

Research Note

***Rhizobium etli* CE3 Carries *vir* Gene Homologs on a Self-Transmissible Plasmid**

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Accepted 15 May 2000.

RosR is a transcriptional regulator important for determining cell-surface characteristics and nodulation competitiveness in *Rhizobium etli* CE3. We identified a 15-kb region that contains genes with similarity to members of the *virB*, *virC*, *virG*, and *virE* operons of *Agrobacterium tumefaciens* and demonstrated that RosR directly regulates one operon in this region. These genes were located on plasmid pa of *R. etli* CE3, which is self-transmissible between *R. etli* and *A. tumefaciens*.

rosR is an important determinant of the cell surface and nodulation competitiveness in *Rhizobium etli* (Bittinger et al. 1997). *rosR* is a member of a family of transcriptional regulators that includes *ros* from *Agrobacterium tumefaciens* and MucR from *Sinorhizobium meliloti* (Cooley et al. 1991; Keller et al. 1995). *ros* is encoded by a chromosomal locus and specifically represses expression of the virulence operons *virC* and *virD* on the Ti plasmid. *mucR* in *S. meliloti* affects the cell surface of *S. meliloti* by repressing expression of genes involved in production of galactoglucan, an alternative exopolysaccharide.

We previously identified a wide array of genes that are transcriptionally regulated by RosR, and one of the RosR-regulated regions that we identified in *R. etli* CE3 displayed sequence similarity to the *virC1* and *virD3* genes of *A. tumefaciens*. *R. etli* mutant MB043 contains a transposon insertion in a region with sequence similarity to the *virC1* and *virD3* genes of *A. tumefaciens* (Bittinger and Handelsman 2000). The presence of *vir* gene homologs in a rhizobial species suggests intriguing possibilities about the evolution and ecology of the symbionts and therefore warranted further study.

In this study, we sequenced 15 kb of the region flanking this transposon insertion, and found that it contained homologs of other *vir* genes of *A. tumefaciens* (Fig. 1). At one end of this region, we identified open reading frames (ORFs) that encode deduced proteins that are 76% identical over 247 amino acids to VirB8, 87% identical over 275 amino acids to VirB9, 67% identical over 406 amino acids to VirB10, and 79% identical

over 343 amino acids to VirB11. Downstream of the *virB* region is an ORF encoding a deduced protein 85% identical over 249 amino acids to VirG (86% identical over 241 amino acids if translation initiates at the TTG codon 228 bp downstream of the ATG codon, which has been observed in *A. tumefaciens*; Pazour and Das 1990). Convergenly oriented to the *virG* homolog are two ORFs that encode deduced proteins that are 83% identical over 230 amino acids to VirC1 and 65% identical over 184 amino acids to VirC2, respectively. The region with nucleotide similarity to the *virD3* gene is not contained in a single ORF, suggesting that it is not a functional gene. ORFs encoding deduced proteins 76% identical over 63 amino acids to VirE1, 70% identical over 557 amino acids to VirE2, and 51% identical over 684 amino acids to VirE3 are located at the other end of the region sequenced. Between the *virC* and *virE* operons is a region with sequence similarity to various insertion elements, including *ISR12*, *ISRm3*, *ISRm10-1*, and *Rmln1*.

We determined that *rosR*, like its homolog *ros* in *A. tumefaciens*, is located on the bacterial chromosome, while the *vir* genes it regulates are plasmid-borne. The physical locations of the *rosR* gene and the *vir* genes in the *R. etli* CE3 genome were identified by Southern blot analysis. We separated the chromosome and plasmids of *R. etli* CE3 by gel electrophoresis with a modified Eckhardt procedure (Eckhardt 1978; Hynes and McGregor 1990), transferred the DNA to membranes, and probed with DNA corresponding to the *virG* ORF (polymerase chain reaction [PCR] amplified with the primers 5'TTT GTC GAC GAT GCT GCT TTG CGG CCC AAC and 5'TTT GAG CTC GTT TAG CAA GGC TTC CCA AAG) or *rosR* ORF (amplified with the primers 5'GAT AGC GCC CGA AAA CGC T and 5'GAA AAC ATA TGA CGG ATA TGG CG) (Fig. 2). In addition to *R. etli* CE3, we analyzed the *A. tumefaciens* strain GMI9023 and a derivative of GMI9023 that carries the plasmid pa from *R. etli* CFN42, the parent strain of CE3 (Flores et al. 1987). The labeled DNA probe specific for the *virG* ORF hybridized to the band corresponding to plasmid pa, the smallest of the *R. etli* plasmids, while the probe specific for *rosR* hybridized to the chromosomal DNA that remained in the well of the gel.

We determined that plasmid pa was self-transmissible and readily transmitted between *R. etli* and *A. tumefaciens*. When *R. etli* MB043, which harbors a spectinomycin resistance gene

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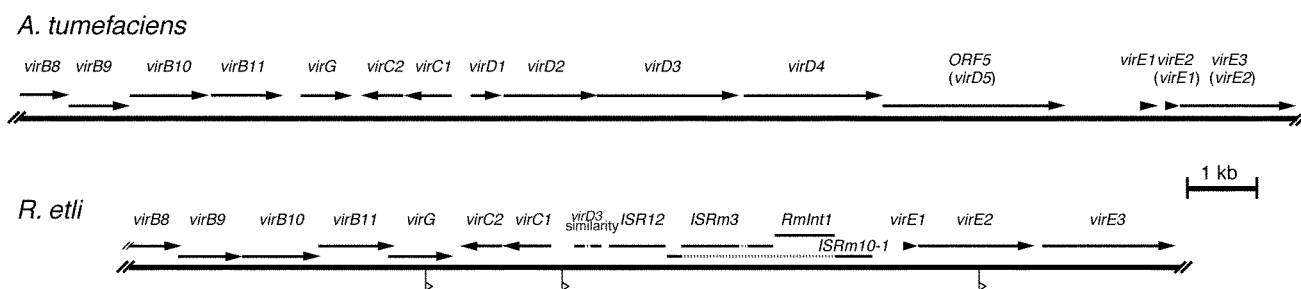


Fig. 1. The *vir* region of *Agrobacterium tumefaciens* and *Rhizobium etli*. Top of figure: diagram of approximately 18 kb of *vir* region of *A. tumefaciens* C58 (sequence from GenBank no. J03320) (Rogowsky et al. 1990). Gene names in parentheses indicate nomenclature in other *Agrobacterium* spp. Beneath *A. tumefaciens* diagram is corresponding 15-kb *vir* region of *R. etli* (GenBank no. AF176227). Regions with similarity to sequences in data bases are indicated with appropriate name above the region. Open reading frames are indicated by arrows; sites of promoterless *gusA* insertions are indicated, with orientation of *gusA* indicated by direction of the flag.

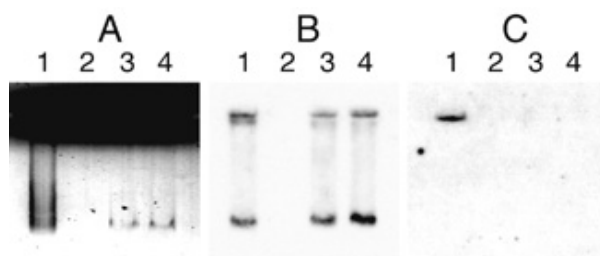


Fig. 2. Southern blot analysis of bacterial chromosome and plasmids of *Rhizobium etli* and *Agrobacterium tumefaciens*. Strains analyzed: (1) CE3; (2) GMI9023; (3) GMI9023pCFN42a; (4) GMI9023p43a. **A**, Gel stained with Sybr green after electrophoresis. Upper portion of panel is black due to intense staining of chromosomal DNA in the wells of the gel. This gel was blotted and probed with **(B)** *virG*, and then **(C)** stripped and re-probed with *rosR*. Hybridization of probes was visualized with the DIG Luminescent Detection Kit (Roche, Indianapolis, IN).

on plasmid *pa*, was mixed with *A. tumefaciens* GMI9023, spectinomycin-resistant derivatives of GMI9023 arose at a frequency of $4.4 (\pm 0.4) \times 10^{-4}$, which is similar to previously reported frequencies of transfer (Noel et al. 1984). The presence of plasmid *pa* in the transconjugants was confirmed by Southern analysis (Fig. 2). Additionally, GMI9023 carrying only plasmid *pa* transmitted spectinomycin resistance back to *R. etli* (data not shown), indicating that plasmid *pa* is self-transmissible between members of this bacterial family.

Purified RosR protein specifically bound DNA sequences in the region upstream of the *virC1* ORF, further demonstrating the functional similarities between RosR and Ros. The 162-bp region immediately upstream of the *virC* ORF was generated by PCR (corresponding to nucleotides 6035 to 6196 of GenBank no. AF176227), labeled with digoxigenin, and used as a substrate for in vitro gel mobility shift assays with the DIG Luminescent Detection Kit (Roche, Indianapolis, IN) to visualize the labeled DNA. RosR protein was purified from *Escherichia coli* with the IMPACT-T7 kit (New England Biolabs, Beverly, MA) (Bittinger 1999). The mobility of this 162-bp fragment decreased in the presence of purified RosR and an excess of unlabeled competitor DNA, and the amount of the shifted DNA fragment was dependent on the amount of RosR added to the binding reaction (Fig. 3).

To determine whether these *vir* genes are involved in the symbiosis of *R. etli* CE3 with common bean, we constructed mutants of CE3 with either the *virG* or *virE2* genes disrupted, and screened those mutants for altered symbiotic phenotypes.

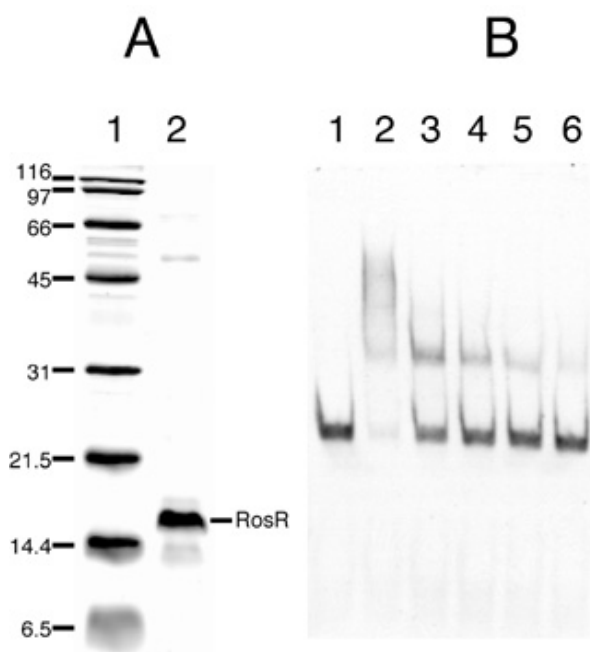


Fig. 3. Gel mobility shift assay. **A**, Sodium dodecyl sulfate–polyacrylamide gel electrophoresis gel of purified RosR protein stained with SYPRO Orange. Lane 1, protein standards (sizes indicated in kDa); lane 2, 500 ng of purified RosR protein. **B**, Electrophoretic gel mobility shift assay with purified RosR protein and labeled 162-bp DNA fragment from upstream of *virC1*. Lane 1, no RosR protein; lane 2, 200 ng of RosR protein; lane 3, 100 ng of RosR protein; lane 4, 50 ng of RosR protein; lane 5, 25 ng of RosR protein; lane 6, 12.5 ng of RosR protein.

A promoterless *gusA* gene was introduced into the ORF of each of these genes by marker exchange with the counterselectable suicide vector pJQ200KS (Quandt and Hynes 1993) (Fig. 1). Southern analysis of genomic DNA from these mutants confirmed the gene disruptions (Bittinger 1999). Both mutants formed nitrogen-fixing nodules on *Phaseolus vulgaris* cv. Black Turtle, and neither mutant displayed altered nodulation competitiveness when coinoculated in a 1:1 ratio with *R. etli* CE3013, a kanamycin-resistant derivative of CE3 unaffected in competitiveness (Beattie and Handelsman 1993; Bittinger 1999).

The findings of this study reinforce the similarity between the role of RosR in *R. etli* CE3 and that of Ros in *A. tumefaciens*

ens. Additionally, this is the first report of a *Rhizobium* species carrying homologs of the *vir* genes. Although it is surprising to find homologs of the *vir* genes in a rhizobial species, they may no longer be involved in pathogenesis due to the transposition of the large insertion element. Because plasmid *pa* is self-transmissible, it seems likely that it is present as a result of horizontal transfer from other members of the *Rhizobiaceae*; however, we do not know if this plasmid provides a selective advantage to the bacterium in its saprophytic life in the soil. It would be interesting to identify the other genes present on this plasmid and ultimately determine whether the rhizobial *vir* homologs can functionally complement *vir* mutants of *A. tumefaciens*.

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