Use of Plasmid pVMG to Make Transcriptional β-Glucuronidase Reporter Gene Fusions in the Rhizobium Genome for Monitoring the Expression of Rhizobial Genes In Vivo

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Abstract

Background: The soil bacterium Sinorhizobium meliloti and its allies are important nitrogen-fixing bacterial symbionts that cause N2-fixing nodules on the roots of legumes. Chromosomal β-glucuronidase gene (uidA) transcriptional fusions are frequently used to monitor the expression of bacterial genes during the symbiosis. However, the construction of the fusions is laborious.

Results: The narrow-host-range, fusion selective plasmid pVMG was constructed and used as a vector for the construction of chromosomal uidA transcriptional fusions in the S. meliloti genome. Translation termination codons were added in all three reading frames upstream of the promoterless uidA in this vector to ensure transcriptional fusions. pVMG replicated to high copy number in Escherichia coli, offering advantages for the isolation of fusion-containing plasmids and the restriction analysis. Genomic locations of uidA fusions were verified in a simple PCR experiment. All these helps reduce the sample processing time and efforts. As a demonstration of its usefulness, the N-acyl homoserine lactone (AHL) signal synthase gene promoter was fused to uidA and shown to be expressed by S. meliloti in the senescence zone of the nodule on the host plant, M. truncatula. This indicates the presence of AHL signals at the late stages of symbiosis.

Conclusions: A simple, pVMG-based method for construction of chromosomal uidA transcriptional fusions has been successfully used in the model rhizobium S. meliloti. It is also applicable for other rhizobial strains.

Keywords: Rhizobia symbiosis, β-glucuronidase gene (uidA), Transcriptional fusions, Chromosomal uidA transcriptional fusions

Background

The chromosomal uidA transcriptional fusions are frequently used for monitoring in vivo expression of bacterial genes for at least three reasons: First, the fusions allow the transcriptional activities of bacterial genes to be monitored at their native levels [1–4]. Second, the fusions do not need antibiotics for maintaining their stability in the genome. Third, they avoid problems that associate with replicating plasmid systems which can disrupt regulation of expression due to copy number effects [1, 3–5]. As higher plants lack β-glucuronidase activity [6], the uidA gene provides a sensitive enzyme assay for which a broad range of substances are available.

Methods for making chromosomal uidA transcriptional fusions in rhizobia involve a bacterial narrow-host-range, plasmid vector with a promoterless uidA gene [1, 2, 4]. Segments of DNA containing gene promoter from rhizobia can be cloned into the multiple cloning sites (MCS) located upstream of the uidA in the vector. The
fusion-containing plasmids are maintained in a suitable *E. coli* strain, isolated and restriction analyzed, and then can be transferred to a rhizobial strain from *E. coli* in bi- and tri-parental matings. As the vector uses an origin of replication (e. g., pUC) that is inactive in rhizobial strains [7], each fusion-containing plasmid co-integrates into the rhizobial host with the rhizobial host genomic DNA. This can create a single copy *uidA* transcriptional fusion in the rhizobial host genome. The genomic locations of the fusions are typically verified by Southern blotting. The integration of fusion-containing plasmid does not disrupt the targeted locus if the cloned DNA fragment in the plasmid will not be internal to the transcription unit [1, 8].

A few plasmids have been used as narrow-host-range *uidA* transcriptional vectors in pioneering studies of rhizobial gene expression: pMH11 [9], pVO155 [1, 2], and pTH1522 [4]. While very fruitful to make *uidA* transcriptional fusions for in vivo studies, some of those vectors lack translational termination codons between the transcription unit [1, 8].

**Table 1** Strains and plasmids

| Strain or plasmid | Relevant features | Source or Reference |
|-------------------|------------------|---------------------|
| DH5a              | a-complementation | Invitrogen          |
| 8530              | *S. meliloti*, expR, sinI+, Sm’ | [10]               |
| 1021              | *S. meliloti*, expR, sinI+, Sm’ | [11]               |
| MG32              | 8530 with sinI deletion expR+, sinI’, Sm’ | [12]               |
| 8530 sinI-VMG495  | sinI-uidA transcriptional fusion, expR+, sinI+, Sm’, Nm’ | This work |
| 1021 sinI-VMG495  | sinI-uidA transcriptional fusion, expR+, sinI+, Sm, Nm | This work |
| MG32 sinI-VMG495  | sinI-uidA transcriptional fusion, expR+, sinI-, Sm’, Nm’ | This work |
| 8530 nop::VMG209  | Non promoter (nop) DNA-uidA, expR+, sinI+, Sm’, Nm’ | This work |
| pVO155            | pUC19-derived integrational *uidA* vector | [1]               |
| pVMG              | pVO155 with stop codons upstream of promoterless *uidA* in all ORFs | This work, [12] |
| pRK600            | pRK2013 Nm::Tn9, Cm’ | [1]               |
| pVMG495           | pVMG, sinI 5’-end, transcriptional fusion | This work |
| pVMG209           | pVMG, nop DNA of *S. meliloti*. | This work |

**Methods**

**Bacterial Strains and Media**

All strains and plasmids used are listed in Table 1. The following media were previously described: LB, a complex medium [13]; TY, a complex medium for *S. meliloti* [14], containing, Per liter, tryptone 6 g; yeast extract 3 g, and CaCl$_2$·H$_2$O 0.5 g. Final concentrations of antibiotics: 100–200 μg mL$^{-1}$ of neomycin (Nm) and 250–500 μg mL$^{-1}$ of spectinomycin (Sm) (for the *S. meliloti* strains).

**Biochemistry**

**DNA Biochemistry**

Restriction endonucleases were purchased from New England Biolabs (New England Biolabs, Inc. Beverly, MA, USA) and used according to the manufacturer’s instructions. A 25-bp linker was created by annealing two complementary oligonucleotides (5’-GATCCCTCGAG CGTACTAACTAGCT-3’; 5’-CTAGAGCTAGTTAGTCAGC TCGAGG-3’). The linker contained a XhoI site and translation termination codons in three different ORFs with termini cohesive to either BamHI I or XbaI. DNA ligations were performed with T4 DNA ligase (Biolabs, # M0320 T). Colony PCR was performed in a sterile 0.5-ml amplification tube containing 1 x Standard *Taq* Reaction Buffer (Biolabs # B9014S), 0.5 μM of four dNTPs, 0.2 μM of two forward and revers primers, 1 unit of *Taq* DNA polymerase (Biolabs # M0320 L) and an individual *S.
meliloti colony. PCR primers are shown in Table 3. The nucleic acids were amplified for 35 cycles. The denaturation, annealing, and polymerization times and temperatures were 1 min at 94 °C, 1 min at 50 °C, and 30 s at 72 °C. DNA sequencing was performed by Rightmire DNA Sequencing Facility at The Ohio State University, Columbus, Ohio, and by the DNA Sequencing Laboratory at the Interdisciplinary Center for Biotechnology Research at University of Florida, Gainesville, Florida.

Genomic DNA extraction was described previously [4] with minor modifications. DNA was prepared from 5 ml of culture grown in TY broth with appropriate antibiotics to saturation with shaking. Bacteria were collected in 2-ml microcentrifuge tubes by centrifugation, washed once with 0.85% NaCl, and then resuspended in 0.5 M, and DNA was precipitated with an equal volume of isopropanol. The optical density at 260 nm (OD 260) of the sample was measured to determine the DNA concentration. The samples were then lyophilized and dissolved to proper concentrations for restriction enzyme digestions.

Acyl Homoserine Lactones (AHL) Biochemistry
C_{16:1}-\Delta^9cis-(L)-homoserine lactone (referred to as “C_{16:1}-HSL” in text) was from Cayman Chemical (Ellsworth Road, Ann Arbor, MI U.S.A.). The molecule was dissolved in ethyl acetate at stock and diluted in methanol. AHL was added in a liquid medium prior to inoculation of bacteria.

β-Glucuronidase (GUS) Biochemistry
Quantitative assay for GUS activity was described previously [6] with modification [15]. Cells were permeabilized with lysozyme (200 μg ml\(^{-1}\), 37 °C for 10 min), and the GUS activity was measured with PNPG (p-nitrophenyl-β-o-glucuronide). GUS activity was calculated in nanomoles per minute per OD\(_{260}\) unit \(\times\) 1000 as in [15]). Color producing substrate X-gluc (5-bromo-4-chloro-3-indolyl-β-D-glucuronide) for GUS enzyme to act was used for visualizing activity of GUS in histochemical assay at working concentration of 40 μg/ml as described before [16].

Conjugations
Recombinant plasmids were maintained in E. coli DH5α and were conjugated into S. meliloti recipient strains with help plasmid pRK 600 by bacterial conjugation method [1] with modifications. Log-phase recipient cells (10\(^{8}\)/ml) were used in the conjugation. The donor and recipient ratio was approx. 8:1. S. meliloti transconjugants were selected on TY agar medium at the present of neomycin.

Sequence Analysis
Sequence assembly was performed with MacVector with Assembler 12.01. Database searches were conducted through the S. meliloti genome web page using blastn.

Plant Growth and Nodulation
Cultivation of M. truncatula A17, root nodulation, and nodule harvesting were described previously [17].

Results
Construction of pVMG
The narrow-host-range plasmid pVO155, with a MCS, a promotorless uidA (gus) reporter gene encoding β-glucuronidase (GUS), and a pUC origin of replication, was used as a base for the construction of pVMG.

pVMG was constructed by replacing a BamHI-XbaI fragment at the end of MCS in pVO155 with the 25-bp BamHI-I-XbaI synthetic DNA linker containing three translation terminations and a XhoI site (see Methods). The structure of pVMG is shown in Fig. 1a. The translation termination codons (referred to as to “stop codons” in Figure) upstream of the uidA in all reading frames ensure transcriptional fusions (Fig. 1b). DNA sequencing and double/triple endonuclease digests of the plasmid confirmed that pVMG retains the original promoterless uidA gene, seven of the 8 restriction enzymes in multiple cloning sites (MCS), a E. coli trpA terminator upstream of the MCS to prevent read-through from the vector, a unique Hind III site adjacent to the trpA, a unique Bgl II and the pUC origin of replication. DNA sequencing data also confirmed that pVMG retains the original oriT origin of transfer, the neomycin (Nm) resistance ORF and the ampicillin (Amp) resistance ORF.

pVMG replicates to high copy number in E. coli DH5α. This provides cloning advantages. The average DNA yield of pVMG in Miniprep experiments was similar to the yield of pUC19 itself (Table 2). Various 200–800 bp DNA fragments from S. meliloti were cloned into pVMG [12, 18, 19]. For an insert size of 200–800 bp, we typically obtained 80–190 Nm\(^{8}\) colonies by using the 1/5 volume of each ligation reaction and of these, approximately 85% contained the expected inserts.
Construction of Transcriptional sinI-uidA Fusions

In order to demonstrate the usefulness of pVMG, we made a transcriptional sinI gene reporter fusion to uidA by using a pVMG-based method (Fig. 2a). The sinI gene of *S. meliloti* encodes the synthase of the bacterial AHL signaling molecules [20]. The sinI gene mutations abolish the transcription of sinI-dependent genes and delay initiation of nodulation on the roots of the host plant, *M. truncatula* [12]. The sinI promoter is inducible by C16:1-HSL and the ExpR protein of *S. meliloti* enhances this induction [10]. The sinI gene is expressed in free-living bacteria and at the time when cells invade the nodule of *M. truncatula* [12]. In our study of the Rhizobium sinI gene function, we wished to examine the expression of sinI gene in late stages of the symbiosis.

The intact 5'-end of sinI gene fragment was cloned into pVMG. This 438-bp fragment contains the sinI promoter and ExpR binding site (nucleotides −416 to +22 with respect to the sinI translation start site) [21]. The fragment was amplified from chromosome of *S. meliloti*.

| Table 2 DNA yields of pVMG, pVO155 and pUC19 grown in medium LB |
|---------------------------|-----------------|-----------------|-----------------|
| Plasmid | Average yield n = exp. (Μg) |
| pUC19 | 18 ± 2 (n = 5) |
| pVMG | 16 ± 2 (n = 75) |
| pVO155 | 16 ± 2 (n = 17) |

QIAprep Spin Miniprep Kit was used to purify DNA from 1.5 ml LB overnight cultures of DH5a containing pUC19, or pVMG or pVO155. Elution was performed according to the standard protocol (50 μl Buffer EB and 1 min incubation). Use of the recommended LB composition (with 10 g/liter NaCl) and Nm resistance provides optimal plasmid yield. Time spent was <15 min. Costs per μg DNA was 7 cents.

pVMG495 was transferred into Invitrogen DH5a competent cells which are neomycin sensitive (Nm$^\text{S}$). pVMG495 was conjugated into the *S. meliloti expR* mutant strain 1021 [11] by a tri-parental matting with pRK600 as a helper. *S. meliloti* conjugants were selected.
for neomycin resistance (Nm\(^R\)), yielding a transcriptional \(\text{sinI-uidA}\) fusion strain called \(S.\ meliloti\) 1021 \(\text{sinI::VMG495}\) (Table 1).

In the experiments reported above, the co-integration frequency of the plasmid pVMG with the 438-bp chromosomal insert in the \(S.\ meliloti\) 1021 was about \(4.3 \times 10^{-5}\) (number of Nm\(^R\) recombinants per total number of receipted cell present). This represents about a 8.6-fold increase over the co-integration frequency obtained in \(A.\ caulinodans\) ORS571 [22] for a pBBR-based replicon with a larger insert introduced by a similar tri-parental matting method. In a control experiment, the vector pVMG was conjugated into the strain 1021, no Nm\(^R\)- transformants were obtained.

Two additional transcriptional \(\text{sinI-uidA}\) fusion strains were constructed by conjugating pVMG495 into \(S.\ meliloti\) sinI mutant strain MG32 [12] and \(S.\ meliloti\) wild-type strain 8530 [10], respectively. They yielded \(S.\ meliloti\) MG32 \(\text{sinI::VMG495}\) and \(S.\ meliloti\) 8530 \(\text{sinI::VMG495}\) (Table 1).
Table 3 Primers used in the study. Sal I and BamH I restriction sites are underlined.

| Name       | Sequence (5′-3′) | usage   |
|------------|-----------------|---------|
| Primer 1-sinl | AGCCGCGATGATGTCCCATACC | snl forward |
| Primer 2-sinl | GGATCCGGGATCCGAGCAGCG | snl reverse |
| Primer 3-sinl | GGATCCGGGATCCGAGCAGCG | snl forward |
| Primer 4   | GGGTTGGGCTTTCTACAGGA | uidA reverse |
| Primer 1-nop | AGCCGCGATGATGTCCCATACC | nop forward |
| Primer 2-nop | ATCGAGGATCGACGGAG | nop reverse |
| Primer 3-nop | AGGTGGGCTTTCTACAGGA | nop forward |

All *S. meliloti* sinl-uidA fusion strains (1021::VMG495, MG32::VMG495, 8530::VMG495) were verified for the site-specific integration of pVMG495 by PCR. Three colonies of each fusion strain candidates were analyzed. The oligonucleotide primers (Primer 3-sinl and Primer 4) used for the amplification are shown in Table 3. The integration specific Primer 3-sinl attaches the genomic DNA region outside and upstream of the cloned 438-bp fragment. The uidA specific primer 4 attaches DNA region inside of the uidA from pVMG and it faces toward the cloned sinl fragment (Fig. 2a). Specific primers amplified the ~720-bp DNA region of integration (Fig. 2b, lanes 1–9) from all tested fusion strains, but not from their parental strains (Fig. 2b, lane 10). The fusion-strain-specific amplification strongly suggests the site-specific integration of pVMG495. The 720-bp product was presumably constituted of the cloned sinl fragment flanked by the upstream *S. meliloti* chromosome and the 5-end of uidA gene. The identity of the 720-bp product was determined by DNA sequencing. This confirmed the presence of all expected DNA segments as well as a termination codon in-frame with the sinl gene (see Additional file 1).

Next, we checked the identity of *S. meliloti* reporter strains (1021::VMG495, MG32::VMG495, and 8530::VMG495) by the method for recovery of integrated plasmid for subsequent DNA sequencing [1, 8]. We cut the entire genome with a restriction enzyme that does not cut within the pVMG495 plasmid. We then circularized the fragments with T<sub>4</sub> DNA ligase and transformed them into *E. coli* strain DH5α. We recovered a plasmid that contains the expected structure in every strain.

Subsequently, we checked the identity of *S. meliloti* reporter strains (1021::VMG495, MG32::VMG495, and 8530::VMG495) by Southern blot [23]. As shown in Additional file 2 Figure S2, Southern blot test detected one fragment of Hind III digestion in the DNA from each sample of the fusion strains of sinl::VMG495. The identical band was visible at approx. 7.7-kb from each fusion sample since the sinl gene integration regions are identical. This band was caused by the chimeric DNA fragment of genome-pVMG495. The Southern blotting test detected no additional second or third copies of the vector in the genomic DNA samples, suggesting no random integrated vector DNA in the genomes of 1021 sinl::VMG495, MG32 sinl::VMG495 and 8530 sinl::VMG495.

By using the pVMG method shown in Fig. 2a, we constructed a control strain called *S. meliloti* 8530 nop::VMG209 (Table 1) for measuring background activity of the GUS. In this experiment, a plasmid called pVMG209 was created by cloning a 209-bp non-promoter (nop) DNA segment of *S. meliloti* to the SalI-BamHI site of pVMG. pVMG209 was integrated into the chromosome of the *S. meliloti* 8530 strain. The primers we used for the construction of 8530 nop::VMG209 strain are shown in Table 3.

Testing Transcriptional sinl-uidA Fusions

The fusion strains and the control strain were tested for responsive changes in GUS activity in free-living bacteria. The rhizobium strains were cultured in (a) TY broth, (b) TY broth containing 7.5 nM of C<sub>16:1</sub>-HSL as we did before [24]. The fusion in sinl promoter was found to have significant changes in GUS activity in response to expR sinl, sinl, expR backgrounds or to added AHL when tested at late log phase (optical density at 600 nm [OD<sub>600</sub>] = 0.98 to 1.08.) (Table 4). Quantitative GUS assay demonstrated that the sinl promoter activity in the presence of C<sub>16:1</sub> was 5.8-fold higher than in the absence of C<sub>16:1</sub>. This induction factor is consistent with the one measured in a *S. meliloti* reporter strain carrying a chromosomal single copy of sinl-lacZ transcriptional fusion [25]. The uidA fusion in non-promoter (nop) DNA had no activity in WT background (Table 4) and was not responsive to the AHL (data not shown).

The wild-type strain that expresses β-glucuronidase (GUS) was also tested for its ability to initiate nodulation on roots of *M. truncatula* plants, using a previously described protocol [17]. The rate and the efficiency of

Table 4 Responses of single copy sinl-uidA transcriptional fusion to expR sinl, sinl, expR, and AHL in medium TY

| Strain       | Genotype         | β-glucuronidase activity<sup>a,b</sup> |
|--------------|-----------------|-----------------------------------|
| 8530 sinl::VMG495 | expR+, sinl+     | 194 ± 4                            |
| MG32 sinl::VMG495  | expR+, sinl-     | 33 ± 3                             |
| MG32 sinl::VMG495 (C<sub>16:1</sub>) | expR+, sinl-     | 192 ± 2                            |
| 1021 sinl::VMG495 | expR-, sinl+     | 95 ± 3                             |
| 8530 nop::VMG209 | expR+, sinl+     | < 0.1                              |

<sup>a</sup>Nanomoles per minute per OD<sub>600</sub> [15].<br>
<sup>b</sup>Shown are averages and standard deviations (n = 3).
nodule initiation by the GUS expressing strain 8530
sinl::VMG495 and the 8530 parent were found similar.
This observation indicated that 8530 sinl::VMG495 was
normal in the ability to initiate nodulation on the host
plant. The normal initiation of nodulation suggested that
sinl activity was not inhibited by the GUS or by the sinl
fragment of 8530 sinl::VMG495 strain.

In order to examine the expression of sinl gene in the
late stages of the symbiosis, roots of M. truncatula
were inoculated with 8530 sinl::VMG495 and 8530 nop::VMG209.
Nodules were harvested and stained with X-Gluc [6].
Stained nodules were examined under a dissecting scope.
The 8530 sinl::VMG495 infected nodules showed a blue
invasion zone and a blue senescence zone (Fig. 3a). The
8530 nop::VMG209 infected nodules did not (Fig. 3b).
While confirming the expression of the sinl by S. meliloti
bacteria at the time of nodule invasion [27], the results
demonstrated the expression of the sinl in the senescence
zone of the M. truncatula root nodule. This indicates the
presence of AHL signals at the late stages of symbiosis.

Discussion
Several reporter gene systems are available to monitor
bacterial gene activity within plant cells. These include
β-galactosidase (lacZ) [28], green fluorescent protein (gfp) [29] and β-glucuronidase (uidA) [6]. uidA reporter
gene system has increased sensitivity relative to lacZ
because most higher plants show large amounts of galactosi-dase activity but they lack β-glucuronidase (GUS) ac-
tivity [6]. uidA reporter system has increased sensitivity
relative to GFP when used within root nodule cells be-
cause molecular oxygen (O2) is low in those cells but
GFP strictly requires O2 for maturation of fluorescence
[30]. Because it has increased sensitivity, uidA has been
extensively used to monitor bacterial gene activity during
the Rhizobium-legume symbiosis [1, 2, 4, 19, 31, 32].

In the course of research in S. meliloti, we have de-
veloped a new transcriptional uidA fusion selective vector
that preserves properties of its parent plasmid pVO155,
i.e., to be a mobilizable narrow-host plasmid and present
at a high copy number in E. coli. The characteristic pre-
sence of translation terminations between the MCS and
the promoterless uidA gene in a pUC-based replicon,
distinguishes pVMG as an effective vector to make
chromosomal uidA transcriptional fusions in Rhizobia.
This effectiveness of making transcriptional fusion, to-
gether with the simple procedure of verifying fusion by
PCR, distinguish pVMG method as an effective method
for various applications in construction gene fusions of
Rhizobium. We have found that Rhizobium DNA frag-
ments with size up to 3.5-kb can be easily cloned in
pVMG [12, 18, 19] and yields of those Rhizobium

![Fig. 3 Histochemical assay of M. truncatula nodules. a 8530 sinl::VMG495 (n = 13) b 8530 nop::VMG209 (n = 5). Nodules were stained with 4 mg
/ml X-Glue in NaH₂PO₄, pH 7.2, 37 °C as described [26] for 3 h (a) or for 48 h (b). Photographs were taken 7 weeks after inoculation with Olympus
MVX10 dissecting scope equipped with a MicroFire camera (Optitronics, Goleta, CA, USA)](image-url)
fusion-containing plasmids from minipreps were similar to the yield of pUC19 which is much higher than those of pBBR-based narrow-host plasmids. Large amount of candidate rhizobial uidA fusion strains can be verified easily by a simple one-step colony PCR which is much easier than Southern blotting or recovery of integrated plasmid for verification of fusion. Additionally, a PCR product could be used to identify the genomic site of integrated plasmid by direct DNA sequencing.

Genomic sites of integrated plasmid have been typically verified with the Southern blotting procedure [23]. Southern blotting is extremely sensitive and specific for detecting DNA in a particular sample and has been used in diagnostic studies to detect genomic sites of integrated plasmid in bacteria [2, 12, 22, 33, 34]. Although sensitive and specific, Southern blotting requires isolation of DNA, digestion of DNA with restriction enzyme(s), separation of DNA by agarose gel electrophoresis, blotting and hybridization with a probe [16]. Making a probe for hybridization involves generating, purifying and labeling DNA. In most cases, radioactive probes are used [16]. While generally effective, these methods are time consuming and create the potential for radiation exposure.

In an effort to reduce sample processing time and efforts, we have verified the genomic site of the integrated plasmid by the simple PCR experiment for subsequent DNA sequencing. Results from this verification method were checked with other two conventional verification methods: 1) the method for recovery of integrated plasmid for genome-vector site identification [8, 35] and 2) Southern blotting [23]. Results from both conventional verification methods supported the conclusion that the PCR verification method has the necessary combination of simple procedure, sensitivity and specificity. Thus, the introduction of the PCR verification method can reduce the use of Southern blotting in diagnostic studies for verification of genomic sites of integrated plasmid.

As shown in Fig. 2a, the genome of S. meliloti uidA reporter strain contains the vector DNA. Because the vector DNA has not affected any known genes for the symbiosis and does not appear to affect the growth of the bacteria, the symbiotic behaviors of the reporter strain are as normal as its parental strain. When one wishes to use pVMG to make a chromosomal uidA fusion while avoiding the vector DNA, one possibility becomes apparent. It is possible to use trpR encoded resolvase [36] to excise the vector DNA from the genome. The resolvase catalyzes excision of the DNA flanked by short DNAs called res sequences. pVMG (Fig. 1a) has a unique HindIII site upstream of the MCS and a unique Bgl II site downstream of uidA, allowing insertion of the res sequences for the resolvase to function. Similar types of work have been successfully carried out in S. meliloti for other target region excision purposes and a version of pVMG carrying a inducible trpR exists [3]. Other techniques might be of use. In CRISPR-Cas9, for example, the class of RNA-guided endonucleases known as Cas9 from the microbial adaptive immune system CRISPR (clustered regularly interspaced short palindromic repeats) can be targeted to virtually any genomic location of choice by a short RNA guide [37]. With two such guides, a CRISPR-Cas9 system has generated target excisions in the genomes of bacteria Streptococcus pneumoniae and E. coli [38]. Given the results of work with those bacteria, CRISPR-Cas9 might be of use to excise the vector DNA from the genome of S. meliloti.

In addition to the generation of transcriptional uidA fusions in a DNA region from S. meliloti, we have been using pVMG for generating genome-wide transcriptional uidA fusions in the bacterium. We have cloned a library of the S. meliloti DNA fragments to the BamHI site of pVMG and screened the resulting strains for the sinl-regulated activity of transcription in the late stages of symbiosis. Preliminary studies of transcription of S. meliloti genes linked expression of some of these genes to the activity of the sinl gene (unpublished results). In fact, the analogous plasmid pVO196 [1] has already been successfully used for generating a library of S. meliloti transcriptional fusions to a promoterless copy of bacA gene for discovering activity of the rhizobial genes in the intermediate stages of symbiosis. According to our preliminary studies and the published study, it is likely that pVMG will be useful for the in planta bacterial transcriptome studies.

Conclusions

A simple method has been developed for making chromosomal uidA transcriptional reporter fusions in S. meliloti. The method is based on the narrow-host-range, high copy number, transcriptional uidA fusion selective pVMG for the effectiveness of the fusion construction. The fusions are verified by a simple colony PCR and a PCR product could be used to identify the fusion site by direct DNA sequencing. The method is successfully used in the model rhizobia S. meliloti. The method is also applicable to many other rhizobia stains, but it is not applicable to those that are resistant to both neomycin and ampicillin.

Additional Files

**Additional File 1:** The DNA sequence of the 720-bp PCR product.

**Additional File 2:** Southern blot of DNAs of S. meliloti strains.
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Availability of Data and Materials
Not applicable.

Authors' Contributions
MG designed this project and analyzed data. MG and TW wrote the manuscript. MG, AB, TW, and RJ conducted the experiments. All authors reviewed and approved the final manuscript.

Ethics Approval and Consent to Participate
reviewed and approved the final manuscript.

Consent for Publication
Not applicable.

Competing Interests
The authors declare that they have no competing interests.

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