TECHNICAL ADVANCE

RIVET—A Tool for In Vivo Analysis of Symbiotically Relevant Gene Expression in *Sinorhizobium meliloti*

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Despite significant advances in the development of sensitive tools for studying genetics and signal exchange in legume–rhizobium symbioses, many uncertainties remain about the in vivo role of bacterial and plant signals in symbiotic gene regulation. In this study, we adapted TnpR recombinase-based in vivo expression technology (RIVET) to document gene regulation in *Sinorhizobium meliloti*. The substrate for TnpR, the *resI-tet-resI* cassette, is stably inherited when cloned into a neutral site of the *S. meliloti* genome. Bicistronic promoterless *tnpR-β-glucuronidase (GUS)* reporters were constructed to track expression (“resolution”) of symbiotically relevant *S. meliloti* genes during different stages of the interaction. In proof of principle experiments, the resolution of the *nodC::tnpR* reporter was detected within 4 h of exposure to micromolar levels of the *nod* operon inducer luteolin and after overnight incubation in the rhizosphere. RIVET demonstrated that cell division gene *ftsZ2* was not strongly expressed in the rhizosphere but was activated inside the nodules and on agar surfaces. Rhizosphere expression of the *N*-acyl homoserine lactone (AHL) synthase *sinI::tnpR-GUS* reporter was modest in prequorate microcolonies, and then increased with time. AHL synthase *sinI* and an AHL-regulated gene, *expG*, were activated inside the nodules.

Additional keywords: exopolysaccharide, ExpR, flavonoid, *Medicago truncatula*, quorum sensing

The symbiosis between *Sinorhizobium meliloti* and *Medicago truncatula* is one of the best-characterized host–bacteria interactions (Barnett and Fisher 2006; Galibert et al. 2001; Jones et al. 2007). The establishment and maintenance of this symbiosis require precisely timed signal exchange and gene expression in both partners. Perception of plant flavonoid signals by the bacteria is thought to be the first step in the symbiosis. In *S. meliloti*, interactions of plant flavonoids with NodD transcriptional activators lead to the induction of *nod* genes (Mulligan and Long 1985; Peters et al. 1986). These genes encode proteins involved in the biosynthesis of chitin lipopolysaccharide signals (Nod factors). Bacterial exopolysaccharides (succinoglycan EPSI and galactoglucan EPSII), lipopolysaccharides, and host lectins play important signaling roles in the infection process (Bauer 1977; Glazebrook and Walker 1989). Nod factors and bacterial exopolysaccharides induce a cascade of changes in host gene expression leading to

nodule development. When invading rhizobia are released into envelopes of host plasma membrane, they multiply to fill most of the plant host cell and differentiate into nitrogen-fixing “bacteroids” (Jones et al. 2007). Despite the fact that many steps in this symbiosis have been well characterized, there remain many questions about the sequence and timing of symbiotic gene expression.

Several elegant strategies have been developed to analyze bacterial gene expression in vivo. In their pioneering study, Oke and Long (1999) developed a promoter trap technique which was based on the activation of a promoterless *bacA*, a gene that is central to the differentiation of free-living rhizobia into symbiotic bacteroids (Oke and Long 1999). This screen identified over 70 promoters specifically activated during bacteroid formation. The corresponding genes are involved in nitrogen and carbon assimilation, stress responses, DNA repair, and cell division (Oke and Long 1999). Another promoter trap experiment, based on the complementation of the *exoY* gene combined with the *gfp*-based fluorescence-activated cell sorting (FACS), identified over 150 promoters expressed early in the nodulation process (Zhang and Cheng 2006). These in vivo expression technology (IVET) promoter traps were invaluable in identifying *S. meliloti* genes induced specifically during targeted stages of the symbiosis.

A recombinase-based IVET (RIVET) approach has the advantage of identifying genes that are transiently expressed during host infections (Camilli et al. 1994; Osorio et al. 2005). The most widely used RIVET is based on the ability of the TnpR recombinase to catalyze site-specific “resolution” at *res* sequences (Camilli et al. 1994). When *tnpR* is expressed from a promoter of interest, activation of this promoter drives the expression of the recombinase gene. TnpR then excises a selectable marker. As a result, DNA sequences flanked by the *res* sites are excised and lost. This creates a heritable molecular record: every time a promoter is expressed, the selectable marker is lost. RIVET also serves to record cumulative gene expression. RIVET is not suited for detecting transcriptional repression. In these respects, RIVET is different from other reporters or high-throughput assays which provide a snapshot of transcript or polypeptide accumulation.

This report describes the construction of a bicistronic promoterless *tnpR-β-glucuronidase (GUS)* RIVET reporter to facilitate quantification and visualization of bacterial gene expression during different steps of the *Sinorhizobium–Medicago* symbiosis. In proof of principle experiments, the regulation of the *nodC::tnpR-GUS* reporter was tested in shake cultures with the inducer luteolin, in the rhizosphere, and in nodules of *M. truncatula*. RIVET then was used to test the

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regulation of a cell-division gene *ftsZ2* and to learn about the contribution of bacterial quorum sensing (QS) to gene regulation during the symbiosis.

RESULTS

The intergenic region Smc00849-Smc00850 is a suitable “neutral” site.

To insert a *resI-tet-resI* cassette into the *S. meliloti* genome, a “neutral” site of the genome is preferred. In searching for a suitable neutral site within the published *S. meliloti* genome, all known and predicted open reading frames (ORF) and promoters were eliminated as candidates. The remaining intergenic regions were scanned in an attempt to rule out sequences that contain known or putative leader regions or small regulatory RNA. The intergenic region between convergently transcribed Smc00849 and Smc00850 did not fit the criteria for sRNA as defined by Wassarman and associates (2001). The intergenic region is relatively short (130 nucleotides), the DNA sequence is conserved only in *Sinorhizobium* spp., and no rho-independent terminator is found within it. The intergenic region contains nucleotides that can form a stem loop (CGCCCTG xxxxxx CAGGGCG), although it is not clear whether this secondary structure has a function. Messages corresponding to Smc00849 and Smc00850 are detected in microarray analyses (M. J. Barnett, *personal communication*). Neither product was differentially accumulated under common assay conditions, although a small (less than twofold) decrease in the accumulation of Smc00849 mRNA was detected in a *cbrA::Tn5* mutant (Gibson et al. 2007; M. J. Barnett, *personal communication*). These ORF encode conserved hypothetical proteins without predicted functions.

To further test the hypothesis that the Smc00849-Smc00850 intergenic region is a neutral site of the genome, the *resI-tet-resI* cassette was inserted into the *SaII* site that occurs naturally in that region (as described below) to create *S. meliloti* strain 8530tr. Growth of this mutant on TY or M9 sucrose and its nodulation ability, colony morphology, and swarming motility were compared with those of the wild-type *S. meliloti* 8530. The phenotypes of the two strains were indistinguishable. Both strains formed a similar number of nodules (1.9 ± 0.5 and 2 ± 0.5 for *S. meliloti* 8530 and 8530tr, respectively). The rate and kinetics of nodulation were indistinguishable for the two

strains. *S. meliloti* 1021tr and MG75tr were constructed and confirmed similarly. These observations suggest that, under common growth and symbiotic assay conditions, the intergenic region encoded between Smc00849 and Smc00850 is a suitable neutral site.

The *resI-tet-resI* cassette is stably maintained in *S. meliloti*.

Homologs of the site-specific recombinase *tnpR* are encoded within several bacterial genomes. Smc0785 and Smc02300 of *S. meliloti* encode homologs of site-specific recombinases. This prompted us to investigate whether this or any other site-specific recombinase can act upon the *resI* sites of the RIVET reporter and interfere with the assay. Two isolates of *S. meliloti* 8530tr and a parental strain (*S. meliloti* 8530) were grown under laboratory conditions and also seeded into the *M. truncatula* rhizosphere as described below. Cells were recovered from log-phase and stationary-phase cultures grown in TY or M9 broth without tetracycline selection, from 48-h-old swarm colonies, from the rhizosphere of 10-day-old seedlings, and from 6- and 14-day-old nodules. Recovered cells were mildly sonicated and plated on TY agar, and colonies then were patched on TYtet5. All *S. meliloti* 8530tr cells recovered from these samples were resistant to tetracycline. The parental *S. meliloti* 8530 was sensitive to tet5 under the same conditions. These observations suggest that *resI-tet-resI* is stably inherited in *S. meliloti* under these conditions. Although endogenous site-specific recombinases may be encoded within the *S. meliloti* genome, it is not clear whether they are expressed under these assay conditions. If functional recombinase proteins are produced under these conditions, they do not seem to interfere with the RIVET assay.

nodC::tnpR-GUS reporter assays in shake culture and in planta.

To test the usefulness of RIVET in studying interactions between *S. meliloti* and its legume hosts, merodiploid *nodC⁺ nodC::tnpR-GUS* reporters were constructed. The bicistronic reporters encode both a recombinase and GUS to quantify and visualize regulation of the *nodC* promoter during different stages of the symbiosis. The reporter allows a direct comparison of the two assays. The responsiveness of the *nodC* promoter to the plant flavone luteolin is well characterized (Barnett et al. 2004; Mulligan and Long 1985; Peters et al. 1986; Sharma and

Fig. 1. Responses of *nodC⁺ nodC::tnpR-β-glucuronidase (GUS)* reporter to 0.1 and 1 μM luteolin. Responses of the *nodC* promoter to the flavonoid inducer were measured as activity of the TnpR recombinase (resolution %) and GUS (Miller units) in triplicate cultures. Resolution was estimated by patching colonies on tetracycline-containing medium, and the activity was calculated as percentage of the tetracycline-sensitive colonies. GUS activity in vitro was measured with 4-methyl-umbelliferyl-glucuronide substrate as described by Gao and associates (2007). Responses to 0.1 and 1 μM luteolin are indicated by a filled diamond or a filled square; responses of the uninduced reporter are indicated by an empty square. Error bars indicate standard errors.

Signer 1990), which makes this gene a suitable candidate for proof of principle experiments.

Prior to the assays, a starter culture of *S. meliloti* MGCR85 (*nodC⁺ nodC::tnpR-GUS res1-tet-res1*) was grown in a medium containing tetracycline, as described below. Cells then were washed and diluted to an optical density at 590 nm = 0.03 in fresh TY or M9 sucrose medium with or without 0.1 to 9 μM luteolin. In rich TY medium, the earliest responses of the TnpR reporter were observed after 2 h of exposure to luteolin (data not shown). In minimal M9 medium, the earliest responses were detected after 4 to 5 h of incubation with luteolin (data not shown). This delay in response approximately corresponds to one cell division cycle, which is needed for a cell to “lose” tetracycline resistance.

Dose-dependent resolution of the reporter during incubation with luteolin consistently was observed by the fourth hour (Fig. 1A). The resolution of the un-induced reporter remained low throughout the experiment. A 4-h-long exposure to 0.1, 1, and 9 μM luteolin stimulated 13 ± 4, 43 ± 3.5, and 58 ± 2% resolution, respectively, compared with 2.5 ± 3% resolution of the un-induced reporter. Only low levels of GUS activity were observed after 4 h of exposure to luteolin. This is consistent with an observation that a chromosomally encoded *nodC::lacZ* reporter is not strongly activated after 3 h of incubation with luteolin (Mulligan and Long 1985). The earliest significant differences in the levels of GUS activity were observed after 8 to 14 h of incubation with luteolin (Fig. 1B). Therefore, it appears that, when directly compared with *lacZ* and *gus*, the *tnpR*-based reporter is a more sensitive indicator of induced gene expression in *S. meliloti*. In laboratory shake cultures, a single copy of *tnpR* was sufficient to document statistically significant responses to biologically relevant levels of the inducer.

To further test the *nodC::tnpR-gus* reporter, its activity was measured in the rhizosphere and in planta. *S. meliloti* MG101 was grown in a tetracycline-containing medium, as described below. The resolution in the inoculum culture was 0% (i.e., all cells were resistant to tetracycline). Following overnight incu-

bation in the rhizosphere, 29 ± 9% of the recovered bacteria were resolved. After growth for 6 days in the rhizosphere, 55 ± 1% of the reporter cells were resolved. The resolution reached 95 ± 5% after 14 days of incubation in the rhizosphere. Activation of *nodC::tnpR* in the rhizosphere is expected based on earlier studies (Mulligan and Long 1985; Peters et al. 1986; Sharma and Signer 1990).

Strong induction of the *nodC* reporter also was observed inside nodules (Fig. 2). Generally, the extent of resolution of the *tnpR* reporter corresponded to the intensity of GUS staining. In the cells recovered from 6-day-old nodules, the resolution was 55 ± 1%. It reached 100% in 14-day-old nodules. Proteins and transcripts corresponding to *nodABC* have been isolated from nodules formed by *S. meliloti* (Capela et al. 2006; John et al. 1988). Sharma and Signer (1990) reported moderate activation of the *nodC::Tn5-gus* reporter in 9- to 11-day-old nodules formed on alfalfa (*M. sativa*) and very strong activation of the same reporter in 15- to 17-day-old nodules.

Regulation of a cell division gene *ftsZ2* during the symbiosis.

Precise regulation of symbiotic cell division in both the plant and bacterial partners is important to maintaining the stability of this interaction. The initiation of cell division is controlled by homologs of the tubulin-like GTP-hydrolase FtsZ. There are two chromosomally encoded copies of the *ftsZ* gene in *S. meliloti* (Galibert et al. 2001; Margolin and Long 1994). A mutation in *ftsZ1* was lethal, whereas disruption of *ftsZ2* was not deleterious to the organism (Margolin and Long 1994). The accumulation of transcripts corresponding to *ftsZ1* and *ftsZ2* was decreased inside bacteroids (Barnett et al. 2004; Becker et al. 2004). To learn about the regulation of bacterial cell division during different steps of the symbiosis, the expression of *ftsZ* homologs was examined using the *tnpR-gus* reporter.

Merodiploid reporters in both *ftsZ1* and *ftsZ2* were constructed and confirmed as described below. When a plasmid carrying *ftsZ1::tnpR* was integrated into *S. meliloti*, all isolates

Table 1. Resolution of the *ftsZ2::tnpR* reporter

Conditions	Resolution (%) ^a				
	0 h	14 to 21 h	48 h	6 days	14 days
TY agar	0	14 ± 5	34 ± 10	80 ± 2	99 ± 1
Rhizosphere	0	15 ± 3	20 ± 10	21 ± 4	40 ± 10
Nodules	n/a	n/a	n/a	n/a	99 ± 1

^a n/a = Not available.

Fig. 2. Activation of the *tnpR-β-glucuronidase* (*GUS*) reporters in planta. To visualize reporter activity in different parts of the nodule, nodules at comparable developmental stages were excised, incubated for 10 min in ice-cold 90:10% (vol/vol) acetone:water, then rinsed three times for 10 min in 0.2 M sodium phosphate buffer (pH 7.2) for 10 min. After the final wash, tissue samples were transferred into 0.2 M phosphate-buffered 5-bromo-4-chloro-3-indolyl-β-D-glucuronide solution (2 mg/ml, pH 7.2). The samples then were vacuum infiltrated for an hour and incubated at 37°C for 1 to 12 h. In order to avoid overstaining, samples were observed under the dissecting microscope every 2 to 3 h. As soon as blue color developed, nodules were washed in phosphate buffer, then stored at 4°C in 50:50% ethanol:water. Stained nodules were photographed with Olympus MVX-TV1XC mounted on a binocular scope (Olympus MVX10, SZX2-ILLT, Tokyo). All images were adjusted for color balance and brightness using the same automatic settings of Adobe Photoshop CS 8.0.

carrying the construct were sensitive to tetracycline. When tetracycline was added to the selection media during reporter construction, no tetracycline-resistant isolates were identified. This failure to isolate a *ftsZ1⁺ftsZ1::tnpR res1-tet-res1* reporter demonstrates an important limitation of RIVET: a promoter that is strongly expressed under common laboratory conditions will most likely get “resolved” during reporter construction.

To test the hypothesis that *ftsZ2* may be expressed differently in shake cultures and during interactions with the host plant, the activity of a chromosomal *ftsZ2⁺ftsZ2::tnpR-gus* reporter (*S. meliloti* MG105) was assayed on TY agar, in the rhizosphere, and in planta. The resolution of the *ftsZ2* reporter in rich medium and in the rhizosphere was significantly different throughout the course of the experiment (Table 1). Resolution of the reporter grown on rich medium agar steadily increased and reached 100% by the second week of the experiment. Resolution of the reporter in the rhizosphere initially was similar to that of the laboratory cultures and then appeared to stabilize (Table 1). The differences in the resolution of the *ftsZ2* reporter on TY agar and in the rhizosphere are not likely due simply to differences in the rate of cell division in these environments. *S. meliloti* divides roughly every 4 h in the plant rhizosphere before reaching a steady-state colonization level of 10⁵ CFU/root segment (Caetano-Anolles et al. 1992). Furthermore, the rate of cell division per se does not affect the resolution percentage, because the ratio of resolved and unresolved cells remains the same for generations if no new gene expression has taken place. The twofold difference between the resolution of the reporter in the rhizosphere and in the nodule-associated bacteria indicates that *ftsZ2* was strongly expressed at some point during bacterial infection of the legume host. It will be interesting to pinpoint the specific stage of infection where expression of *ftsZ2* was stimulated.

QS machinery is activated during the symbiosis.

Genes regulated by QS are promising targets for in vivo expression analysis because QS is broadly implicated in symbiotic interactions between rhizobia and legumes (Sanchez-Contreras et al. 2007). In *Rhizobium etli*, the *N*-acyl homoserine lactone (AHL) synthase *cinI* was shown to be expressed in the rhizosphere and inside the isolated bacteroids (Daniels et al. 2002), indicating that AHL-mediated gene expression takes place during different stages of the symbiosis. In *S. meliloti* 8530, QS contributes to the regulation of hundreds of genes, including genes that potentially are regulated during symbiosis (Chen et al. 2003; Gao et al. 2005, 2007; Hoang et al. 2004; Teplitski et al. 2004), although QS signal-mediated regulation of these genes has not yet been demonstrated in planta. QS of *S. meliloti* is effected by AHL signals produced by the SinI synthase and by two (to perhaps six) AHL receptors (Gao et al. 2005; Marketon et al. 2002; Pellock et al. 2002; Teplitski et al. 2003). In the rhizosphere, bacterial QS could be manipulated by the array of *Medicago* host plant compounds that mimic AHL signals (Gao et al. 2003, 2007; Teplitski et al. 2000). To learn more about QS regulation during symbiotic interactions, merodiploid *tnpR-gus* reporters were constructed with promoters of the *sinI* gene encoding AHL synthase and of a QS-regulated gene *expG*. Their regulation in laboratory cultures, in the rhizosphere, and inside nodules was then tested.

The resolution of the *sinI⁺sinI-tnpR* reporter (*S. meliloti* MG104) in the rhizosphere was variable during the first week after inoculation, and ranged between 20 and 30% and 70 and 90%, depending on the sample. This variability may be biologically significant because rhizosphere colonization by *S. meliloti* is not uniform (Caetano-Anolles et al. 1992; Fujishige et al. 2006; Villaceros et al. 2003). During early stages of rhizosphere colonization, sinorhizobia exist as individual cells,

microcolonies, and even biofilm-like structures (Fujishige et al. 2006; Villaceros et al. 2003). We hypothesize that individual cells have not yet reached “quorum” and the expression of QS genes is at low, pre-quorate level. In microcolonies and biofilms, bacteria are at quorum and QS gene expression is fully autoinduced. In support of this hypothesis, we observed that, after 2 weeks, the resolution of *sinI-tnpR* was uniformly high (approximately 90%) in all rhizosphere samples, which suggests that either all microcolonies have reached quorum or stimulatory plant AHL-mimics have contributed to the synchronization of bacterial gene expression in the rhizosphere.

The *sinI* gene also was expressed inside the nodules. In 6-day-old nodules, the *tnpR*-mediated resolution was low (approximately 25 to 30%); however, the resolution of the reporter reached 92% in 14-day-old nodules. Staining of the nodules carrying the *tnpR-gus* reporter indicates differences in the staining intensity consistent with the differences in the resolution (Fig. 2).

The *expG* gene, which contributes to regulation of EPSII production and is induced by SinI AHLs via the ExpR receptor (Hoang et al. 2004; Pellock et al. 2002), also was expressed inside the nodules, as indicated by the resolution of the *expG⁺expG::tnpR-gus* reporter (Fig. 2). As expected for a QS-regulated gene, *expG* expression required the presence of *sinI* and *expR*. These results provide evidence that AHLs are synthesized inside the nodules. *expG* also was expressed in the rhizosphere (40 ± 3% resolution 48 h after inoculation and 42 ± 2% 14 days after inoculation). Essentially no resolution of the *expG* merodiploid *tnpR-GUS* reporter occurred in the strain that carries null *sinI* and *expR* (AHL receptor) alleles (*S. meliloti* MG102 versus MG103) (Fig. 2). These observations suggest that the levels of QS signals in the rhizosphere are sufficient to induce *expR*-mediated QS gene expression.

Fig. 3. Recombinase-based in vivo expression technology (RIVET) reporter system in *Sinorhizobium meliloti*. **A**, Map of pVMGTnpR. MCS of pVMGTnpR contains unique *SpeI* and *BamHI* available for cloning promoters of interest upstream of the stop codons encoded in three reading frames (CTGACTAAGTAG) cloned upstream of an *XbaI* site. A promoterless *tnpR*- β -glucuronidase (*GUS*) reporter (wild-type ribosome binding site) is cloned into the *XbaI* site directly downstream from the stop codons. *uidA* encodes GUS. The plasmid has ColE1 origin of replication and an origin of transfer; *bla* (β -lactamase) and *nptII* (neomycin phosphotransferase) determine resistance to ampicillin and neomycin or kanamycin. GenBank accession number EU232661. **B**, A *res1-tet-res1* cassette is inserted into the intergenic region between open reading frames *Smc00849* and *Smc00850*. The *res1* sequences are a substrate for the TnpR recombinase. When RIVET reporter is activated, TnpR acts upon *res1* sites and excises the tetracycline cassette. As a result, the antibiotic marker is lost and a *res1* scar remains in its place (Camilli et al. 1994).

DISCUSSION

The need to understand genetics and signal exchange during interactions between rhizobia and their plant hosts has led to the development of several strategies which allow the detection and visualization of changes in patterns of symbiotic gene regulation. High-throughput transcriptomic and proteomic studies provided “snapshots” of changes in bacterial gene expression during the symbiosis (Barnett et al. 2004; Becker et al. 2004; Djordjevic et al. 2003). IVET selections have “trapped” bacterial promoters that are expressed specifically during early stages of the symbiosis or during bacteroid formation (Oke and Long 1999; Zhang and Cheng 2006). The adaptation of RIVET expands the research tool set for studying legume–bacteria symbioses and makes it possible to focus on niche-specific gene expression.

TnpR-based IVET was adapted to analyze induced gene expression in *Sinorhizobium* spp. A transmissible plasmid that carries a promoterless *tnpR-gus* reporter, pVMGTnpR, was constructed (Fig. 3A). The *res1-tet-res1* cassette was cloned into the Smc00849–Smc00850 intergenic region of two commonly used type strains of *S. meliloti* (Fig. 3B). In the genetic background that carries a *res1-tet-res1* cassette encoded within a neutral site of the genome, TnpR serves as a sensitive reporter of cumulative gene expression. The sensitivity of the RIVET reporters is indicated by the rapid and substantial resolution of the *nodC::tnpR* constructs following induction by luteolin (Fig. 1). Even modest activation of a promoter was detected by the *tnpR*-based reporter. This sensitivity of *tnpR* is also a disadvantage: we were unable to construct reporters in several promoters that were activated during strain construction.

To overcome this limitation of RIVET, Osorio and associates (2005) developed a series of promoterless *tnpR-lacZ* cassettes with point mutations in their ribosome binding sites. These tunable reporters can be useful in the RIVET studies of strongly expressed genes.

These studies document symbiotic gene regulation of *nodC*, *sinI*, *ftsZ2*, and *expG*. The functions of these genes are known, although their regulation in the rhizosphere and within nodules was not quantified before. Patterns of *nodC* activation in the rhizosphere and inside the nodules were consistent with earlier work. Regulation of *ftsZ2* is different from earlier reports. Levels of resolution of the *ftsZ2* reporter on TY agar and inside the nodules were similar. This may seem inconsistent with earlier reports which showed that accumulation of *ftsZ2* mRNA is decreased inside the nodules compared with the bacteria grown in TY shake cultures (Barnett et al. 2004; Becker et al. 2004). This inconsistency can be explained by technical differences between the studies (host plant, nodule age, and growth conditions) and by inherent differences between the transcriptomic and RIVET analyses. In transcriptomic studies, mRNA was extracted from both the differentiated bacterioids and saprophytic bacteria that reside within nodules (Barnett et al. 2004; Becker et al. 2004). In this RIVET study, the recovered bacteria are almost certainly undifferentiated saprophytic rhizobia and not bacterioids. It is intriguing to speculate that, inside the nodule, *ftsZ2* may be regulated differently in bacterioids and in their undifferentiated sibling saprophytes.

Because RIVET requires that the recovered cells be culturable, the value of RIVET in studying bacteroid-specific gene expression may be uncertain. An opportunity to characterize gene expression in the saprophytic sinorhizobia that occupy nodules is, perhaps, the most significant advantage of RIVET over any other reporter system. Undifferentiated saprophytic sinorhizobia colonize nodules in small numbers (Timmers et al. 2000) and are predicted to reap the evolutionary benefits of the symbiotic nitrogen fixation.

MATERIALS AND METHODS

Bacterial culture and reporter assay conditions.

Bacterial strains and plasmids used in this study are listed in Table 2. *Escherichia coli* strains were grown at 37°C in Luria-Bertani (LB) medium or on LB agar (Fisher Scientific). Strains of *S. meliloti* were routinely propagated at 28 to 30°C in TY (Barnett et al. 2004). M9 minimal medium with sucrose was prepared as described (Gao et al. 2005). When necessary, media were supplemented with streptomycin (500 µg/ml), neomycin (50 to 200 µg/ml), tetracycline (2.5 to 5 µg/ml), gentamicin (30 µg/ml), or the GUS substrate 5-bromo-4-chloro-3-indolyl-β-D-glucuronide (25 µg/ml). For *sacB* selection, TY with 10% (wt/vol) sucrose was used.

The “resolution” of RIVET reporters was measured in bacteria recovered from shake cultures, TY agar, root surfaces, and the inside of nodules. Culture suspensions or root segments were mildly sonicated (output power 5W) using Sonic Dismembrator, Model 100 to separate bacteria. The effectiveness of sonication was confirmed by observing representative samples under the microscope. Nodules were surface sterilized in 20% Clorox bleach for 5 min, then rinsed in sterile water and crushed with a glass bar. To determine the percentage of bacterial cells that were resolved and, therefore, had lost their resistance to tetracycline, samples were streaked on a medium with neomycin and streptomycin but without tetracycline. As soon as colonies appeared, they were patched onto a tetracycline-containing medium. Selective plates with tetracycline were scored visually after 36 to 48 h and the percentage of tetracycline-sensitive (i.e., resolved) colonies was calculated.

Nodulation and plant growth conditions.

Seeds of *M. truncatula* cv. Jemalong A17 were surface sterilized by a 60-min agitation in 95% ethanol followed by a 30-min incubation in 30% Clorox bleach. Seeds then were rinsed in sterile water and then imbibed for 4 to 8 h at room temperature and then overnight at 10°C. Seeds were germinated in the dark and then transferred individually into growth pouches (Northrup King, Minneapolis, MN, U.S.A.), which were prewetted with 9 ml of sterile two-fold diluted N-free Hoagland solution. Pouches were incubated in the upright position at 24°C under a cycle of 16 h of light (100 µE) and 8 h of dark in an Advanced Intellus Environmental Controller growth chamber (Percival Scientific, Inc, Perry, IA, U.S.A.). Each day, pouches were restored to the original moisture levels with sterile water. Two-day-old seedlings were inoculated along the length of the root with 100 µl of inoculum containing approximately 10⁷ CFU/ml of *S. meliloti* cells. The position of the root tip (RT1) was marked at the time of inoculation and then again 24 h postinfection as described earlier (Caetano-Anolles et al. 1992; Gao et al. 2005).

DNA manipulation and genetic techniques.

Total DNA was extracted from cells of *S. meliloti* 8530 or 1021 by beating with acid-washed glass beads (Sigma-Aldrich, St. Louis). The resulting cell lysates were treated with RNase I, and DNA then was purified using standard solvent partitioning protocols followed by ethanol precipitation. DNA samples were stored as aqueous solutions at –20°C, and aliquots were used as templates for polymerase chain reaction (PCR) as described in the text.

Unless otherwise indicated in the text, all PCR products first were cloned into appropriate pCR vectors (Invitrogen, Carlsbad, CA, U.S.A.) and then excised from the pCR vectors with restriction enzymes. Ligation reactions typically were carried out overnight at 14°C and then transformed into chemically competent *E. coli* DH5α or an *E. coli* S17λpir (for propagation of R6K-ori plasmids). Plasmids were mobilized into *S. meli-*

loti cells by triparental conjugation using helper plasmid pRK600 (Finan et al. 1986).

Plasmid and strain construction.

To construct pVMG85CTR (a *nodC::tnpR-gus* reporter plasmid that also carries a *resI-tet-resI* cassette), a 600-bp fragment corresponding to the *nodBC* region was amplified by Pfu-catalyzed PCR with primers MGnodCF1 and MGnodCR1 using total DNA of *S. meliloti* 8530 as a template. The amplified PCR products were cloned into pCR2.1-TOPO. The insert was excised with *Bam*HI and cloned into pVMG. A 3.5-kb *Kpn*I fragment carrying *resI-tet-resI* cassette was cloned from

pIVET6 into the resulting plasmid. The *Xba*I fragment containing *tnpR* gene with its wild-type ribosome-binding site then was cloned from pMM11 into the *Xba*I site of this construct. The resulting construct was confirmed by sequencing (GenBank accession number EU232662). The *E. coli* DH5 α strain carrying pVMG85CTR was propagated in LB containing tetracycline at 2.5 to 5 μ g/ml (a higher concentration of tetracycline reduced growth of several *E. coli* strains carrying pVMG85CTR). Because pVMG85CTR carries a bicistronic promoterless *tnpR-gus* reporter and a *resI-tet-resI* cassette, it may be adapted for use in other bacteria, although this possibility was not explored further.

Table 2. Strains and plasmids^a

Strains	Genotype or description	Source, construction of reference
<i>Escherichia coli</i> DH5 α	ϕ 80 <i>lacZAM15 deoR endA1 gyrA96 hsdR17 recA1 relA1 supE44 thi-1 Δ(lacZYA-argF)U159</i>	Life Technologies, Gaithersburg, MD, U.S.A.
<i>Sinorhizobium meliloti</i>		
1021	<i>expR101::ISRm2011-1</i> , strep ^f	Galibert et al. 2001
8530	1021 <i>expR</i> +, strep ^f	Pellock et al. 2002
1021rtr	1021 <i>Smc00849::resI-tet-resI</i>	This study
8530rtr	8530 <i>Smc00849::resI-tet-resI</i>	This study
MG101	8530rtr <i>nodC</i> ⁺ <i>nodC::tnpR-gus Smc00849::resI-tet-resI</i>	8530rtr mated with DH5 α pVMG-nodCTnpR, integration confirmed by PCR with NodCR2 and PVO155R2
MG102	<i>expR</i> ⁺ <i>sinI</i> ⁺ <i>expG</i> ⁺ <i>expG::tnpR-gus Smc00849::resI-tet-resI</i>	8530rtr mated with DH5 α pVO3-TnpR24, integration confirmed by PCR with MT7expD and PVO155R2 primers
MG103	<i>expR101::ISRm2011-1 ΔsinI expG</i> ⁺ <i>expG::tnpR-gus Smc00849::resI-tet-resI</i>	MG75rtr mated with DH5 α pVO3-TnpR24, integration confirmed with MT7expD and PVO155R2 primers
MG104	<i>expR</i> ⁺ <i>sinI</i> ⁺ <i>sinI::tnpR-gus Smc00849::resI-tet-resI</i>	8530rtr mated with DH5 α pVMGsinITnpR, integration confirmed with 168com and PVO155R2 primers
MG105	1021rtr <i>ftsZ2</i> ⁺ <i>ftsZ2::tnpR-gus</i>	1021rtr mated with DH5 α pVMGftsZ2TnpR, integration confirmed by PCR with Ftsz2outF1 and PVO155R2 primers
MG32	8530 <i>ΔsinI</i>	Gao et al. 2005
MG75	<i>expR101::ISRm2011-1 ΔsinI</i>	Gao et al. 2005
MG75rtr	<i>expR101::ISRm2011-1 ΔsinI Smc00849::resI-tet-resI</i>	This study, MG75 mated with DH5 α pJQ200SK-8530RTR10
MGCRT85	8530 <i>nodC</i> ⁺ <i>nodC::tnpR-gus resI-tet-resI</i>	8530 mated with DH5 α pVMGCRT85, integration confirmed by PCR with MGnodCR2 and PVO155R2 primers
Plasmids		
p168L	pBluescript II SK carrying a 494-bp <i>Sal</i> II/ <i>Bam</i> HI fragment encoding upstream region of <i>sinI</i>	Gao et al. 2005
pCR2.1-TOPO	PCR cloning vector, pUC <i>ori</i> , amp ^r , kan ^r	Invitrogen Inc., San Diego, CA, U.S.A.
pGOA1193	pIVET5n <i>tnpR</i>	Source of <i>tnpR</i> with wild-type RBS (Osorio et al. 2005)
pIVET6	<i>ori6K mobRP4 T7t tnpR resI-tet-resI amp</i> ^r <i>neo</i> ^r	Source of <i>resI-tet-resI</i> (Camilli et al. 1994)
pJQ200SK	<i>ori P15A traI oriT sacB gent</i> ^r	Vector for gene replacement in <i>S. meliloti</i> , Quandt and Hynes 1993
pJQ200SK-8530RTR10	pJQ200 with a <i>resI-tet-resI</i> cassette in the <i>Sal</i> II site of the <i>Smc00849-Smc00850</i> intergenic region	This study
pMM11	<i>tnpR</i> with wild-type RBS, flanked by <i>Xba</i> I sites in pCR2.1	Source of <i>tnpR</i> , <i>tnpR</i> was amplified with MT22F and MT26RTnpR using pMM1193-1 as a template
pMM11931-1	Fragment containing <i>tnpR</i> with wild-type RBS from pGOA1193 in pUC19	<i>Bgl</i> III- <i>Sal</i> II fragment from pGOA1193 was cloned into <i>Bam</i> HI, <i>Xho</i> I-treated pUC19.
pVMG	pVO155 with CTGACTAAGTAG upstream of the <i>Xba</i> I site, amp ^r <i>neo</i> ^r	Gao et al. 2005, stop codons in three reading frames are inserted immediately downstream from the MCS upstream of <i>GUS</i>
pVMGCRT85	<i>nodC::tnpR-gus</i> that also carries <i>res-tet-res</i> cassette in pVMG	This study, GenBank accession no. EU232662
pVMG-ftsZ1TnpR	<i>ftsZ1::tnpR-gus</i> in pVMGTnpR	<i>ftsZ1</i> promoter amplified by PCR with MG3ftsZ1F and MG4ftsZ1R, and cloned into <i>Bam</i> HI site of pVMG-TnpR, orientation was confirmed with the MG21ftsZ1IN
pVMG-ftsZ2TnpR	<i>ftsZ2::tnpR-gus</i> in pVMGTnpR	<i>ftsZ2</i> promoter PCR amplified with FtsZ2F and FtsZ2R, and cloned into <i>Spe</i> CI/ <i>Xho</i> I site of pVMG-TnpR, orientation confirmed with FtsZ2Out1 and DNA sequencing
pVMG-nodCTnpR	<i>nodBC::tnpR-gus</i> in pVMGTnpR	This study
pVMGsinI-7	<i>sinI::gus</i> in pVMG	<i>Sal</i> II/ <i>Bam</i> HI fragment from p168L cloned into pVMG
pVMGsinITnpR	<i>sinI::tnpR-gus</i> in pVMG	<i>Xba</i> I fragment containing <i>tnpR</i> from pMM11 cloned downstream of the <i>sinI</i> promoter in pVMGsinI-7, confirmed by PCR with 168com and PVO155R2
pVMGTnpR	Carries promoterless <i>tnpR-gus</i> in pVMG background	This study, GenBank accession no. EU232661
pVO155	Vector for constructing <i>gus</i> promoter probes, ColE1 <i>oriT</i> amp ^r <i>neo</i> ^r	Oke and Long, 1999
pVO3	<i>expG-gus</i> in pVMG	<i>expG-gus</i> construct isolated by screening a library of <i>S. meliloti</i> promoters responsive to AHLs, M. Gao, unpublished data
pVO3-TnpR24	<i>expG::tnpR-gus</i> in pVMGTnpR	<i>tnpR</i> gene was inserted after <i>expG</i> promoter in pVO3 as <i>Xba</i> I fragment from pMM11

^a GUS = β -glucuronidase; PCR = polymerase chain reaction; RBS = ribosome binding site; AHL = *N*-acyl homoserine lactone; strep^f, amp^r, kan^r, gent^r, and neo^r = resistant to streptomycin, ampicillin, kanamycin, gentamicin, and neomycin, respectively.

pVMG85CTR was mobilized in *S. meliloti* 8530 by triparental conjugation. The single crossover reporter carrying *nodC::tnpR-gus res1-tet-res1* was selected on M9-sucrose agar containing neomycin, streptomycin, and tetracycline. The insertion was confirmed by diagnostic PCR (using appropriate primers listed in Table 3) and by sequencing of the corresponding PCR products. The resulting strain *S. meliloti* MGCRT85 is nodulation proficient because neither *nodB* nor *nodC* ORF were disrupted. An intact copy of the *nodC* gene is downstream of the site of pVMG85CTR integration; its expression probably is driven by a plasmid-encoded *bla* promoter. *S. meliloti* MGCRT85 was grown in TY with tet5, or in LB with tet2.5. No growth defects are observed under these conditions. The parental strain *S. meliloti* 8530 was sensitive to these levels of tetracycline.

pVMGTnpR, a plasmid carrying promoterless *tnpR-gus*, was constructed by first excising the 3.5-kb *KpnI* fragment carry-

ing *res1-tet-res1* cassette from pVMG85CTR. This yielded pVMG-nodCTnpR (*nodBC::tnpR-gus*). The *nodBC* fragment was removed from pVMG-nodCTnpR by *BamHI* digest. The plasmid then was self-ligated, yielding pVMGTnpR. Features of pVMGTnpR are shown in Figure 3A (GenBank accession number EU232661).

Strains of *S. meliloti* carrying the *res1-tet-res1* cassette were generated individually using suicide construct pJQ200SK-850-RTR10. pJQ200SK-850-RTR10 was made as follows. First, a 1.1-kb fragment containing a presumed neutral site between convergently transcribed ORF Smc00850 and Smc00849 was amplified using primers Sm850F and Sm849R and genomic DNA of Sm1021 as a template. The amplified PCR fragment was cloned into pCR2.1, yielding pCR2.1-850.849. The construct was sequenced. A native *SalI* site was encoded within the Smc00849-Smc00850 intergenic region. A 3,650-bp DNA carrying *res1-tet-res1* cassette was amplified with Pfu poly-

Table 3. Primers used in this study

Name	Sequence	Description ^a
168com	GAAGAAATCGGGCTTTCCACCGAA	Binds nt 1996293 of <i>S. meliloti</i> chromosome sequence (NC_003047), used to check the orientation of the pVMGSinITnpR integration into the chromosome
Ftsz2F	AGCTTTTCGAACGTTTCGTCGAACCGCT	Binds nt 2215532 of NC_003047, used to amplify promoter of <i>ftsZ2</i>
Ftsz2outF1:	TCCGTCTCACCGGATGCGCTCAAGGA	Binds nt 2216156 of NC_003047, used to confirm integration of pVMG-ftsZ2TnpR into the chromosome
Ftsz2outR1:	ATCCTACTGCAGCGTACGCGAAGGAAC	Binds nt 2214113 of NC_003047, used to confirm integration of pVMG-ftsZ2TnpR into the chromosome
Ftsz2R	ATGCCTGCCGTGACGAAGCACATA	Binds nt 2214860 of NC_003047, used to amplify promoter of <i>ftsZ2</i>
In849R	ATACGTTCAAAATTTTATCAT	Binds nt 938660 of NC_003047, used to check the insertion of <i>res1-tet-res1</i> in the intergenic region between Smc00849 and Smc00850.
In850F	ATTTCTTCAATGACGTTCTCGTCA	Binds nt 937263 of NC_003047, used to check the insertion of <i>res1-tet-res1</i> in the intergenic region between Smc00849 and Smc00850.
MG21		
ftsZ1Fin	TTTTCCACCACGGCCGTCGGGCTGATGAT	Used to check orientation of the <i>ftsZ1</i> construct
MG24	ATCGTCGGCAAGCGCATCGTCTTGA	Binds nt 2336547 of NC_003047, used to check the orientation of the pVMG-ftsZ1TnpR integration into the chromosome
MG3ftsZ1F	TCGGCGGATCCGGCGCTGCCGGCCGCTGCCAA	Binds nt 2336441 of NC_003047, used to amplify promoter of <i>ftsZ1</i> , has an introduced <i>BamHI</i> site.
MG4ftsZ1R	TCGGGGATCCAGGCGACGCCGAATAAAGA	Binds nt 2335562 of NC_003047, used to amplify promoter of <i>ftsZ1</i> , has an introduced <i>BamHI</i> site.
MGnodCF1	CTCGAGAACCATCATCAACGACATAG	Binds nt 480016 of the NC_003037 pSymA sequence, used to amplify the region upstream of <i>nodC</i>
MGnodCF2	AGAATGAAGCTGAACCTCGGAT	Binds nt 479952 of NC_003037 pSymA sequence, binds within <i>nodB</i> , upstream of MGnodCF1, to confirm integration of pVMG-nodCTnpR into pSymA
MGnodCR1	GGATCCAAGGCGATTGTTGATGCAGT	Binds nt 480497 of NC_003037 pSymA sequence, used to amplify the region upstream of <i>nodC</i> , has an introduced <i>BamHI</i> site.
MGnodCR2	TACATTGGTCGGCAGATCCGCGAGATT	Binds nt 480539 of NC_003037 pSymA sequence, used to confirm orientation of the pVMG-nodCTnpR integration into pSymA
MT22F	TCTAGATCTCAATTGAGAATTTAGGATA	Binds at nt 6 of pGOA1193, upstream of RBS of <i>tnpR</i> , has <i>XbaI</i> site
MT26RtnpR	TCTAGAGTCGACTTTCCTCCTTAGTTGCTT	Binds at nt 600 (bottom strand) of pGOA1193 to amplify entire <i>tnpR</i> , has an <i>XbaI</i> site
MT7expD	AGAAGTTGATGCCCTTGATGATCGG	Binds nt 480731 of NC_003047 (within <i>expD</i>), used to check the orientation of the pVO3-TnpR24 integration into the chromosome
PVMGkpnIF1	AAATCCGTCGGCATCCAGGAAA	Binds nt 3406 (top strand) of pVMG, upstream of unique <i>KpnI</i> and <i>SacI</i> sites, used to check insertion of <i>res1-tet-res1</i> in the <i>KpnI</i> site of pVMG
PVMGkpnIR1	TTGTAAAACGACGCGCCAGTGAATT	Binds 3545 (bottom strand) of pVMG downstream of unique <i>KpnI</i> and <i>SacI</i> sites, used to check insertion of <i>res1-tet-res1</i> in the <i>KpnI</i> site of pVMG
PVO155F	AAGCTTGCATGCTGCAGGT	Binds nt1 of pVMG (top strand), used to confirm orientation of inserts in pVMG and pVMGTnpR
PVO155R2	TTTCGCGATCCAGACTGAAT	Binds nt120 within <i>gus</i> of pVMG (bottom strand), used to confirm orientation of inserts in pVMG and pVMGTnpR
ResTeF2	TTGCTCGAGATCCCCGGGTA	Binds nt 55 of pVMGCRT85, used to amplify <i>res1-tet-res1</i> cassette, has 5' <i>XhoI</i>
ResTeR3	TAAAACGACGGCCAGTGAATTCGAGCTCGAGA	Binds nt 4755 of pVMGCRT85, used to amplify <i>res1-tet-res1</i> cassette
SinIF	GGGGTACCGGGCCGAAACGGAGG	Binds nt 1996515 of NC_003047, used to amplify promoter of <i>sinI</i> , has an introduced <i>KpnI</i> site
SinIR	CCGCTCGAGTTTTTCGCTCCATGCGT	Binds nt 1996866 of NC_003047, used to amplify promoter of <i>sinI</i> , has an introduced <i>XhoI</i> site
Sm849R	ATCACAGCCGCTCTCGTCTAT	Binds nt 938562 of NC_003047, used to amplify Smc00849-Smc00850 intergenic region
Sm850F	ATGACCGCCAGGATTCTCGTCAGT	Binds nt 937447 of NC_003047, used to amplify Smc00849-Smc00850 intergenic region

^a nt = Nucleotides.

merase using pVMG85CRT as a template and primers ResTeF2 and ResTeR3. The amplified *res1-tet-res1* PCR product was cloned into pCR-ZeroBlunt and sequenced. The *res1-tet-res1* cassette was cut from the pCR-ZeroBlunt vector with *XhoI* and cloned into the *Sall*-treated pCR2.1-850.849, so that the *res1-tet-res1* cassette was inserted into the Smc00849-Smc00850 intergenic region. The 4.7-kb *SpeI-XhoI* fragment carrying the *res1-tet-res1* cassette flanked by sequences homologous to the Smc00850-Smc00849 then was excised and cloned into pJQ200SK. The pJQ200SK-850-RTR10 was conjugated into *S. meliloti* strains 8530, 1021, and MG75 as described above. The transconjugants were selected on TY agar with streptomycin, tetracycline, and gentamicin. Several transconjugants then were grown overnight in TY broth to allow for the second crossover event. The mutants then were cultured on TY with tetracycline and sucrose. Gentamicin-sensitive isolates that resulted from the second recombination event were identified. The insertion of the *res1-tet-res1* cassette was confirmed by PCR using primers In850 and In849.

To construct *S. meliloti* RIVET reporter strains, promoter::*tnpR-gus* in pVMGTnpR (Table 2) was mobilized into *S. meliloti* 8530tr or *S. meliloti* 1021tr by triparental mating and selected on TY medium with streptomycin, tetracycline, and neomycin. Details of constructions are summarized in Table 2. All constructs were designed in such a way that the integration of the reporter into the host chromosome generated a merodiploid strain, which contained an intact wild-type copy of the gene and a promoter::*tnpR-gus* reporter. Orientation of inserts was confirmed by PCR using appropriate primers that bind to the chromosome upstream of the predicted insertion site of each cloned fragment paired with the PVO155R primer that binds at the beginning of the *uidA* gene. A complete list of relevant primers used in this study is in Table 3.

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