sucrose does not act as a signal in ChvE-mediated *vir* gene induction (7) and that low phosphate levels increase transcription of *virG*, a transcriptional activator of *vir* gene expression (66). The probable reason why autoclaving the sucrose medium increased *vir* gene expression (Table 2) is that autoclaving can cause sucrose cleavage resulting in the release of glucose that acts as a signal in ChvE-mediated *vir* gene induction. We found that under these conditions, when AB sucrose medium was employed, the induction of the *virB::lacZ* fusion gene in *A. tumefaciens* At44#37 and At44#38 was not as greatly reduced as it was in *A. tumefaciens* At44 (Table 2). In addition, we found that the reduced expression of the *virB::lacZ* gene fusion in *A. tumefaciens* At44#37 varied from an approximate ninefold difference after 6 h of induction to a four- to fivefold difference at later time periods. In subsequent experiments, an induction period of 12 to 14 h was used.
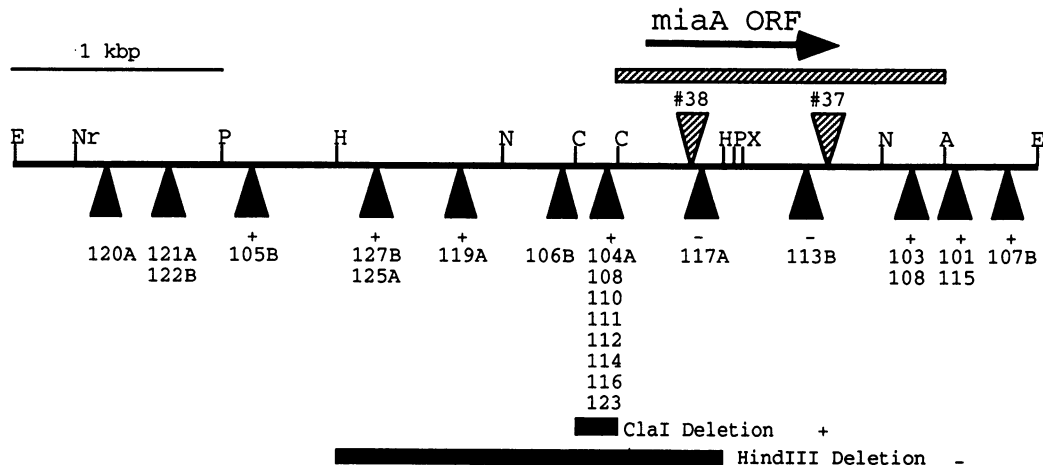
FIG. 1. Genetic and molecular analysis of the A. tumefaciens miaA chromosomal region. In the restriction map, restriction endonuclease sites are shown within the 4.9-kbp EcoRI fragment that contains the A. tumefaciens miaA locus. Abbreviations: A, AccI; C, ClaI; E, EcoRI; H, HindIII; N, NarI; Nr, NruI; P, PstI; X, XhoI. Hatched triangles above the line indicate the sites of Tn5 insertion for A. tumefaciens At44#37 and At44#38. Dark triangles below the line indicate sites of transposon insertion for 23 pJG100 Tn5Seq-1 derivatives. Insertions mapping within 150 bp of each other are represented by a single triangle, and different orientations of transposon insertion are indicated by the letter A or B where determined. Dark bars below the line indicate regions of the cloned insert deleted from pJG100 in pJG100ΔClaI and pJG100ΔHindIII. The effect of each Tn5Seq-1 insertion or internal deletion on the ability of the cloned region to complement in trans the reduced vir gene induction phenotype of A. tumefaciens At44#37 and At44#38 is indicated by plus or minus signs above the plasmids. The hatched bar above the line indicates the 1.49-kbp ClaI-AccI DNA fragment used for sequencing purposes. miaA ORF represents the proposed miaA ORF determined from sequence analysis of the cloned ClaI-AccI fragment.

Tn5 insertions in the mutants A. tumefaciens At44#37 and At44#38 occur in the same chromosomal region. The Tn5 insertions in A. tumefaciens At44#37 and At44#38, along with their respective flanking DNA regions, were cloned to generate pJG1 and pJG2, respectively. Restriction endonuclease mapping of the cloned EcoRI fragments indicated that chromosomal fragments 5.0 and 5.5 kbp in size had been disrupted in A. tumefaciens At44 to generate A. tumefaciens At44#37 and At44#38, respectively. In addition, a 5-kbp EcoRI fragment was isolated from A. tumefaciens At44. This DNA fragment exhibited the expected restriction endonuclease pattern of the corresponding EcoRI restriction endonuclease fragment from A. tumefaciens At44#37, but it lacked the Tn5 insertion. Southern blot analysis revealed that this same chromosomal DNA fragment was disrupted by Tn5 in both A. tumefaciens At44#37 and At44#38. This paradoxical result was explained by further Southern blot analysis, which revealed that the Tn5 insertion in A. tumefaciens At44#38 had undergone an internal 500-bp duplication (data not shown). Thus, the mutated EcoRI fragment of A. tumefaciens At44#38 appeared larger than that of At44#37. We further established that the two Tn5 insertion sites in A. tumefaciens At44#37 and At44#38 lie approximately 700 bp apart on the chromosome (Fig. 1). This result suggested that we had isolated two independent Tn5 insertions in the same chromosomal locus and that both these mutations resulted in a similar reduction in virB::lacZ expression.

Decrease in the induction of other vir genes in mutant Agrobacterium strains. Having established the observed mutant induction phenotype described above for virB, we asked whether or not this phenotype extends to other members of the vir regulon. We therefore reconstructed the At44#38 Tn5 mutation in A. tumefaciens A136, a strain lacking a Ti plasmid. The resulting strain, At587, was analyzed by Southern blot analysis to ensure that no rearrangements of the original mutation had occurred during the marker exchange

recombination process. pTiR10 plasmids bearing Tn3-HoHo1 lacZ fusions in the virB, virC, virD, virE, virG, and pinF operons were mobilized into the reconstructed mutant A. tumefaciens At587. We used the Ti plasmid donor strains as wild-type controls in this set of experiments. The chromosomal histories of these donor strains and of A136 (the strain in which the mutant was reconstructed) differ only in that the latter strain exhibits rifampin resistance, but control experiments indicated that the rifampin allele did not affect vir expression (data not shown).

Table 3 shows that the induction phenotype for virB in the original mutant strains was also observed for the reconstructed mutant strain At559. A reduced expression of virB was also observed when a different virB::Tn3-HoHo1 fusion was used as a reporter (At558), although in this case induction was 47% that of the wild type, compared with 32% that of the wild type for At559. A reduced induction phenotype was also observed for each of the other vir::lacZ reporter fusions. This reduction in vir gene activity ranged from 49% that of the wild type for a virE::lacZ fusion to 28% that of the wild type for a pinF::lacZ fusion. We also tested whether this reduced vir gene expression was an artifact due to poorer growth of the mutant strains by plotting vir gene expression (Miller units) versus the number of cell doublings following induction. For strains At605 and At612, marginally reduced growth rates relative to those of the wild-type strains At558 and At353, respectively, were observed. This growth reduction did not account for the observed reduction in vir gene expression, however (data not shown).

The reduction of expression of the virD, virE, and pinF genes in the mutant Agrobacterium chromosomal background might readily be explained if the expression of the positive transcriptional activator virG was specifically reduced in the mutant background. To examine virG expression in the mutant chromosomal background, a merodiploid strain was constructed. This merodiploid strain allowed the

TABLE 3. Expression of various *vir::lacZ* gene fusions on the Ti plasmid in mutant (At587) and wild-type (donor) strains

vir::lacZ fusion on Ti plasmid	pH	AS[a]	*vir::lacZ* expression[b]		
			Wild-type strain (U)	Mutant *miaA* strain (U)	As % of wild type
virB::lacZ (mx234)	5.6	+	At559 (500 ± 91)	At606 (160 ± 40)	32 ± 8
virB::lacZ (mx30)	5.6	+	At558 (158 ± 13)	At605 (74 ± 14)	47 ± 9
virD::lacZ (mx304)	5.6	+	At560 (470 ± 97)	At607 (173 ± 14)	37 ± 3
virE::lacZ (mx358)	5.6	+	At561 (1,779 ± 258)	At608 (872 ± 107)	49 ± 6
pinF::lacZ (mx219)	5.6	+	At353 (321 ± 71)	At612 (90 ± 32)	28 ± 10

[a] Presence (+) of acetosyringone (AS).
[b] β-Galactosidase activity was determined as described in Table 2. Results are the average of at least three independently induced cultures. Assays were performed following 12 to 14 h of induction with acetosyringone.

examination of both noninduced and induced *virG* expression that occurs because of the positive autoregulation exhibited by this gene. The plasmid pSM363 harboring a Tn*3*-HoHo1 insertion in *virG* was mobilized into the reconstructed mutant strain containing pTiR10 (At627), thus creating the merodiploid strain At613. pSM363 was also mobilized into At488 to create At614 for use as a wild-type control. A similar merodiploid strain was made for a *virB* fusion (At618). The induction of these strains was examined, and the results are presented in Table 4. For strain At617, *virB::lacZ* expression was reduced only to 70% that of the wild type. The expression of *virG*, however, was reduced to approximately 37% that of the wild type. A similar reduction in *virG* expression was noted whether or not acetosyringone was present. The reduction of *virG* expression was not altered further when the pH of the induction medium was reduced to 5.2. The above results extend the mutant induction phenotype originally observed for *virB* to at least four more operons of the *vir* regulon. The possibility exists, however, that the reduced expression of *virG* is the cause of reduced expression of the other *vir* genes.

Analysis of virulence in mutant (At627) and wild-type (At488) strains. The reduced expression of at least five members of the *vir* regulon could result in an altered virulence of the mutant strain. The possible effects upon virulence might be general or host specific. Virulence assays were carried out with the reconstructed mutant strain *A. tumefaciens* At627 that carries the Ti plasmid pTiR10. Assays were performed on red potato disks, tobacco leaf disks, kalanchoe leaves, tomato stems, and sunflower stems. The results of these assays are summarized in Table 5. The only situation in which a marginal reduction in virulence was observed was when a low number of bacteria was used for infection on potato disks.

Complementation of mutant induction phenotype by pJG100. In order to prove linkage of the Tn*5* insertions to the

reduced induction phenotype of *A. tumefaciens* At44#37 and At44#38, we tested whether the cloned wild-type fragment corresponding to the Tn*5* insertion site could complement this phenotype. To this end, we cloned the wild-type 5-kbp *Eco*RI fragment from pJW100 into the broad-host-range vector pRK290 to make plasmid pJG100. pJG100 was introduced into *A. tumefaciens* At44, At44#37, and At44#38 by triparental mating. *virB::lacZ* expression was examined with each of these strains, and the results are presented in Table 6. The presence of pJG100 in At44 did not cause any further increase in *virB* expression, indicating that the gene product from this locus is not limiting for the process of *vir* gene expression in wild-type cells. We did find, however, that pJG100 partially restored *virB::lacZ* activity in both *A. tumefaciens* At44#37 and At44#38 to 63 to 73% that of wild-type activity. These assays were repeated many times, but full restoration of activity was never observed. The partial restoration of *virB::lacZ* activity in the mutant strains harboring pJG100 proves linkage of the Tn*5* insertions in At44#37 and At44#38 with the mutant induction phenotype exhibited by both of these strains.

Identification of the region of pJG100 that complements the reduction of vir gene expression. The 5-kbp *Eco*RI DNA fragment cloned in pJG100 partially restores the induction of *vir* gene expression (Table 6). In order to define further the genetic unit responsible for this complementation, we mutated pJG100 with Tn*5*Seq-1. Twenty-three Tn*5*Seq-1 insertions were obtained and mapped within the 5-kbp *Eco*RI insert (Fig. 1). A subset of these derivatives was used in complementation studies. Two plasmids (pJG117 and pJG113) failed to complement the induction phenotype of both mutant strains. Furthermore, the deletion derivative pJG100Δ*Cla*I continued to complement the original mutants, whereas the deletion derivative pJG100Δ*Hind*III failed to do so. These results defined a complementation unit with a

TABLE 4. Expression of *virG::lacZ* and *virB::lacZ* gene fusions on multicopy plasmids in mutant (At627) and wild-type (At488) strains

vir::lacZ fusion on multicopy plasmid	pH	AS[a]	Expression[b]		
			Wild-type strain (U)	Mutant *miaA* strain (U)	As % of wild type
virG::lacZ (pSM363)	5.6	−	At614 (100 ± 9)	At613 (37 ± 8)	37 ± 8
virG::lacZ (pSM363)	5.6	+	At614 (1,900 ± 74)	At613 (665 ± 57)	35 ± 3
virG::lacZ (pSM363)	5.2	−	At614 (127 ± 14)	At613 (52 ± 8)	41 ± 6
virG::lacZ (pSM363)	5.2	+	At614 (2,253 ± 145)	At613 (856 ± 45)	38 ± 2
virB::lacZ (pSM30)	5.6	−	At618 (18 ± 4)	At617 (7 ± 2)	39 ± 11
virB::lacZ (pSM30)	5.6	+	At618 (629 ± 74)	At617 (440 ± 31)	70 ± 5

[a] Presence (+) or absence (−) of acetosyringone (AS).
[b] β-Galactosidase activity was determined as described in Table 2. Results are the average of at least three independently induced cultures. Assays were performed following 12 to 14 h of induction with acetosyringone.

TABLE 5. Characterization of virulence in wild-type (At488) and mutant (At627) strains

Plant	No. of bacteria[a]	Virulence[b] $(n)^c$ in strain:	
		At488	At627
Potato	10^7	112 ± 43 (47)	94 ± 43 (45)
	10^6	60 ± 33 (30)	61 ± 28 (31)
	10^5	22 ± 18 (22)	5 ± 5 (33)^d
Tobacco	10^7	7.3 ± 5.4 (48)	8.2 ± 4.5 (48)
	10^6	5.5 ± 3.4 (44)	4.5 ± 3.6 (47)
	10^5	3.1 ± 1.9 (40)	2.5 ± 2.4 (46)
	10^4	1.2 ± 1.3 (19)	0.8 ± 1.3 (18)
Kalanchoe	10^6	90 (120)	76 (120)
	10^5	55 (120)	27 (120)
	10^4	14 (120)	10 (120)
Tomato	10^5	100 (30)	96 (26)
	10^4	93 (29)	93 (27)
	10^3	79 (34)	56 (32)

[a] Values are cells per milliliter for potato and tobacco and cells per wound for kalanchoe and tomato.

[b] Virulence is in tumors per disk for potato and tobacco and as percent of wounds with tumors for kalanchoe and tomato.

[c] *n* represents number of disks for potato and tobacco and number of wounds for kalanchoe and tomato.

[d] Means are significantly different at 1% level by using Student *t* test statistical analysis.

TABLE 6. Partial complementation of induction phenotype in mutants At44#37 and At44#38 by pJG100^a

Bacterial strain	Plasmid	*virB::lacZ* expression (as % of wild-type At44)[b]
At44	pRK290	100
At44	pJG100	102 ± 10
At44#37	pRK290	31 ± 6
At44#37	pJG100	73 ± 20
At44#38	pRK290	22 ± 7
At44#38	pJG100	63 ± 16

[a] Cells were assayed after a 12-h induction in AB glucose minimal medium, pH 5.6, with 30 mM MES, 2 mM phosphate, and 100 μM acetosyringone.

[b] Results are the averages of at least nine independently induced cultures.

maximum size of 1.4 kbp. This region spans the sites of both of the original Tn5 insertions (Fig. 1).

Identification of an ORF with homology to the *miaA* locus of *E. coli*. Following the definition of the complementation unit, we cloned a 1.5-kbp *ClaI-AccI* fragment spanning this region into pUC119 in both orientations, thus creating the plasmids pJG5 and pJG6. Both strands of this fragment were sequenced. The entire sequence is 1,490 bp in length and is presented in Fig. 2. We analyzed the DNA sequence and identified an open reading frame (ORF) extending from nucleotides 203 to 1096 (Fig. 2). This ORF spans the region delineated by the Tn5 insertions in the mutants *A. tumefaciens* At44#37 and At44#38 but does not extend as far as the insertion sites of pJG104 and pJG103 (Fig. 1). A homology search, using the programs FASTA and TFASTA (48), was conducted by using both the DNA sequence and the predicted amino acid sequence of this ORF. Highly significant homology between this ORF and the *miaA* gene of *E. coli* was found. Fifty-two-percent nucleic acid identity and 37% amino acid identity, plus a further 38% conservative amino acid replacement, was observed over the entire length of the ORF. The ORF also had significant homology to the *mod5* locus of *Saccharomyces cerevisiae* (30% amino acid identity plus 41% conservative amino acid replacement). TFASTA optimal scores for these alignments with the *E. coli* and *S. cerevisiae* genes were 517 and 336, respectively. Each of these loci encodes a protein with a known tRNA:isopentenyladenine transferase activity (12, 47). The predicted amino acid sequences for the *Agrobacterium* protein (A. tum. MiaA) and for the *E. coli* MiaA and yeast Mod5 proteins are aligned in Fig. 3 by using the PILEUP alignment program. The homology among each of the three proteins is extensive and is present in both local domains as well as being spread evenly throughout the protein. Particularly well conserved among all three proteins is a putative ATP/GTP-binding site close to the amino termini of the proteins (12,

65). A second well-conserved region among the three proteins (amino acids 207 to 233) is a possible isopentenylpyrophosphate-binding site (12). The possible significance of other regions that are well conserved among all three proteins (e.g., residues 98 to 114 and 273 to 282) is unknown. The yeast Mod5 protein has an additional 98 amino acids present at the carboxy terminus of the protein that may comprise another functional domain not present in the procaryotic proteins (12). The *Agrobacterium* MiaA protein has two methionine residues at the amino terminus, although the first methionine may not be translated. This is suggested by the existence of a possible Shine-Dalgarno ribosome-binding site with the correct spacing from the second methionine residue but too close to the first methionine residue. The *miaA* sequence predicts a protein 298 amino acids in length (33 kDa) that is slightly smaller than the MiaA protein of *E. coli* (34 kDa). The apparent molecular mass of native MiaA transferase in *E. coli* is approximately 55 kDa (1), indicating that the active enzyme probably exists as a homodimer (12). The evolutionary comparison favors the view that one of the noted AUG codons is a translational start codon, but alternative in-frame GTG and TTG start codons are present further upstream. The sequenced region of the *A. tumefaciens miaA* gene includes 202 bp upstream of the proposed translational start site. A second possible partial ORF in this region, extending from nucleotide 1 to nucleotide 168 (Fig. 2), was also identified. We did not observe for this region an acceptable fit to known transcriptional start consensus sequences. For the region 3' of the translational stop codon, we observed a possible weak rho-independent transcription terminator signal between nucleotides 1129 and 1142. We also conducted homology searches by using either the 5' or 3' portion of the sequenced region not including the MiaA ORF but did not find significant homology to known genes or proteins.

Homology searches conducted with the ORF revealed some homologies of possible significance between the *A. tumefaciens miaA* locus and the *A. tumefaciens tzs* and *ipt* (*tmr*) loci (genes involved in cytokinin biosynthesis). These homologies occurred over smaller regions of the gene than did those with the *E. coli miaA* or *S. cerevisiae* loci. Two examples include 33.3% amino acid identity over 57 amino acids with the *A. tumefaciens tzs* gene (2) and 23% amino acid identity over 163 amino acids with the *A. tumefaciens ipt* gene (37). The homology to this set of genes occurs near the amino terminus of the protein and includes the highly conserved putative ATP/GTP-binding site. This motif may more likely correspond to an adenosine-binding site than a phosphate-binding site, since an adenosine-binding site might be expected for all of these enzymes.

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  1  GATGGGTTTTCCGATAAGCGCGGCTGCAATCGCGCTGCGCGCCTGTTCGGCTGCGGTGCCCAGGGGCAGGGCTAT   75
     AspGlyPheSerAspLysArgGlyCysAsnArgAlaAlaArgLeuPheGlyCysGlyAlaGlnGlyGlnGlyTyr
     Putative ORF

 76  ATCGCAGGCGATGCCGTGGGCCAGCCAATAAAGGCCCGATGCATTGACCGCCTTTGCGGCGGCTTCGCCGAGGGC  150
     IleAlaGlyAspAlaValGlyGlnProIleLysAlaArgCysIleAspArgLeuCysGlyGlyPheAlaGluGly

151  TGCTGTCAGAACAGGATTTGACGGATTGGCGATAAGCGTGGCAACCAGAGCCATGATGAAAAACCTTGATCAGAA  225
     CysCysGlnAsnArgIleEnd                                  MetMetLysAsnLeuAspGlnAs
                                                        --- Proposed miaA ORF

226  TTTTGATGCGATCCTGATAACCGGCCCGACGGCAAGCGGCAAGTCCGCGCTTGCGCTTCGTCTGGCGCGGGAGCG  300
     nPheAspAlaIleLeuIleThrGlyProThrAlaSerGlyLysSerAlaLeuAlaLeuArgLeuAlaArgGluAr

301  GAACGGCGTCGTCATCAATGCCGACAGCATGCAGGTTTACGACACGCTGCGGGTGCTGACCGCCCGGCCTTCCGA  375
     gAsnGlyValValIleAsnAlaAspSerMetGlnValTyrAspThrLeuArgValLeuThrAlaArgProSerAs

376  CCACGAAATGGAGGGGGTGCCACACCGTCTCTACGGCCATGTGCCCGCTGGCAGCGCCTATTCGACCGGCGAATG  450
     pHisGluMetGluGlyValProHisArgLeuTyrGlyHisValProAlaGlySerAlaTyrSerThrGlyGluTr

451  GCTGCGGGATATTTCCGGACTGCTTTCGGATCTGCGCGGTGAGGGGCGTTTTCCTGTCATTGTCGGCGGTACGGG  525
     pLeuArgAspIleSerGlyLeuLeuSerAspLeuArgGlyGluGlyArgPheProValIleValGlyGlyThrGl

526  GCTTTATTTCAAGGCGCTGACCGGCGGCCTTTCCGATATGCCCGCCATTCCCGATGACCTCCGCGAGGGGCTGCG  600
     yLeuTyrPheLysAlaLeuThrGlyGlyLeuSerAspMetProAlaIleProAspAspLeuArgGluGlyLeuAr

601  CGCCCGGTTGATCGAAGAGGGAGCGGCAAAGCTTCACGCCGAATTGGTGAGCCGCGATCCGTCCATGGCGCAGAT  675
     gAlaArgLeuIleGluGluGlyAlaAlaLysLeuHisAlaGluLeuValSerArgAspProSerMetAlaGlnMe

676  GCTGCAGCCGGGAGATGGCCAGCGCATCGTCCGGGCACTGGAGGTGCTCGAGGCGACGGGGAAATCGATCCGCGA  750
     tLeuGlnProGlyAspGlyGlnArgIleValArgAlaLeuGluValLeuGluAlaThrGlyLysSerIleArgAs

751  TTTCCAGCGCGCCAGCGGCCCGATGATCATCGATCCCGAGCGGGCGCAGAAATTCATCGTCCTGCCGGAGAGGCC  825
     pPheGlnArgAlaSerGlyProMetIleIleAspProGluArgAlaGlnLysPheIleValLeuProGluArgPr

826  GGTGCTGCATGACCGTATCAACCGCCGTTTTGAGGCGATGATGGACAGTGGTGCGGTGGAGGAGGTCCAGGCGCT  900
     oValLeuHisAspArgIleAsnArgArgPheGluAlaMetMetAspSerGlyAlaValGluGluValGlnAlaLe

901  TCTTGCGCTCAATCTAGCGCCCGATGCAACGGCGATGAAGGCGATTGGCGTTGCTCAGATCGCCGATATGCTGAC  975
     uLeuAlaLeuAsnLeuAlaProAspAlaThrAlaMetLysAlaIleGlyValAlaGlnIleAlaAspMetLeuTh

976  TGGGCGCATGGGTGCGGCGGAGGTGATAGAAAAATCGGCGGCTGCGACCCGCCAATATGCCAAACGGCAGATGAC 1050
     rGlyArgMetGlyAlaAlaGluValIleGluLysSerAlaAlaAlaThrArgGlnTyrAlaLysArgGlnMetTh

1051 CTGGTTCCGCAACCAGATGGGGGATGACTGGACGCGCATCCAGCCGTGAAAGGGGCCTAATCCTTGCGGTAGAGG 1125
     rTrpPheArgAsnGlnMetGlyAspAspTrpThrArgIleGlnProEnd

1126 CTGCCGAGCGGTTTTTTCAACAGGCTTTCGCGCAACGAGGGGCGCGTCTCACCGGGCGGCGGGCCATTCTGCGCC 1200
     ***  ********

1201 TGCATCTGGAAACCCGCCTTGCGCAGATCGTTGACGGCGCCGGCGGTGGGGTCCGTTGACGCCGCATTGGATGGC 1275

1276 ACAGGCGGTGTGCGTCCGGGCAGCATATCCGGGCGGTAGGGTTCGTATCCCCCCGTTGCGGGCGTTCCCAATTCT 1350

1351 CGCTTCCAGCGATCGATGTCGTCTGCCGTCTGGAATACGGACGGTTCTGGCTGGTCGGAAAAAGCCGCTTCCACG 1425

1426 CTTTGCTGGAAGCCGGTGATAACGGGTTTGGCCGTGGCGCGCATGCGGGTGACTATGCTGTTCAG 1490
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FIG. 2. Nucleotide sequence of the 1,490-bp ClaI-AccI DNA fragment. The proposed A. tumefaciens miaA translational reading frame is between nucleotides 203 and 1099. A putative ribosome-binding site, GAG, is underlined upstream of two possible methionine translational initiation codons. A weak rho-independent terminator sequence is underlined with asterisks between nucleotides 1129 and 1142. A possible partial ORF is indicated between nucleotides 1 and 168.

Complementation of E. coli miaA mutants with pJG5 and pJG6. The strong similarity of the A. tumefaciens At44#37 and At44#38 mutant locus to the miaA gene of E. coli provided an opportunity to define a function for the gene we had isolated. We sought to complement an E. coli miaA mutant by using the wild-type 1.5-kbp EcoRI fragment (pJG5 and pJG6) that had been sequenced. To assay for the

presence of a functional miaA gene in E. coli, Petrullo and Elseviers (49) constructed a strain [DEV15lacZ(UGA)] that contains an ochre stop codon in the lacZ gene. In a wild-type strain, readthrough of this stop codon occurs so that lactose can be utilized by the bacterium. Such colonies appear red on 0.4% lactose-MacConkey plates. In miaA strains, however, tRNA undermodification leads to increased transla-

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A.tum. MiaA  MMKNLDQNF.....DAILIIGPTASGKSALALRLARERNGVVINADSMQV45
E.coli MiaA  .MSDISKASL...PKAIFIMGPTASGKTALAIELRKILPVELISVDSALI
Yeast Mod5   MIKGPLKGCLNMSKKVIYIAGTTGVGKSQLSIQLAQKFNGEVINSDSMQV

A.tum. MiaA  YDTLRVLTAKPSDHEMEGYPHRLYGHVPAGSAYSTGEWLRDISGLLSDLR95
E.coli MiaA  YKGMDIGTAKPNAEELLAAPHRLLDIRDPSQAYSAADFRRDALAEMADIT
Yeast Mod5   YKDLPIITNKHPLQEREGIPHHVMNHVDWSEEYYSHRFETKCMNAIEDIH

A.tum. MiaA  GEGRFPVIVGGTGLYFKALTGGLSDMPAIPDLLREGIRARLIEEGAAKLH145
E.coli MiaA  AAGRIPLIVGGTMLYFKALLEGLSPIPSADPEVRARIEQQAAEKGWESLH
Yeast Mod5   RRGKIPLVVGGTHYYLQTLFNKRVDTKSSERKLTRKQLDILESTDPDVIY

A.tum. MiaA  AELVSRDPSMAQMLQPGDGQRIVRALEVLEATGKSIRDFQRASGPMIIDP195
E.coli MiaA  RCLQEVDPVAAARIHPNDPQRLSRALEVFFISGKTLTELTQTSGDAL..P
Yeast Mod5   NTLVKCDPDIATKYHPNDYRRVQRMLETYYKTGKKPSE...TFNEQKITL

A.tum. MiaA  ERAQKFIVLPE.RPVLHDRINRRFEAMMDSGAVEEVQALLA......L.N237
E.coli MiaA  YQVHQEAIAPASREILHQRIEQRFHQMLASGFEAEVRALFA......RGD
Yeast Mod5   KFDTLEIWLYSKPEPLFQRLDDRVDMLERGALQEIKQLYEYYSQNKFTP

A.tum. MiaA  LAPDATAMKAIGVAQIADMLTGR.....MGAAEVIEKSAAATRQYAKRQM282
E.coli MiaA  LHTDLPSIRCVGYRQMWSYLEGE.....ISYDEMVYRGVCATRQLAKRQI
Yeast Mod5   EQCENGVWQVIGFKEFLPWLTGKTDDNTVKLEDCIERMKTRTRQYAKRQV

A.tum. MiaA  TWFRN......QMGDDWTRIQP*.........................
E.coli MiaA  TWLRG......WEGVHWLDSEKPEQARDEVLQVVGAIAG*...........
Yeast Mod5   KWIKKMLIPDIKGDIYLLDATDLSQWDTNASQRAIAISNDFISNRPIKQE

A.tum. MiaA  ............................
E.coli MiaA  ............................
Yeast Mod5   RAPKALEELLSKGETTMKKLDDWTHYTRNVCRNADGKNVVAIGEKYWKIH

A.tum. MiaA  ............................
E.coli MiaA  ............................
Yeast Mod5   LGSRRHKSNLKRNTRQADFEKWKINKKETVE
```

FIG. 3. Alignment of the amino acid sequences of the *A. tumefaciens miaA*, *E. coli miaA*, and *S. cerevisiae mod5* gene products. Alignment was completed by using the PILEUP multiple alignment program (15). Amino acid similarities as determined by the BOXSHADE program are shown (15). Dark shading indicates identical amino acids, and light shading indicates conservative amino acid replacements between sequences. Numbering is with reference to the *A. tumefaciens miaA* sequence. The first underlined region, amino acids 16 to 25, indicates a putative ATP/GTP-binding motif highly conserved among all three proteins. The third underlined region, amino acids 207 to 233, indicates a proposed isopentenyl substrate binding domain. The second and fourth underlined regions, amino acids 98 to 114 and 273 to 284, respectively, indicate regions highly conserved among the three proteins but of unknown significance.

tional proofreading and readthrough of the stop codon is reduced, resulting in the growth of white colonies on the same indicator plates. *E. coli* DEV15*su⁺9* also contains a suppressor tRNA that allows readthrough of the *lacZ*(UGA) codon, with a resultant metallic green colony color on 0.4% lactose–EMB plates. In contrast, a DEV15*su⁺9miaA* strain exhibits a pinkish black colony color on these plates.

We first transformed the plasmid pJG6 into both DEV15 and DEV15*miaA*, using the vector pUC119 as a control. The result of this experiment is shown in Fig. 4. Both pUC119 and pJG6 (pUC119 plus 1.5-kbp insert) did not alter the red colony color of DEV15 on MacConkey-lactose plates. pJG6 could restore the red colony color to DEV15*miaA*, whereas pUC119 could not. This result indicated that the 1.5-kbp insert in pJG6 contains a gene that exhibits *miaA* activity. There remained the possibility that this plasmid did not contain a native *Agrobacterium* promoter and that the gene was being expressed from the *lac* promoter of pUC119. We tested for this by transforming both pJG5 and pJG6 (which contain the insert cloned in opposite orientations) into DEV15*su⁺9* and DEV15*su⁺9miaA*. We found that the Lac⁺ phenotype was restored to DEV15*su⁺9miaA* by both plas-

mids, whereas the vector plasmid pUC119 alone could not restore it (data not shown). This result indicates that the cloned gene from *Agrobacterium* strains is indeed a *miaA* gene and contains a promoter that can function in *E. coli*. This promoter, however, is not necessarily the native promoter that functions in *Agrobacterium* strains.

DISCUSSION

We initiated this study to identify chromosomal loci in *A. tumefaciens* that play a role in *vir* gene expression. In this paper, we report the isolation of a gene on the *Agrobacterium* chromosome that, when mutated, results in a two- to tenfold reduction in the induced expression of *vir* genes. We isolated two Tn*5* mutant strains with this phenotype that mapped in the same chromosomal locus. The locus was defined genetically and cloned, and the DNA sequence was determined. The predicted amino acid sequence of an ORF within the defined region exhibited strong homology to both the MiaA protein of *E. coli* (12) and the Mod5 protein of *S. cerevisiae* (47). This relationship was confirmed by comple-

DEV 15
pUC119 +
1.5kb
insert

DEV 15
pUC119

DEV 15 *miaA*
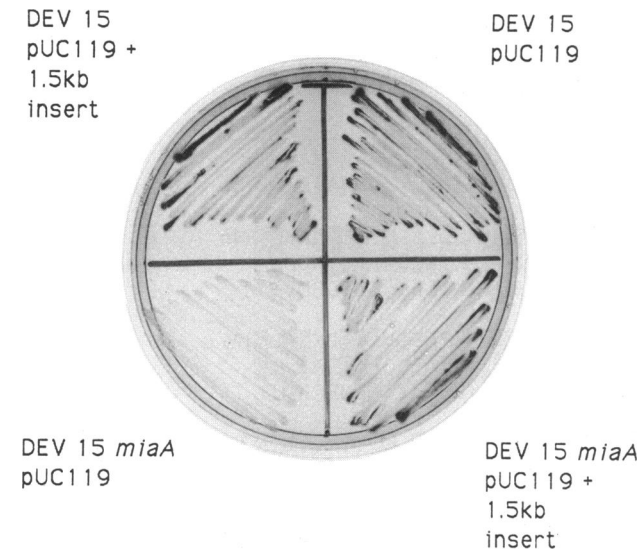pUC119

DEV 15 *miaA*
pUC119 +
1.5kb
insert

FIG. 4. Functional complementation of *E. coli* DEV15*miaA* by pJG5. pJG5 restores the red colony color to DEV15*miaA* growing on 0.4% lactose-MacConkey plates, whereas the vector alone, pUC119, fails to restore the wild-type MiaA function.

mentation of a mutant *E. coli miaA* strain with the gene isolated from *A. tumefaciens*.

The *miaA* gene of *E. coli* has been extensively studied (3). The MiaA protein is a tRNA:isopentenyltransferase. The specific function of this protein is to catalyze the addition of an isopentenyl moiety onto the N-6 atom of adenosine in position 37 of UNN species tRNAs (18). This adenosine residue occurs immediately 3' to the anticodon sequence. Further modification of the i^6-A-37 by methylation, thiolation, and hydroxylation can occur subsequent to the isopentenylation reaction. Similar modifications have been shown to occur in the tRNA of *A. tumefaciens* (10, 45). Modification of the A-37 residue appears to be necessary for the stabilization of the codon-anticodon interaction during protein translation (20). Other investigations have indicated that nonmodification of tRNA in a *miaA* mutant strain results in increased translational proofreading, a reduced protein elongation rate, and increased peptidyl-tRNA release (49). The lack of tRNA modification also results in decreased transcription termination at the *phe* and *trp* operon attenuators in *E. coli* (24, 69) and reduced *leu* operon expression in *Salmonella typhimurium* (4). In summary, modification of A-37 plays an important role in the efficiency and fidelity of translation as well as codon context sensitivity.

We have isolated a *miaA*+ homolog in *A. tumefaciens* and proved its function by complementing an *E. coli miaA* mutant. Mutations in this locus should result in nonmodification of UNN tRNAs in *Agrobacterium* strains and could thus result in a reduced translation of *vir* gene transcripts. We observed a two- to threefold reduction in the expression of *virB*, *virD*, *virE*, *pinF*, and *virG* under AB glucose induction conditions and as much as a five- to tenfold reduction in *virB* expression under AB sucrose induction conditions. We complemented the reduced *vir* expression in the *A. tumefaciens* strains At44#37 and At44#38 by using the plasmid pJG100, which contains the wild-type *Agrobacterium miaA* gene. In these studies, we never observed full restoration of *vir* gene activity. This partial complementation may result from the possible formation of heterodimers

between the wild-type MiaA protein encoded on pJG100 and truncated mutant proteins caused by aberrant expression from Tn5 insertions in the mutant chromosomal loci. The native MiaA transferase in *E. coli* is apparently a dimer (1). An alternative explanation for partial complementation is the possibility that pJG100 may not contain the full promoter for the *A. tumefaciens miaA* gene (i.e., if *miaA* were part of a larger operon). This possibility is lessened by the fact that an *E. coli miaA* mutant was complemented by both pJG5 and pJG6. These two plasmids contain a short DNA region containing the *miaA* gene in opposite orientations. Our results suggest that a native *Agrobacterium* promoter was present and that it could function in *E. coli*. The color change indicating *E. coli* complementation is not quantitative, however, and it may well be that full promoter activity is not specified by both of these plasmids. If the *A. tumefaciens miaA* gene is monocistronic, its genetic organization differs from that of the *miaA* gene in *E. coli*, which is the third member of a three-gene operon that includes a *mutL* (mismatch repair protein) gene immediately upstream of the *miaA* gene (11, 12). We did not observe any homology to *mutL* in the 202 bp upstream of the *A. tumefaciens miaA* gene, but this finding does not rule out the possibility of a *mutL* analog being farther upstream or downstream in a possible operon. A possible ORF (nucleotides 1 to 168 in Fig. 2) that ends close to the MiaA ORF was observed, indicating that the *miaA* gene may be part of an operon in *Agrobacterium* strains. The question of whether or not the *A. tumefaciens miaA* gene is part of an operon is not answered by the present study, however.

The observed reduction in *virB*, *virD*, *virE*, and *pinF* expression may readily be explained if tRNA undermodification has a direct effect upon the activity of *virG*, a positive activator of gene expression for these other loci. It will be necessary to extend our study to genes outside of the *vir* regulon in order to determine whether the reduced gene expression that we have observed is *vir* gene specific. We note that in this study, *vir* gene expression was measured from *lacZ* fusions; further studies using alternative reporter genes will be needed to determine if the observed effects could be specific to these fusions. There is evidence that modification of A-37 in tRNA plays a role in global control of gene expression in cells and that the expression of only a subset of total proteins in *E. coli miaA* mutant strains are affected (11). If there is a subset of proteins whose expression is influenced strongly by this type of tRNA modification, it will be of interest to determine why the *vir* regulon belongs to such a group.

Despite the observed reduction in *vir* gene expression in *A. tumefaciens miaA* strains, we observed only a marginal reduction in virulence in the *miaA* strain At627 on potato plants (Table 5). The four- to fivefold reduction in the number of tumors per potato disk induced by the *miaA* mutant strain appears very minor compared with the effect of mutations in the *chvE* (30) or *ivr* loci (41). We did not observe a decreased virulence in the mutant strain with any of the other virulence assays, although these assays are generally perceived not to be as sensitive as the potato disk assay and thus may not allow the detection of minor virulence determinants. One other possible interpretation of our results is that the reduced expression of at least five members of the *vir* regulon, to between 30 and 50% of wild-type levels, does not alter virulence because these products do not limit the rate of the infection process when expressed in these amounts.

Finally, we observed a significant degree of amino acid

homology among the A. tumefaciens miaA, tzs, and ipt genes. The tzs gene is involved in trans-zeatin synthesis and secretion in the bacterium (52), while the ipt gene is involved in cytokinin biosynthesis in the host plant following T-DNA transfer and integration (6). We considered whether or not the MiaA protein may have a role in free trans-zeatin synthesis in Agrobacterium strains. MiaA functions to transfer an isopentenyl group to adenosine previously incorporated into tRNA, and Tzs functions to transfer an isopentenyl group to free 5' AMP. In plants, much evidence exists to suggest that the presence of cytokinins in tRNAs is unrelated to free cytokinin biosynthesis (35). The tzs gene in Agrobacterium strains is inducible by acetosyringone and can catalyze trans-zeatin formation in E. coli (2, 50). An earlier study (52), however, indicated that free isopentenyladenine (iP) was found in the culture filtrate of a strain cured of its Ti plasmid at levels only half that of a strain harboring a nopaline-type Ti plasmid. The production of this free iP would appear to be a chromosomally encoded process and is of unknown significance. It also appears that the generation of iP is not related to tRNA breakdown (34, 52). It is conceivable that the miaA gene is involved in the synthesis of free iP in Agrobacterium strains. The availability of the mutant miaA A. tumefaciens strains that we have isolated allows this subject to be investigated.

ACKNOWLEDGMENTS

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