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RESEARCH ARTICLE

Transcriptional Activation of Virulence Genes of *Rhizobium etli*

Luyao Wang,[a,b] Benoît Lacroix,[a] Jianhua Guo,[b] Vitaly Citovsky[a]

Department of Biochemistry and Cell Biology, State University of New York, Stony Brook, New York, USA[a]; Department of Plant Pathology, College of Plant Protection, Nanjing Agricultural University, Key Laboratory of Monitoring and Management of Crop Diseases and Pest Insects, Ministry of Agriculture, Engineering Center of Bioresource Pesticide in Jiangsu Province, Nanjing, Jiangsu Province, China[b]

ABSTRACT Recently, *Rhizobium etli*, in addition to *Agrobacterium* spp., has emerged as a prokaryotic species whose genome encodes a functional machinery for DNA transfer to plant cells. To understand this *R. etli*-mediated genetic transformation, it would be useful to define how its *vir* genes respond to the host plants. Here, we explored the transcriptional activation of the *vir* genes contained on the *R. etli* p42a plasmid. Using a reporter construct harboring *lacZ* under the control of the *R. etli virE* promoter, we show that the signal phenolic molecule acetosyringone (AS) induces *R. etli vir* gene expression both in an *R. etli* background and in an *Agrobacterium tumefaciens* background. Furthermore, in both bacterial backgrounds, the p42a plasmid also promoted plant genetic transformation with a reporter transfer DNA (T-DNA). Importantly, the *R. etli vir* genes were transcriptionally activated by AS in a bacterial species-specific fashion in regard to the VirA/VirG signal sensor system, and this activation was induced by signals from the natural host species of this bacterium but not from nonhost plants. The early kinetics of transcriptional activation of the major *vir* genes of *R. etli* also revealed several features distinct from those known for *A. tumefaciens*: the expression of the *virG* gene reached saturation relatively quickly, and *virB2*, which in *R. etli* is located outside the *virB* operon, was expressed only at low levels and did not respond to AS. These differences in *vir* gene transcription may contribute to the lower efficiency of T-DNA transfer of *R. etli* p42a than of T-DNA transfer of pTiC58 of *A. tumefaciens*.

IMPORTANCE The region encoding homologs of *Agrobacterium tumefaciens* virulence genes in the *Rhizobium etli* CE3 p42a plasmid was the first endogenous virulence system encoded by the genome of a non-*Agrobacterium* species demonstrated to be functional in DNA transfer and stable integration into the plant cell genome. In this study, we explored the transcriptional regulation and induction of virulence genes in *R. etli* and show similarities to and differences from those of their *A. tumefaciens* counterparts, contributing to an understanding and a comparison of these two systems. Whereas most *vir* genes in *R. etli* follow an induction pattern similar to that of *A. tumefaciens vir* genes, a few significant differences may at least in part explain the variations in T-DNA transfer efficiency.

KEYWORDS *Rhizobium etli*, plant genetic transformation, virulence genes

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Address correspondence to Benoît Lacroix, benoit.lacroix@stonybrook.edu.

Agrobacterium tumefaciens genetically transforms the host plants by transferring and integrating a segment of its own DNA into the genome of its host cells (1, 2). This virulence is conferred by a large plasmid (Ti plasmid) that harbors both the genes required for the DNA transfer, i.e., the virulence (*vir*) genes, and the DNA sequence to be transferred (transfer DNA [T-DNA]). Whereas in nature *A. tumefaciens* transfers specific genes that cause plant cell proliferation and the production of opines, i.e., small amino acid derivatives used as sources of carbon and nitrogen by the bacteria (3), the

DNA transfer mechanism *per se* is not sequence specific, making *A. tumefaciens* the most widely used vector for plant genetic transformation (4). Traditionally, the ability of *A. tumefaciens* to mediate the bacterium-to-eukaryote DNA transfer has been considered unique in the living world. Indeed, other bacterial species within the *Rhizobiales* could acquire this ability only if they are provided with the *vir* region from a virulent strain of *A. tumefaciens*. For example, several species of *Rhizobium*, *Sinorhizobium*, and *Mesorhizobium* (5–7) and, later, *Ensifer adhaerens* (*Ensifer* and *Sinorhizobium*, in fact, represent the same genus) (8, 9) have been shown to become capable of plant genetic transformation when provided with the *A. tumefaciens* Ti plasmid functions. Only very recently, we demonstrated that another bacterial species, *Rhizobium etli* strain CE3, known to be a symbiotic plant-associated bacterium able to fix nitrogen like many other members of the *Rhizobiales* family, has evolved its own functional bacterium-to-plant DNA transfer machinery (10). Although *R. etli* can potentially be associated with different host plant species, this bacterium represents the predominant species nodulating common beans (*Phaseolus vulgaris*), particularly in its region of origin, Latin America (11). Specifically, a region of *R. etli* plasmid p42a contains genes with extensive homology to all the essential *vir* genes of *A. tumefaciens*. Complete genome sequencing of *R. etli* CFN42 revealed that it harbors six plasmids, designated p42a to p42f, with different functions. Among these plasmids, p42a is a 194-kb self-conjugative plasmid containing a segment of 30 kb that carries genes involved in conjugative transfer (12), similar to the *A. tumefaciens* Ti plasmid. The similarity between these two plasmids, however, is restricted to the *vir* region, and no T-DNA-like sequences have been detected in *R. etli* (10, 12). The activity of the p42a *vir* region does not appear to affect symbiosis directly, as normal nodulation was observed with mutants with mutations in the *virG* and *virE2* genes (13); however, p42a is involved in the conjugative transfer of the symbiotic plasmid p42d/pSym (14). When supplied with a binary plasmid that contains a T-DNA sequence but not the *vir* region, *R. etli* was able to genetically transform plant cells (10). In *A. tumefaciens*, *vir* genes are transcriptionally activated in response to signaling molecules, such as the phenolic compound acetosyringone (AS) or small phenolics contained in plant exudates (15, 16). Here, we investigated whether the *vir* genes of *R. etli* also are induced by similar signals and examined potential similarities and differences in transcriptional activation of the virulence systems encoded by *R. etli* and *A. tumefaciens*.

RESULTS

Sequence analysis of *vir* promoter regions in *R. etli* p42a. We analyzed the sequences of the *vir* genes and operons contained within the *R. etli* p42a plasmid (Table 1) for the presence of *vir* box motifs in the promoter regions. These motifs, first identified in both octopine and nopaline strains *A. tumefaciens* (17, 18), comprise a short sequence of 10 to 12 nucleotides with a loose consensus sequence of 5′-dPu (T/A)TDCAATTGHAAPy (where dPu is a deoxypurine; H is A, C, or T; D is A, G, or T; and Py is a pyrimidine) (19) and are found in promoters of *vir* operons, usually between 50 and 200 bp upstream of the translation initiation codon of the first gene of each *vir* operon. The *vir* boxes are directly recognized by the transcriptional activator VirG, which binds to their sequences (19, 20). We performed a manual search for conserved motifs in the promoter regions of *R. etli vir* operons (Table 1). Putative *vir* boxes were found for all *vir* operons of *R. etli* on the basis of the comparison with their closest counterparts identified in *A. tumefaciens* (17). Similar to the *vir* box sequences in different *Agrobacterium* strains, the identified *vir* boxes of *R. etli* are not strongly conserved, but each *vir* operon in the p42a plasmid contains at least one *vir* box homolog.

Activation of the *R. etli* p42a *virE* promoter and T-DNA transfer capability in *R. etli* and *A. tumefaciens* backgrounds. To assess and compare the activation of a *vir* promoter from the *R. etli* p42a origin in the *R. etli* and *A. tumefaciens* backgrounds, we designed a construct, designated pREp-lacZ (Table 2), that harbors the β-galactosidase reporter gene under the control of the promoter region of the *virE* operon of *R. etli*

TABLE 1 Sequence homologs of the *A. tumefaciens vir* box in *vir* promoter regions of *R. etli*/p42a

R. etli CFN42		*A. tumefaciens*		No. of nucleotide hits/ total no. of nucleotides
Operon	Sequence (position)[a]	Operon	Sequence (position)[b]	
virB	**AA**A**AAT**C**GAAA**A (−102)	*virB*	A**GCAATTGAAA**A* (−110)	9/12
		virG	**TA**A**AATTGAAAT** (−78)	9/12
virD	CG**AATT**CA**AAAT** (−415)	*virC*	C**G**AA**TTTGAAAT*** (−116)	10/12
	TTAA**TTTGCAA**G (−188)	*virD*	**TT**A**AATTGCAAT** (−151)	10/12
	A**GCA**G**TT**C**AAT**G (−178)	*virB*	A**GCAATTGAAA**A* (−110)	9/12
	AATG**ATTGC**G**AT** (−143)	*virG*	**AA**CG**ATTGA**G**AA** (−99)	9/12
	TGCG**ATTGTAAT** (−137)	*virD*	**TTCAATTGTAAT** (−141)	10/12
	ATA**AAC**T**GAAAT** (−122)	*virB*	**TTCAATTGAAAT*** (−130)	9/12
		virB	**TTCAA**A**TGAAAT** (−132)	9/12
	A**TA**A**AC**T**GAAAT** (−123)	*virC*	**TA**-A**AATTGAAAT** (−117)	11/12
		virE	**TACA**TA-**TGAAAC** (−126)	10/12
	TAAACTGAAATT (−125)	*virC*	**TACAAT**AA**AAT**T (−122)	9/12
	TATAAT**ATTGA**T (−102)	*virD*	**TAT**T**TTCAAT** (−147)	9/12
virE	**TTCA**GA**TGAA**GC (−436)	*virB*	**TTCAA**A**TGAAAT** (−132)	9/12
	TTCAC**TTCAAT**T (−376)	*virA*	**TTCA**C**TTGAAAC** (−117)	9/12
		virD	**TTCAATTT**T**ATT** (−163)	10/12
virB2	**TTC**GC**TTC**A**AAT** (−537)	*virA*	**TTCA**C**TTGAAAC** (−117)	10/12
		virB	**TTCAATTGAAAT*** (−130)	10/12
	TGAG**ATTGAAAT** (−526)	*virC*	C**G**AA**TTTGAAAT*** (−116)	9/12
		virC	**TA**A**AATTGAAAT** (−117)	10/12
	AGA**AAT**C**GAAA**A (−512)	*virB*	A**GCAATTGAAA**A* (−110)	10/12
virF1	**TG**TG**ATTGAAA**G (−33)	*virE*	**TTCAATTGAAAT*** (−130)	10/12
virF2	AC**CA**T**TTGAAA**G (−210)	*virB*	A**GCAATTGAAA**A* (−110)	9/12
		virA	**TTCA**T**TTGAAA**C (−117)	9/12
virC	**TTCAA**AG**G**G**A**T**T** (−742)	*virB*	**TTCAA**A**TGAAAT** (−132)	9/12
	A**ACAA**G**TGC**TAC (−613)	*virC*	**TA**T**AATTGC**TAC* (−147)	10/12
	CCATC**TTGAAAT** (−460)	*virC*	CGA**A**T**TTGAAAT*** (−116)	11/12
	AAAT**AT**A**AAAT**T (−109)	*virC*	**TACAA**TA**AAAT**T (−122)	10/12
	TTCG**ATT**A**A**T**A**G (−99)	*virB*	**TTCAATTGAAAT*** (−130)	9/12
virG	**TA**TGT**TTGTAAC** (−59)	*virD*	TTTT**ATTGTAAT** (−158)	10/12
		virE	**TACA**TA**TGAAAC** (−126)	10/12

[a]Consensus bases of the *vir* box sequences (19) are indicated by boldface. Position coordinates are relative to the position of the annotated translation initiation codons.

[b]Sequences that have been validated experimentally are indicated by asterisks.

p42a, which includes the sequence ca. 500 bp upstream of the translation initiation codon of the *virE1* gene. *R. etli* and *A. tumefaciens* strains transformed with this reporter plasmid were then used to measure promoter activity in response to challenge with AS (Fig. 1) under the conditions known to favor AS induction in *A. tumefaciens*, i.e., a low pH, a low phosphate concentration, and the presence of glucose (21). In *R. etli* harboring the wild-type p42a plasmid, the β-galactosidase activity increased significantly upon treatment with 50 and 100 μM AS, whereas the basal expression level was observed in the absence of AS. In contrast, the expression levels remained very low in the *R. etli* Δ*virG* mutant strain, showing that activation of the *virE* promoter depends on the VirA/VirG two-component system. Furthermore, in the absence of AS, the *R. etli* Δ*virG* mutant exhibited lower reporter expression levels than the wild-type *R. etli* strain (Fig. 1), suggesting that the basal, uninduced level of *vir* gene expression in *R. etli* also requires VirG. As expected, when the pREp-lacZ reporter construct was introduced into the *A. tumefaciens* C58 strain cured of its Ti plasmid (C58NT1), only residual β-galactosidase activity was observed (Fig. 1). However, when C58NT1 was cotransformed with p42a and pREp-lacZ, AS induced reporter expression to levels similar to those observed in the wild-type *R. etli* background (Fig. 1). Thus, the *vir* gene induction machinery encoded by the p42a plasmid remains functional in the *A. tumefaciens* background.

TABLE 2 Plasmids and bacterial strains used in this study

Plasmid or bacterial strain	Description	Source and/or reference
Plasmids		
pBp	For gene expression in *A. tumefaciens*	This study
pE2431	For gene expression in *A. tumefaciens* from the *A. tumefaciens virB* promoter	S. B. Gelvin (Purdue University)
pREp-lacZ	For *lacZ* expression in *A. tumefaciens* and *R. etli* from the *R. etli virE* promoter	This study
pAEp-lacZ	For *lacZ* expression in *A. tumefaciens* and *R. etli* from the *A. tumefaciens* C58 *virE* promoter	This study
pREp-*virB2*	For *R. etli virB2* expression in *R. etli* from the *R. etli virE* promoter	This study
pE2431-Re*virG*	For *R. etli virG* expression in *A. tumefaciens* and *R. etli* from the *R. etli virG* promoter	This study
p302T-GFP	Binary plasmid with a GFP expression cassette in its T-DNA	41
p42a Spec^r	*R. etli* CFN42 p42a plasmid with a spectinomycin resistance cassette inserted in the RHE_PA00165 locus	S. Brom (University of Mexico) (35)
Strains		
CE3	*R. etli* with wild-type p42a plasmid	R. Carlson (University of Georgia)
CFN42 Δ*virG*	*R. etli* Δ*virG* mutant	J. Handelsman (Yale University) (13)
C58	*A. tumefaciens* strain with wild-type pTiC58 plasmid	Lab collection
C58NT1	*A. tumefaciens* strain C58 with no Ti plasmid	Lab collection

Next, we examined whether the *vir* region of the p42a plasmid is fully functional, i.e., able to mediate T-DNA transfer to host cells, in *A. tumefaciens*. To this end, we introduced both p42a and p302T-GFP, a binary reporter construct with a green fluorescent protein (GFP) expression cassette in its T-DNA, into the disarmed C58NT1 strain (Table 2). When the resulting cultures were infiltrated into *Nicotiana benthamiana* leaves, multiple GFP-expressing plant cells were observed (Fig. 2), indicating transient genetic transformation and, consequently, the ability of the *R. etli* p42a *vir* region to mediate T-DNA transfer from the *A. tumefaciens* background. In positive-control experiments, infiltration of *N. benthamiana* leaves with *A. tumefaciens* strain C58, carrying the wild-type Ti plasmid pTiC58 and the p302T-GFP reporter construct, resulted in high levels of transformation, whereas in negative-control experiments, no GFP expression was observed after infiltration with C58NT1 carrying p302T-GFP but no Ti plasmid (Fig. 2A). Quantification of the transient transformation efficiency indicated that *A. tumefaciens* carrying its native, wild-type Ti plasmid was approximately 10 times more efficient than *A. tumefaciens* carrying the *R. etli* p42a plasmid (Fig. 2B).

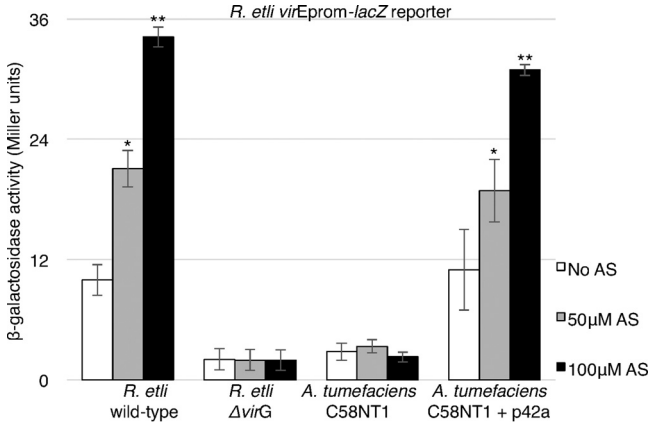

FIG 1 AS-dependent induction of p42a *virE* promoter activity in the *R. etli* and *A. tumefaciens* backgrounds. Wild-type *R. etli* carrying p42a, *R. etli* Δ*virG*, *A. tumefaciens* C58NT1, and *A. tumefaciens* C58NT1 carrying the p42a-Spec^r plasmid were transformed with the pREp-lacZ reporter plasmid (*R. etli virE*prom-*lacZ*). An assay measuring *β*-galactosidase activity was used to measure *virE* promoter activity upon induction by the indicated concentrations of AS. All data represent average values from three independent experiments with the indicated standard deviations. Asterisks indicate the statistical significance (*, $P < 0.05$; **, $P < 0.01$) of the difference between data from noninduced control and AS-induced cells within each experiment, as determined by the two-tailed Student's *t* test.

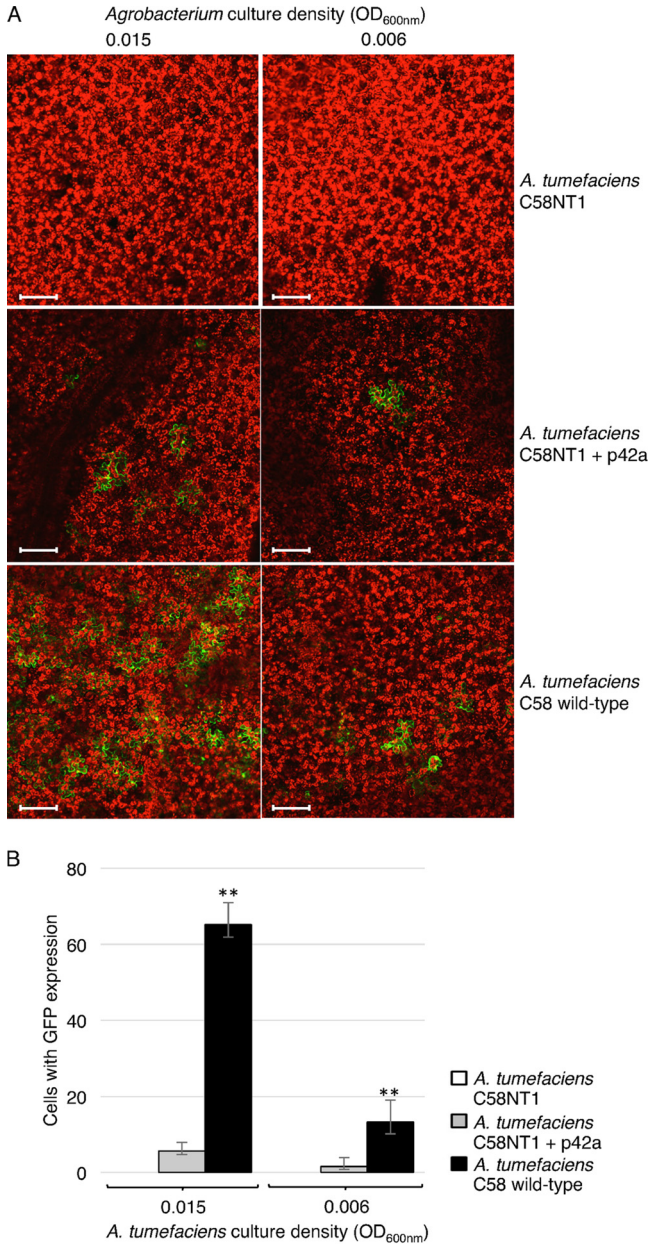

FIG 2 Comparison between T-DNA transfer efficiencies mediated by *vir* genes from *R. etli*/p42a or *A. tumefaciens* C58/pTiC58 in the *A. tumefaciens* background. *N. benthamiana* leaves were infiltrated with *A. tumefaciens* C58NT1, *A. tumefaciens* C58NT1 carrying *R. etli* p42a, and wild-type *A. tumefaciens* strain C58 carrying pTiC58. All bacterial strains also harbored the pCB302T-GFP reporter plasmid. (A) Representative confocal images of the infiltrated leaf areas. Green, GFP; red, plastid autofluorescence. All images are single confocal sections. Bars = 100 μm. (B) Efficiency of T-DNA transfer estimated by scoring the number of GFP-expressing cells per leaf surface area. All data represent average values from three independent experiments with the indicated standard deviations. Asterisks indicate the statistical significance (**, $P < 0.01$) of the difference between experimental and control data, as determined by the two-tailed Student's *t* test.

***R. etli* VirG is required for *R. etli virE* promoter activation.** To explore further the compatibility between induction of the *R. etli virE* promoter and the *A. tumefaciens* VirA/VirG *vir* gene induction system, we utilized the pREp-lacZ construct, which expresses the *lacZ* reporter from the *R. etli virE* promoter (Table 2). Unexpectedly, when the pREp-lacZ reporter construct was introduced into the *A. tumefaciens* C58 strain, only a very weak expression of the β-galactosidase reporter activity, which was not inducible by AS, was observed (Fig. 3A). These data suggest that the *R. etli virE* promoter cannot

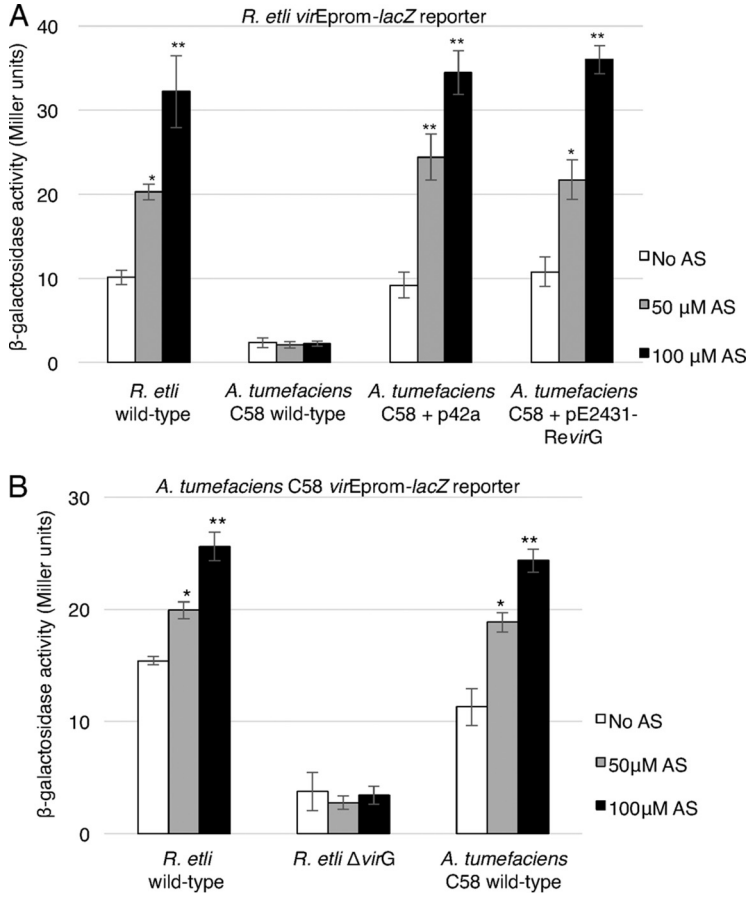

FIG 3 *R. etli* VirG is required for activation of the *R. etli virE* promoter. (A) Expression of the *lacZ* reporter under the control of the *R. etli virE* promoter (*R. etli virE*prom-*lacZ*) in wild-type *R. etli* carrying p42a, wild-type *A. tumefaciens* strain C58 carrying pTiC58, *A. tumefaciens* C58 carrying pTiC58 and the *R. etli* p42a plasmid, and *A. tumefaciens* C58 carrying pTiC58 and the *R. etli virG* expression construct pE2431-Re*virG*. (B) Expression of the *lacZ* reporter under the control of the *A. tumefaciens* C58 *virE* promoter (*A. tumefaciens* C58 *virE*prom-*lacZ* reporter) in wild-type *R. etli*, *R. etli* Δ*virG*, and *A. tumefaciens* C58 carrying wild-type pTiC58. Reporter expression was induced by the indicated concentrations of AS, and the level of expression was determined by measuring β-galactosidase activity. All data represent average values from three independent experiments with the indicated standard deviations. Asterisks indicate the statistical significance (*, $P < 0.05$; **, $P < 0.01$) of the difference between the experimental data for noninduced control and AS-induced cells, as determined by the two-tailed Student's *t* test.

be induced by the *A. tumefaciens* VirG protein and that the induction of the *R. etli vir* genes most likely is species specific in regard to the VirA/VirG system. As a positive control, substantial and AS-dependent induction of the *lacZ* reporter gene was observed in wild-type *R. etli* cells containing pREp-lacZ (Fig. 3A). Because VirG functions as a transcriptional activator that directly binds *vir* promoters, we reasoned that it is the native VirG protein of *R. etli* that the *R. etli virE* promoter requires for activation. To test this hypothesis, we generated a plasmid, designated pE2431-Re*virG* (Table 2), which expresses the *R. etli virG* gene from its own promoter, and introduced it into wild-type *A. tumefaciens* strain C58 together with the pREp-lacZ reporter construct. Figure 3A shows that the AS-inducible expression of the *R. etli virE* promoter activity was restored to levels similar to those observed with the p42a plasmid.

Next, we examined a reciprocal scenario, i.e., whether *R. etli* VirG can activate the *A. tumefaciens virE* promoter. Figure 3B shows that when the pAEp-lacZ construct that expresses the *lacZ* reporter from the *A. tumefaciens* C58 *virE* promoter (Table 2) was introduced into the wild-type *R. etli* strain, significant levels of AS-dependent induction of the *lacZ* reporter were observed. This induction depended on the presence of the *R. etli virG* gene because it did not occur in the *R. etli* Δ*virG* mutant cells (Fig. 3B). Similar

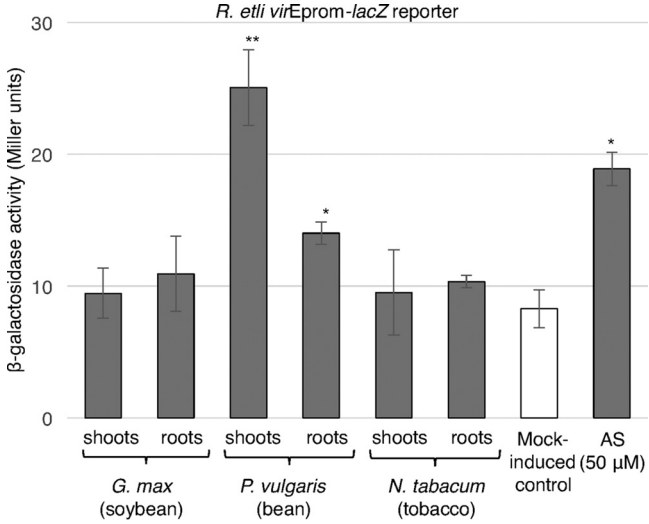

FIG 4 Induction of *R. etli virE* promoter activity by extracts from host and nonhost plant species. The level of expression of the *lacZ* reporter under the control of the *R. etli virE* promoter (*R. etli virE*prom-l*acZ*) was determined by measuring the β-galactosidase activity in the cell culture of wild-type *R. etli* carrying p42a supplemented with identical volumes of ethanol (mock-induced control), AS, and ethanolic extracts of the roots and shoots of plantlets from the indicated species. All data represent average values from three independent experiments with the indicated standard deviations. Asterisks indicate the statistical significance (*, $P < 0.05$; **, $P < 0.01$) of the difference between the experimental data for noninduced control and AS-induced cells, as determined by the two-tailed Student's *t* test.

levels of induction by increasing concentrations of AS were achieved by the native *A. tumefaciens* VirG protein when pAEp-lacZ was introduced into the wild-type *A. tumefaciens* C58 strain (Fig. 3B).

Activation of the *R. etli virE* promoter by extracts from host and nonhost plant species. Besides AS, plants secrete numerous other phenolic compounds that can activate the VirA/VirG two-component system and, thus, *vir* gene expression (22, 23). Because *A. tumefaciens* and *R. etli* differ in their host range, it was interesting to investigate how the virulence induction system of *R. etli* responded to *vir* gene-inducing phenolics contained in extracts from host and nonhost plant species. For the host species, we selected *Phaseolus vulgaris* (the common bean), and for the nonhost species, we utilized *Glycine max* (soybean) and *Nicotiana tabacum* (tobacco). *R. etli* cells containing p42a and the pREp-lacZ reporter construct were challenged with concentrated ethanolic extracts of the roots and shoots of these plants. Among these plant species, only extracts from *P. vulgaris* displayed a significant ability to induce the *virE* promoter; the highest levels of this induction were obtained with the extract of plant shoots, and they were comparable to the induction levels achieved with AS. Roots of *P. vulgaris* had modest but significant *vir* gene-inducing activity, whereas both the roots and shoots of nonhost plants had little or no *vir* gene-inducing activity (Fig. 4).

Kinetics of early transcriptional activation of different *vir* genes of *R. etli*. Next, we expanded our analysis of transcriptional activation to additional *R. etli vir* genes representing four major *vir* operons. Specifically, we measured the kinetics of *vir* gene expression directly by reverse transcription (RT)-quantitative real-time PCR (qPCR) amplification of the specific transcripts of the *virB4*, *virB5*, *virD2*, *virE2*, *virG*, and *virB2* genes (see Table S1 in the supplemental material) at 6, 16, and 24 h following the beginning of treatment with AS. The *virB4*, *virB5*, *virD2*, and *virE2* genes exhibited similar patterns of transcriptional activation by AS, ranging from a 2-fold to a 4-fold increase at 6 h of AS treatment to a 8-fold to 12-fold increase after 24 h of AS treatment (Fig. 5A to D). This is unlike the transcription of *virG*, which, having increased ca. 4-fold after 6 h of AS treatment, remained relatively stable thereafter, still displaying a 5-fold increase after 24 h of treatment (Fig. 5E). Thus, the temporal pattern of transcriptional regulation

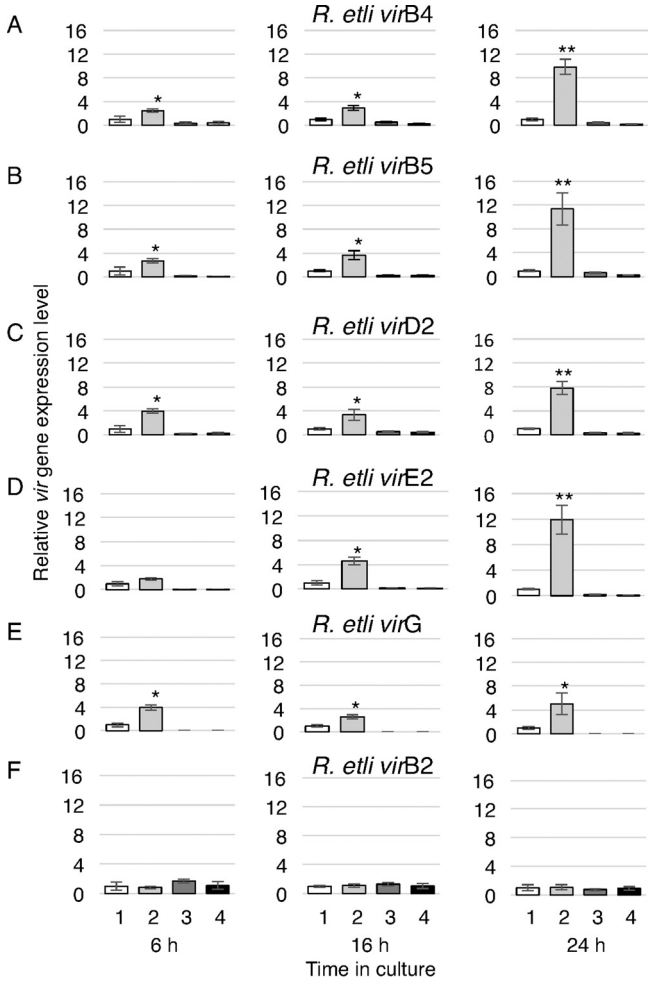

FIG 5 RT-qPCR analysis of early activation of *R. etli vir* gene transcription. Relative transcript levels were determined for the *R. etli virB4* (A), *virB5* (B), *virD2* (C), *virE2* (D), *virG* (E), and *virB2* genes (F) after 6, 16, and 24 h of growth of the following bacterial cultures: wild-type *R. etli* without AS induction (bars labeled 1), wild-type *R. etli* with 50 μM AS induction (bars labeled 2), *R. etli* Δ*virG* without AS induction (bars labeled 3), and *R. etli* Δ*virG* with 50 μM AS induction (bars labeled 4). All data represent average values from two independent biological replicates and three technical replicates with the indicated standard deviations. Asterisks indicate the statistical significance (*, $P < 0.05$; **, $P < 0.01$) of the difference between the experimental data for noninduced control and AS-induced cells, as determined by the two-tailed Student's *t* test.

of the *R. etli* AS-sensing VirA/VirG two-component system most likely differs from that of other major *vir* operons.

One of the *R. etli vir* genes, *virB2* is located separately from the other *virB* genes in a discrete locus on the p42a plasmid, unlike its *A. tumefaciens* homolog, which is a part of the *virB* operon. Consistent with this positional uniqueness, *R. etli virB2* did not respond to the presence of AS, showing no transcriptional activation even 24 h of treatment with AS (Fig. 5F).

Next, we examined whether this weak and uninducible expression of *virB2* might contribute to the lower efficiency of transient genetic transformation of plants mediated by the *R. etli* p42a plasmid (Fig. 2) (10). We produced a pREp-*virB2* plasmid that expresses *R. etli virB2* from the *R. etli virE* promoter (Table 2), which is inducible by AS, and introduced it into the wild-type *R. etli* cells carrying p42a together with the p302T-GFP reporter plasmid. Quantification of the transient expression of GFP detected no differences in this transient transformation efficiency between wild-type *R. etli* and the *R. etli* strain that expresses the inducible *virB2* (Fig. 6).

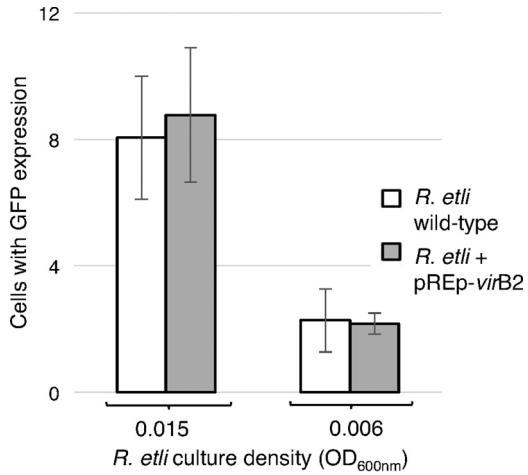

FIG 6 Effect of enhanced expression of *virB2* on efficiencies of T-DNA transfer from *R. etli* CFN42. *N. benthamiana* leaves were infiltrated with wild-type *R. etli* carrying p42a and *R. etli* carrying the pREp-*virB2* construct, which expresses *R. etli virB2* under the control of the *R. etli virE* promoter. Both bacterial strains also harbored the pCB302T-GFP reporter plasmid. The efficiency of T-DNA transfer was estimated by scoring the number of GFP-expressing cells per leaf surface area. All data represent average values from three independent experiments with the indicated standard deviations.

DISCUSSION

Recently, *R. etli* has emerged as only the second known prokaryote, in addition to *Agrobacterium* spp., that encodes a functional protein machinery for prokaryote-to-eukaryote DNA transfer. Here, we demonstrate that this virulence machinery, the *vir* genes, is transcriptionally activated by AS, a paradigm of plant phenolic signals that activate the *vir* genes of *A. tumefaciens*. Furthermore, also similar to the *A. tumefaciens vir* genes, the AS-induced expression of the *R. etli vir* genes required the presence of the VirG constituent of the VirA/VirG two-component system.

One interesting aspect of *R. etli vir* gene induction is its requirement for the native *vir* gene transcriptional activator VirG, such that the *R. etli* VirG but not the *A. tumefaciens* VirG can activate the *R. etli vir* promoters in the heterologous background of *A. tumefaciens* cells. Previously, similar compatible interactions between VirA/VirG and the regulatory sequences of other *vir* genes have been reported for different *A. tumefaciens* strains (24). On the other hand, the *vir* region of the *R. etli* p42a plasmid can function autonomously in a heterologous cellular background of disarmed *A. tumefaciens* C58 that lacks its own Ti plasmid-based virulence machinery. Importantly, this functionality of p42a *vir* genes was essentially complete, as it supported both *vir* gene induction and the transfer of T-DNA into the plant cells, followed by its transient expression. Quantitatively, however, the level of induction of the *R. etli vir* promoter observed here using the β-galactosidase reporter was 3 to 25 times lower than the level of induction of the same reporter gene measured in earlier studies of the *vir* genes of *A. tumefaciens* (24, 25). However, our reporter system utilizes the specific 527-bp sequence of the *R. etli virE* promoter fused to *lacZ*, whereas most induction assays with *A. tumefaciens* have been done using *lacZ* insertion into the Ti plasmid or a larger segment of the *vir* region (19, 25). Thus, it is possible that other untranslated sequences of the *vir* region contribute to the induction efficiency. For example, these regions may encode regulatory small RNA molecules that have been shown to activate *A. tumefaciens* virulence (26–28). This notion is lent support by our observations that AS induction of the defined, 1,384-bp-long *A. tumefaciens virE* promoter by the *A. tumefaciens* VirG was comparable to that of the *R. etli virE* promoter by the *R. etli* VirG. Furthermore, the *R. etli* VirG activated the *virE* promoter from both *A. tumefaciens* and *R. etli*, suggesting the broader interspecies recognition of *vir* promoter sequences by the *R. etli* VirG than by the *A. tumefaciens* VirG, which was species specific.

RT-qPCR analysis of the expression kinetics of major *vir* operons, which represents

the first use of this approach to quantify *vir* gene expression, revealed two variations in AS-induced transcriptional activation of the tested *vir* genes. First, whereas the three main *vir* operons, *virB*, *virD*, and *virE*, exhibited a continuous increase in expression over the period of at least 24 h of AS treatment, expression of *virG* already reached apparent saturation after 6 h of treatment. In *A. tumefaciens*, on the other hand, induction of expression of *virG* was observed to increase for at least 24 h of AS treatment (29), although its differences with the induction patterns of other *vir* genes have not been examined. Second, in *R. etli*, the *virB2* gene is located outside the *virB* operon, is expressed constitutively at relatively low levels, and shows no detectable inducibility by AS. Although the *virB2* promoter region contains several potential *vir* boxes, it may be lacking nonconserved sequences downstream of the *vir* box, known to be required for efficient VirG binding and transcriptional activation (30). In contrast, *A. tumefaciens virB2* is a part of the *virB* operon and is induced by AS. *A. tumefaciens virB2* is considered essential for virulence (31), but *A. tumefaciens* mutants unable to form VirB2 pili still can transfer DNA (32). Thus, in *R. etli*, the weak expression of *virB2* most likely is sufficient for the native bacterial virulence.

Another interesting aspect of the transcriptional activation of the *R. etli vir* genes is its differential response to signal molecules originated from different plant species. Generally, besides AS, *vir* genes respond to numerous other plant phenolic signals, and the nature of the inducer molecules produced by a given plant species might affect the host specificity for different bacterial strains. Our data suggest that the *R. etli vir* genes respond differently to cell extracts from host and nonhost species. Specifically, cell extracts from the common bean, which is nodulated by *R. etli* in nature, elicited significant levels of *vir* gene expression comparable to those elicited by synthetic AS, while extracts from the nonhosts tobacco and soybean had no effect. Whereas the induction was particularly strong with the shoot extract of the common bean, it was lower, but still significant, with the root extract, which may simply reflect differences in the concentrations of the *vir* gene-inducing compounds between the root and shoot extracts. Collectively, our data demonstrate that (i) the *vir* genes of *R. etli*—which, together with their *A. tumefaciens* homologs, encode the only known functional bacterial machineries for DNA transfer to eukaryotic cells—are transcriptionally induced by plant phenolic signals, (ii) the *R. etli vir* genes are induced by signals from the natural host species of this bacterium, and (iii) the induction pattern and extent exhibit small but most likely functionally relevant differences between *R. etli* and *A. tumefaciens*.

The cumulative differences between *R. etli* and *A. tumefaciens* in the transcriptional activation efficiency of one or more of their *vir* operons may also underlie the significantly lower efficiency of T-DNA transfer by the chimeric *A. tumefaciens*/p42a system than by the conventional *A. tumefaciens*/pTiC58 system. This is because the only difference between these two systems is the plasmid carrying the *vir* region, i.e., p42a and pTiC58, rather than other functions of the bacterial cell, such as attachment to the host cell surface, which relies on chromosomal factors (33, 34). Obviously, the involvement of factors other than *vir* gene expression, e.g., functional differences in specific *vir* gene products, such as the ability of VirE2 to bind single-stranded DNA, cannot be ruled out.

MATERIALS AND METHODS

***R. etli* and *A. tumefaciens* strains.** Three different strains of *R. etli* were used in this study. The wild-type strain *R. etli* CE3, a streptomycin-resistant isolate of strain CFN42, was kindly provided by Russell Carlson, University of Georgia, Athens, GA. The *virG* mutant of *R. etli* CFN42 (13) was a gift from Jo Handelsman, Yale University, New Haven, CT. The modified p42a plasmid carrying the spectinomycin resistance gene (35) located in the RHE_PA00165 locus was extracted from an *R. etli* strain provided by Susana Brom, National Autonomous University of Mexico, Cuernavaca, Mexico. These *R. etli* strains were grown in TY medium, composed of 5 g · liter^{-1} tryptone, 3 g · liter^{-1} yeast extract, and 10 mM $CaCl_2$. *A. tumefaciens* strains C58, a nopaline strain carrying the wild-type pTiC58 plasmid, and its derivative, C58NT1, i.e., the C58 strain cured of its TiC58 plasmid, were grown as described previously (36). The different plasmids used in this study and summarized in Table 2 were introduced into the *R. etli* and *A. tumefaciens* strains using the classical $CaCl_2$ protocol, with minor modifications being used in the case of *R. etli* (37).

Plasmid construction. The pREp-lacZ plasmid, which expresses *lacZ* from the *R. etli virE* promoter, was produced in three steps. First, the *virB* promoter from the *A. tumefaciens* pTiA6 plasmid was amplified with the primer pair 5′-ATGCCATGGCGATCCGTCTTGCGCTGACAG-3′/5′-CCGCTCGAGGTCGACACTAGTAG ATCTAAGCCTACCTTATCTCCTTAGCTCGCAAC-3′ and introduced into the NcoI-SalI sites of the pCB302 (38) backbone amplified with the primer pair 5′-CGCGTCGACATCGATGGTACCGGATCCGAATTCCGCTCA CCGGGCTGGTTG-3′/5′-ATGCCATGGAGTAAAGCGCTGGCTGAACCC-3′, resulting in pBp. Then, the *R. etli virE* promoter region, comprising 527 bp upstream of the translation initiation codon of the *virE1* gene, was amplified from p42a with the primer pair 5′-GGACCATGGTCCACCTCTCCGCTGTGGA-3′/5′-GGAAAG CTTGTCGTTGTTTCTCCTGCAAAACTTGC-3′ and inserted into the NcoI-HindIII sites of pBp, replacing the *virB* promoter and resulting in pREp. Finally, the *lacZ* gene, encoding β-galactosidase, was amplified from the pET30b:β-gal plasmid (Novagen) with the primer pair 5′-GGAAAGCTTATGACCATGATTACGGATTCA CTGG-3′/5′-GGAGGATCCTTATTTTTGACACCAGACCAACTGGTAATG-3′ and inserted into the HindIII-BamHI sites of pREp, resulting in pREp-lacZ. To produce the pAEp-lacZ plasmid, which expresses *lacZ* from the *A. tumefaciens virE* promoter, the promoter region of 1,384 bp upstream of the translation initiation codon of *virE1* was amplified from pTi58 with the primer pair 5′-CATGTCATGAGTCGACCGCTGAGGTTG AATATAC-3′/5′-CCCAAGCTTATCATTGTTTCTCCTACAGA-3′ and inserted into the NotI-HindIII sites of pREp-lacZ, replacing the *R. etli virE* promoter. For expression of the *R. etli virG* gene from its own promoter, the segment containing *virG* and its promoter region, comprising the sequence 98 bp upstream of the translation initiation codon, was amplified from the p42a plasmid with the primer pair 5′-GGCGAGCTCATGTTGCTCAACTTTCAAGCAGGC-3′/5′-CGGGGTACCTCAGGCAGCCATCACTCCT-3′ and inserted into the SacI-KpnI sites of the pE2431 plasmid (kindly provided by Stanton B. Gelvin, Purdue University, West Lafayette, IN), replacing the *virB* promoter with the *R. etli virG* expression cassette and resulting in pE2431-Re*virG*. For expression of the *R. etli virB2* gene from the *R. etli virE* promoter, the *virB2* coding sequence was amplified from the p42a plasmid with the primer pair 5′-CCCAAGCTTATGATGCG ATGCTTTGAGAGATAC-3′/5′-CGCGGATCCTCAACCACCTCCAGTCAGCG-3′ and inserted into the HindIII-BamHI sites of pREp-lacZ, replacing *lacZ* and producing pREp-*virB2*.

Ethanolic plant extracts. The shoots and roots of 3- to 4-week-old plants grown *in vitro* on Murashige and Skoog basal medium (Sigma) supplemented with 30 g · liter^{-1} sucrose and 4 g · liter^{-1} agar were flash-frozen in liquid nitrogen and ground with a mortar and pestle, resuspended in 100% ethanol, and extracted for 5 min at 4°C. The extract was then concentrated 100 times in a SpeedVac concentrator (50 rpm at room temperature) and stored at −20°C before use.

β-Galactosidase assay. The β-galactosidase assay was performed as described previously (25, 39, 40) with modifications. Bacteria were grown for 24 h at 28°C and 240 rpm in induction medium [1× AB salts, 2 mM phosphate buffer (pH 5.6), 50 mM 2-(4-morpholino)ethanesulfonic acid (MES), 0.5% glucose] (21) without inducer, with AS (50 or 100 μM), or with 10 μl of ethanolic plant extract, and the cell density of the culture (optical density at 600 nm [OD_{600}]) was determined. Then, 0.2 ml of the bacterial suspension was added to 1.8 ml of Z buffer (60 mM Na_2HPO_4, 40 mM NaH_2PO_4, 10 mM KCl, 1 mM $MgSO_4$, 50 mM β-mercaptoethanol), combined with 100 μl chloroform and 50 μl 0.1% SDS, and vigorously vortexed, after which 0.4 ml of medium A [60 mM K_2HPO_4, 33 mM KH_2PO_4, 7.5 mM $(NH_4)_2SO_4$, 2.0 mM Na citrate] containing 4 g · liter^{-1} *ortho*-nitrophenyl-β-galactoside (ONPG) was added, and the mixture was incubated at 28°C and 240 rpm for approximately 40 min. When a light yellow color appeared upon visual examination, the reaction was stopped by the addition of 1 ml of 1 M Na_2CO_3. After centrifugation (10 min, 20,000 × *g*) to pellet the cell debris, the OD_{420} of the supernatant was determined. The resulting measurements of the β-galactosidase activity, expressed in Miller units, were calculated using the following equation: $(1{,}000 \times OD_{420})/(OD_{600} \times v \times t)$, where *v* is the volume of the bacterial suspension (in milliliters) and *t* is the reaction time (in minutes).

Transient GFP expression in plant cells. *A. tumefaciens* or *R. etli* strains carrying pCB302T-GFP (10) were grown for 24 h at 28°C and infiltrated into intact *N. benthamiana* leaves as described previously (41). The bacterial suspension was first adjusted to an OD_{600} of 0.6 and then diluted 40 or 100 times, i.e., to OD_{600}s of 0.015 and 0.006, respectively, in MES buffer before infiltration. Three days after infiltration, the expression of the GFP reporter was analyzed under a Zeiss LSM 5-Pa confocal microscope at low magnification with a 10× objective; the number of GFP-expressing cells per square centimeter of infiltrated leaf surface was scored as described previously (41).

RNA extraction and RT-qPCR. Different bacterial strains were grown for 6, 16, or 24 h, as described above for the β-galactosidase assays, without AS or with 50 μM AS. Two milliliters of bacterial suspension was first mixed with 4 ml of RNAprotect Bacteria reagent (Qiagen), and the mixture was incubated for 5 min at room temperature to stabilize the RNA. After centrifugation, total RNA was extracted from the pellet using an RNeasy minikit (Qiagen) and treated with DNase I (New England BioLabs) to eliminate potential DNA contamination. The resulting total RNA preparation (1.0 μg) was used for cDNA synthesis with a RevertAid RT kit (Thermo) with random hexamer primers. The cDNA preparation was diluted 1:100 for the reference 16S RNA gene or 1:10 for the tested *vir* genes, and 0.5 μl of the resulting cDNA solution was analyzed by qPCR with the Maxima SYBR green–carboxy-X-rhodamine qPCR master mix (Thermo-Fisher) in a Mini Opticon real-time PCR system (catalog number PTC1148; Bio-Rad). The primer sequences for the different *vir* and control genes are summarized in Table S1 in the supplemental material. Data were analyzed with the CFX Manager software program (Bio-Rad), using 16S RNA as an internal control, and are presented as relative gene expression levels, with the values obtained for uninduced wild-type *R. etli* being set equal to 1.

SUPPLEMENTAL MATERIAL

Supplemental material for this article may be found at https://doi.org/10.1128/JB.00841-16.

TEXT S1, PDF file, 0.06 MB.

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