β-Glucuronidase (GUS) transposons for ecological and genetic studies of rhizobia and other Gram-negative bacteria

Kate J. Wilson,¹ Angela Sessitsch,² Joseph C. Corbo,²† Ken E. Giller,³ Antoon D. L. Akkermans⁴ and Richard A. Jefferson¹

Author for correspondence: Kate J. Wilson. Tel: +61 6 246 5302. Fax: +61 6 246 5303.
e-mail: wilson@cambia.org.au

A series of transposons are described which contain the gusA gene, encoding β-glucuronidase (GUS), expressed from a variety of promoters, both regulated and constitutive. The regulated promoters include the tac promoter which can be induced by IPTG, and nifH promoters which are symbiotically activated in legume nodules. One transposon contains gusA with a strong Shine-Dalgarno translation initiation context, but no promoter, and thus acts as a promoter-probe transposon. In addition, a gus operon deletion strain of Escherichia coli, and a transposon designed for use in chromosomal mapping using PFGE, are described. The GUS transposons are constructed in a mini-Tn5 system which can be transferred to Gram-negative bacteria by conjugation, and will form stable genomic insertions. Due to the absence of GUS activity in plants and many bacteria of economic importance, these transposons constitute powerful new tools for studying the ecology and population biology of bacteria in the environment and in association with plants, as well as for studies of the fundamental molecular basis of such interactions. The variety of assays available for GUS enable both quantitative assays and spatial localization of marked bacteria to be carried out.

Keywords: GUS transposons, microbial ecology, rhizobial competition, rhizosphere colonization

INTRODUCTION

Reporter genes are powerful molecular biological tools with a diversity of applications. They may be used to substitute for a structural gene-of-interest and hence to report on regulation of gene expression through creation of a gene fusion. They are used in microbial ecology to facilitate the detection of individual marked strains of bacteria (Drahos, 1991; Wilson, 1995). Additionally they can be used to report on properties of the surrounding environment, e.g. bioavailability of phosphate (de Weger et al., 1994) or naphthalene (Heitzer et al., 1992).

The key advantage of reporter genes as tools in microbial ecology is that they enable closely related strains of bacteria to be readily distinguished, and provide a rapid means of identifying the strain of interest (Wilson, 1995). The extent to which these advantages are realized depends largely on the properties of the reporter gene used. To date, reporter genes used as markers for Gram-negative bacteria in microbial ecology have included lacZ, encoding β-galactosidase, the xylE gene, encoding catechol 2,3-dioxygenase, and the different sets of luciferase genes—the bacterial luxAB genes or the luc gene from fireflies. Each has different advantages and limitations (reviewed in Drahos, 1991; Wilson, 1995).

The gus A gene, encoding β-glucuronidase (GUS), is the most widely used reporter gene in plant molecular biology (Jefferson et al., 1987). It has the major advantages that there is no background activity in plants, and the wide variety of GUS substrates available enable both quantitative assays and spatial localization of reporter gene activity (Jefferson, 1987). Although the gus A gene was
isolated from *Escherichia coli* (Jefferson et al., 1986), GUS activity is not found in many bacteria of economic and agricultural importance, including *Rhizobium*, *Bradyrhizobium*, *Agrobacterium*, *Pseudomonas* and *Azospirillum* species (Wilson et al., 1992) nor in many fungi, including *Saccharomyces*, *Schizosaccharomyces*, *Aspergillus*, *Neurospora* or *Ustilago*. In addition to its widespread use in plant molecular biology, *gusA* is therefore also of great utility as a reporter gene in microbes.

To date, use of *gusA* as a marker gene in bacteria has been largely restricted to analysis of regulation of gene expression (Sharma et al., 1994) or in fungi, including *Rhizobium* or *Xanthomonas*, *Emericella* and *Pseudomonas*. Use of *gusA* as a marker gene in microorganisms has been largely restricted to analysis of regulation of gene expression (Sharma et al., 1994) or in fungi, including *Rhizobium* or *Xanthomonas*, *Emericella* and *Pseudomonas*. Use of *gusA* as a marker gene in bacteria has been largely restricted to analysis of regulation of gene expression (Sharma et al., 1994) or in fungi, including *Rhizobium* or *Xanthomonas*, *Emericella* and *Pseudomonas*.

**METHODS**

**Bacterial strains, plasmids and media.** Bacterial strains are given in Table 1 and plasmids are given in Table 2. Media used for growth of *E. coli* were: LB (Miller, 1972) supplemented as appropriate with ampicillin (50 µg ml⁻¹), tetracycline (10 µg ml⁻¹), kanamycin (50 µg ml⁻¹), spectinomycin (50 µg ml⁻¹), X-GlcA (5-bromo-4-chloro-3-indolyl-β-d-glucuronide; 50 µg ml⁻¹), X-Gal (20 µg ml⁻¹) or IPTG (100 µM). Minimal medium for growth of *E. coli* was M9 salts (I⁻¹: 3 g Na₂HPO₄, 1·5 g KH₂PO₄, 0·5 g NH₄Cl, 0·25 g NaCl) with 0·2% glucose, 0·2% casamino acids, 1 mM MgSO₄, 7H₂O, 0·5 µg thiamine hydrochloride ml⁻¹. For growth of strain KW1, minimal medium was supplemented with 15 µg hypoxanthine ml⁻¹ and 15 µg adenine ml⁻¹. Agar was added to 1·5% (w/v) for solid media.

*Rhizobia* were grown in yeast-mannitol (YM) medium (Vincent, 1970) or in modified minimal BD medium (Brown & Dilworth, 1975) which contains (I⁻¹): 0·7 g KNO₃, 0·25 g MgSO₄·7H₂O, 0·02 g CaCl₂, 0·2 g NaCl, 0·36 g KH₂PO₄, 1·4 g K₂HPO₄, 0·02 g MgCl₂, 0·15 mg EDTA, thiamine HCl (1 µg ml⁻¹), biotin (1 ng ml⁻¹), calcium pantothenate (2 µg ml⁻¹), and glucose or glycerol 0·2% (w/v) as carbon source.

**Construction of *E. coli* strain KW1.** Two successive phage P1 transductions (Miller, 1972) were used to convert *E. coli* strain SO200 to an *bsdR* genotype. First, strain SO200 was infected with a P1 lysate of *E. coli* strain TPC48 and colonies that grew on LB/tet plates at 32 °C were checked for temperature-sensitivity due to co-transduction of the temperature-sensitive *dnaC235* allele with the tetracycline resistance marker from transposon Tn10. One such derivative was infected with a second P1 lysate and cloned on strain K802 and transductants that regained the ability to grow at 37 °C were shown to be tetracycline sensitive, indicating replacement of the region containing the Tn10 with the corresponding region from strain K802.

Isolates were checked for acquisition of the linked *bsdR* genotype from strain K802 by examining the efficiency of transformation with *pUC18* DNA prepared from strain DH5α (rK⁻ mK⁺) and from strain NM522 (rK⁻ mK⁻). The efficiency of transformation of strain SO200 was three orders of magnitude higher with DNA prepared from strain DH5α than with DNA.

**Table 1. Bacterial strains**

<table>
<thead>
<tr>
<th>Strain</th>
<th>Relevant characteristics</th>
<th>Source/reference</th>
</tr>
</thead>
<tbody>
<tr>
<td><em>E. coli</em></td>
<td>endA1 bsdR17 (rK⁻ mK⁺) supE44 thi-1 recA1 gyrA96 (Nal') relA1 Δ(lacZYA-argF) U169 Φ80d lac(lacZ)M15</td>
<td>Woodcock et al. (1989)</td>
</tr>
<tr>
<td>SO200</td>
<td>metB strA purB Δ(add-gus-man)</td>
<td>Jochimsen et al. (1975)</td>
</tr>
<tr>
<td>K802</td>
<td>bsdR⁻ bsdM⁻ gal− met− supE merA− mcrB−</td>
<td>Noreen Murray, University of Edinburgh, Edinburgh, UK</td>
</tr>
<tr>
<td>TPC48</td>
<td>dnaC 325 Zij::Tn10</td>
<td>Millie Masters/Noreen Murray, University of Edinburgh, Edinburgh, UK</td>
</tr>
<tr>
<td>NM522</td>
<td>F' lacP3 Δ(lacZ)M15 proA1 B' supE thi Δ(lac-proAB) Δ(bsdMS− mcrB−) (rK⁻ mK− MbcBC)</td>
<td>Woodcock et al. (1989)</td>
</tr>
<tr>
<td>KW1</td>
<td>metB strA purB Δ(add-gus-man) bsdR⁻ bsdM⁺</td>
<td>This work</td>
</tr>
<tr>
<td>S17-1</td>
<td>thi pro bsdR⁺ bsdM⁺ recA RP4 2- Tc::Mu-Km::Tn7(Tp' Sn')</td>
<td>Simon et al. (1983)</td>
</tr>
<tr>
<td>S17-1 λ-pir</td>
<td>λ-pir lysogen of S17-1</td>
<td>Victor de Lorenzo, Centro de Investigaciones Biologicas, Madrid, Spain</td>
</tr>
<tr>
<td><em>Rhizobium</em></td>
<td>R. tropici; nodulates <em>P. vulgaris</em> and <em>Leucaena leucocephala</em></td>
<td>Trinick (1980)</td>
</tr>
<tr>
<td>NGR234</td>
<td><em>Rhizobium</em> sp.; nodulates broad range of tropical legumes</td>
<td>Martinez-Romero et al. (1991)</td>
</tr>
<tr>
<td>CIAT 899</td>
<td><em>Rhizobium</em> sp.; nodulates broad range of tropical legumes</td>
<td>Trinick (1980)</td>
</tr>
</tbody>
</table>
Table 2. Plasmids used or constructed during this work (intermediate plasmids are not shown)

<table>
<thead>
<tr>
<th>Name</th>
<th>Relevant characteristics</th>
<th>Reference/source</th>
</tr>
</thead>
<tbody>
<tr>
<td>pUT/mini-Tn5 Sm/Sp</td>
<td>Ap, Sm, Sp; mini-Tn5 encoding Sm’/Sp’ with a unique NotI site for insertion of cloned fragments on broad-host-range suicide plasmid.</td>
<td>de Lorenzo et al. (1990)</td>
</tr>
<tr>
<td>pUC18Not</td>
<td>Ap; pUC18 derivative with NotI sites flanking the polylinker</td>
<td>Herrero et al. (1990)</td>
</tr>
<tr>
<td>pWM74</td>
<td>Ap; 1·2 kb lacF gene as an EcoRI fragment</td>
<td>W. Margolin, Stanford University, CA, USA</td>
</tr>
<tr>
<td>pCQ15</td>
<td>niFH from R. etli strain CFN42</td>
<td>Quinto et al. (1985)</td>
</tr>
<tr>
<td>pBN370</td>
<td>Ap; 2·8 kb HindIII fragment in pBR322 containing niFH from Bradyrhizobium sp. (Parasponia) strain Rp501</td>
<td>B. Tracy Nixon, Pennsylvania State University, PA, USA</td>
</tr>
<tr>
<td>pBKuidA</td>
<td>Ap; Tc; 6·5 kb EcoRI–HindIII insert in pBR325 containing gusABC and downstream convergently transcribed ORF</td>
<td>Jefferson et al. (1986)</td>
</tr>
<tr>
<td>pTTQ18</td>
<td>Ap; high copy vector with the tac promoter and lacF enabling regulated expression of cloned genes</td>
<td>Stark (1987)</td>
</tr>
<tr>
<td>pBI101.1</td>
<td>Km; gusA plus nos polyA site in pBIN19; reading frame 1</td>
<td>Jefferson et al. (1987)</td>
</tr>
<tr>
<td>pBI101.2</td>
<td>Km; gusA plus nos polyA site in pBIN19; reading frame 2</td>
<td>Jefferson (1987)</td>
</tr>
<tr>
<td>pBI101.3</td>
<td>Km; gusA plus nos polyA site in pBIN19; reading frame 3</td>
<td>Jefferson (1987)</td>
</tr>
<tr>
<td>pRAJ289</td>
<td>Ap; 6·2 kb insert in pTTQ18 containing promoterless gusABC and downstream convergently transcribed ORF</td>
<td>This work</td>
</tr>
<tr>
<td>pRAJ294</td>
<td>Ap; promoterless gusA gene with bacterial Shine–Dalgarno sequence in pTTQ18</td>
<td>This work</td>
</tr>
<tr>
<td>pTacter</td>
<td>Ap; tac promoter and trpA terminator flanking unique cloning sites in pUC8</td>
<td>This work</td>
</tr>
<tr>
<td>pKW28</td>
<td>Ap, Km; Tn5-containing EcoRI fragment from a Bradyrhizobium mutant in pUC13</td>
<td>Wilson (1987)</td>
</tr>
<tr>
<td>pKW106</td>
<td>Ap; 2·3 kb Papb–gusA–ter HindIII cassette in pUC13</td>
<td></td>
</tr>
<tr>
<td>pKW117</td>
<td>Ap; gusA plus trpA ter for translational fusions: reading frame 1 in pTacter</td>
<td></td>
</tr>
<tr>
<td>pKW118</td>
<td>Ap; gusA plus trpA ter for translational fusions: reading frame 2 in pTacter</td>
<td></td>
</tr>
<tr>
<td>pKW119</td>
<td>Ap; gusA plus trpA ter for translational fusions: reading frame 3 in pTacter</td>
<td></td>
</tr>
<tr>
<td>pKW120</td>
<td>Ap; promoterless gusA fragment from pRAJ294 in pUC18Not</td>
<td></td>
</tr>
<tr>
<td>pKW121</td>
<td>Ap; 2·4 kb XhoI–Srl fragment from pKW111 with Papb–gusA–ter cassette and adjacent SdrI site in pUC18Not</td>
<td></td>
</tr>
<tr>
<td>pJC63</td>
<td>Ap; 1·2 kb EcoRI lacF fragment in pUC18Not</td>
<td></td>
</tr>
<tr>
<td>pJC64</td>
<td>Ap; 2·2 kb PtaC–gusA–ter fragment plus 1·2 kb EcoRI lacI fragment in pUC18Not</td>
<td></td>
</tr>
<tr>
<td>pJC66</td>
<td>Ap; 2·2 kb PtaC–gusA–ter fragment in pUC18Not</td>
<td></td>
</tr>
<tr>
<td>pJC67</td>
<td>Ap; 2·3 kb Papb–gusA–ter cassette from pKW106 in pUC18Not</td>
<td></td>
</tr>
<tr>
<td>pAS12</td>
<td>Ap; R. etli niFH–gusA translational fusion in pUC18Not with ~ 1 kb of upstream sequence including the UAS</td>
<td>This work</td>
</tr>
<tr>
<td>pAS22</td>
<td>Ap; Bradyrhizobium niFH–gusA translational fusion in pUC18Not with the niFH promoter but no UAS</td>
<td></td>
</tr>
<tr>
<td>pCAM110</td>
<td>Sm/Sp, Ap; mTn5SSgusA10 (PtaC–gusA–trpA ter translational fusion and lacF gene) in pUT/mini-Tn5 Sm/Sp</td>
<td></td>
</tr>
<tr>
<td>pCAM111</td>
<td>Sm/Sp, Ap; mTn5SSgusA11 (PtaC–gusA–trpA ter translational fusion) in pUT/mini-Tn5 Sm/Sp</td>
<td></td>
</tr>
<tr>
<td>pCAM120</td>
<td>Sm/Sp, Ap; mTn5SSgusA20 (Papb–gusA–trpA ter translational fusion) in pUT/mini-Tn5 Sm/Sp</td>
<td></td>
</tr>
<tr>
<td>pCAM121</td>
<td>Sm/Sp, Ap; mTn5SSgusA21 (Papb–gusA–trpA ter translational fusion with adjacent unique Sdrl site) in pUT/mini-Tn5 Sm/Sp</td>
<td></td>
</tr>
<tr>
<td>pCAM130</td>
<td>Sm/Sp, Ap; mTn5SSgusA30 (R. etli niFH–gusA–trpA ter translational fusion) in pUT/mini-Tn5 Sm/Sp</td>
<td></td>
</tr>
<tr>
<td>pCAM131</td>
<td>Sm/Sp, Ap; mTn5SSgusA31 (Bradyrhizobium niFH–gusA–trpA ter translational fusion) in pUT/mini-Tn5 Sm/Sp</td>
<td></td>
</tr>
<tr>
<td>pCAM140</td>
<td>Sm/Sp, Ap; mTn5SSgusA40 (promoterless gusA for translational fusions) in pUT/mini-Tn5 Sm/Sp</td>
<td></td>
</tr>
</tbody>
</table>
prepared from strain NM522, due to restriction of the unmodified DNA prepared from strain NM522. By contrast, equal transformation efficiencies of both modified and unmodified DNA were obtained in the isolates derived from the two successive P1 transductions, indicating co-transduction of the hsdR marker with the wild-type dnaC gene. One of these isolates was named strain KW1. The physical absence of the gus operon in strain KW1 was confirmed by Southern hybridization analysis using a 6.2 kb EcoRI-HindIII fragment from pRAJ289 containing gusABC as a probe.

Strain KW1 was used as the recipient strain in all subsequent DNA manipulations involving the gusA gene (except for manipulations involving pUT/mini-Tn5 Sm/Sp and derivatives, which had to be carried out in a λ-pir lysogen), as a presence of a gusA insert could be unambiguously detected by formation of blue colonies on media containing 50 μg ml⁻¹ X-GlcA.

DNA manipulations. Routine DNA manipulations were carried out as described by Wilson et al. (1994). Restriction digestions were performed according to manufacturers’ instructions and, where appropriate, sticky ends were blunted using the Klenow fragment of DNA polymerase I or T4 polymerase. Oligonucleotides were from Pharmacia LKB. DNA amplification was done on a Corbett FTS-1 thermocycler. The buffer was 50 mM KCl, 10 mM Tris/HCl, pH 8.4, 200 μM dNTPs, 1.5 mM MgCl₂, 1 μM primers. Tag polymerase was from Perkin Elmer. The amplification programme used was: (95 °C, 1 min) × 1, (95 °C, 10 s; 55 °C, 20 s; 72 °C, 1 min) × 30.

Construction of general plasmids of use in GUS expression constructs

(i) pRAJ289. To create a plasmid containing the entire gus (formerly uid) operon under the control of a regulatable vector promoter, gusABC plus 1.8 kb downstream sequence were obtained from pBKuidA on two fragments, a 539 bp HincII-BamHI fragment lacking any promoter sequences but containing the rest of the operon and downstream sequences. These two fragments were combined in SmaI-HindIII-digested pTCTC placing the operon under control of the tac promoter in the vector. The resulting construct was digested with EcoRI and KpnI, blunt-ended and re-cloned, thus regenerating the EcoRI site but eliminating the KpnI site to form pRAJ289.

(ii) pRAJ294. To create a derivative containing gusA on its own, pRAJ289 was digested with AatII which cleaves 49 bp downstream of the gusA terminator codon (inside gusB), blunt-ended and a HindIII linker (CAAGCTTG, New England Biolabs) was added. The remaining gusABC and downstream sequences were then eliminated by digestion with HindIII and ligation of the linker and 3' polynucleotidate HindIII sites to form pRAJ294.

(iii) pTacter. A 350 bp EcoRI-BamHI fragment containing the tac promoter from pDR540 was inserted into pUC8 to create pUCTac. The trpA terminator was then added by attaching Ncol linkers (AGATGCACTCT, New England Biolabs) to the trpA transcription terminator GenBlock (AGCCCGGCTAAT-GAGCCGGGCTTTTTTTTTTT, Pharmacia), cleaving with Ncol and then inserting this fragment into the SalI site of pUCTac to create pTacter.

(iv) pKW117, pKW118 and pKW119. The upstream polynucleotidate and gusA gene (without the 3' nos polyadenylation site) was removed from the gusA translational fusion vectors pBI101.1, pBI101.2 and pBI101.3, respectively, as 1.9 kb PstI-SstI fragments which were blunted and inserted into the blunt-ended SalI site of pTacter.

Construction of plasmids and transposons with constitutive Paph-gusA fusions. These constructs contain the promoter sequences from the aph gene from Tn5 driving an aph-gusA translational fusion.

(i) pKW106. To construct this fusion, the aph gene was first isolated as a 1.8 kb HindIII-XbaI fragment from pKW28 and inserted into HindIII/SalI-digested pUC13 to give pKW101. To create a fusion to gusA, pKW101 was digested with EagI which cleaves at nucleotide 35 of the aph gene, blunted, and subsequently digested with S Aval prior to inserting a 1.9 kb SmaI-SalI gusA fragment from pBI101.3 to make a translational fusion with aph in pKW102. The aph-gusA fusion from pKW102 was then inserted as a blunt-ended 2.3 kb HindIII-SalI fragment into the blunt-ended SalI site of pTacter. In the resultant plasmid, pKW103, a HindIII site was regenerated at the 5' end and a SalI site at the 3’ end of the insert, with the trpA transcriptional terminator downstream of gusA. To separate this Paph-gusA-ter cassette from the tac promoter in pTacter, the whole cassette was inserted into pUC13 as a 2.3 kb HindIII fragment to create pKW106.

(ii) mTn55gusA20. The 2.3 kb aph-gusA-ter HindIII cassette from pKW106 was inserted into pUC18Not to create pJC67, and the resulting 2.4 kb Ncol cassette was inserted into pUT/mini-Tn5 Sm/Sp to create pCAM120 containing mTn55gusA20.

(iii) mTn55gusA21. This transposon was created to contain a unique SpeI site. The 2.3 kb HindIII cassette from pKW106 was inserted into the blunt-ended Ncol site in pCAM120 containing mTn55gusA21. To clone this cassette plus adjacent SpeI site into pKW111, this was digested with XbaI and SalI and the 2.4 kb Paph-gusA-ter SpeI cassette plus adjacent SpeI site was inserted into SalI-SalI digested pUC18Not, creating pKW121. Finally, pCAM121 containing mTn55gusA21 was constructed by cloning the Ncol cassette from pKW121 into NotI-digested pUT/mini-Tn5 Sm/Sp. It should be noted that there are three Ncol sites in pKW121, and that one of the sites bounding the NotI cassette in mTn55gusA21 therefore derives from the pBluescript SKII(+) polynucleotidate, which contains an adjacent SpeI site, to create pKW111. This was digested with XbaI and SalI and the 2.4 kb Paph-gusA-ter cassette plus adjacent SpeI site was inserted into SalI-SalI digested pUC18Not, creating pKW121. Finally, pCAM121 containing mTn55gusA21 was constructed by cloning the Ncol cassette from pKW121 into NotI-digested pUT/mini-Tn5 Sm/Sp. It should be noted that there are three Ncol sites in pKW121, and that one of the sites bounding the NotI cassette in mTn55gusA21 therefore derives from the pBluescript SKII(+) polynucleotidate.

Construction of gusA transposons using the regulatable tac promoter

(i) mTn55gusA10. The promoterless gusA gene from pRAJ294 was inserted as a 1.9 kb blunt-ended EcoRI-HindIII fragment into the HindIII site of pTacter to create pKW104. The resulting 2 kb HindIII cassette (PTac-gusA-ter) was inserted into the HindIII site of pJC63, which contains the lacI gene from pWM74 as a 1.2 kb EcoRI fragment in pUC8Not, to create pJC64. Ptac-gusA-ter plus lacI was then moved as a 3.3 kb Ncol fragment into pUT/mini-Tn5 Sm/Sp to create pCAM110 containing mTn55gusA10.

(ii) mTn55gusA11. A transposon with gusA driven consti-
A gene was oriented so that it would be transcribed from the assays, 1-5 ml of a mid-exponential phase culture was counted using the Miles count method, prior to carrying out quantitative GUS assays inducible by IPTG (strains marked with mTn5SSgusA). The primer was also used to confirm junctions of translational with WIL2 downstream of the ATG codon) 5` GAATGCCCACAG-CTGACTCTTATACACAAGTGC 3`; WIL2 (homologous to the interposon) 5` GCTCAATCAATCACCGGATCC 3`; and was inserted into pUT/mini-Tn5 Sm/Sp. A clone in which the pCAM140, containing mTn5SSgusA40. pUCl8Not forming pKW120. The resulting 2 kb NotI fragment was inserted into pUT/mini-Tn5 Sm/Sp to create pCAM130 containing mTnSSgusA30.

Construction of a promoter-probe transposon pUT/mTnSSgusA40. The promoterless gusA gene from pRAJ294 was inserted as a 1.9 kb EcoRI-HindIII fragment into pUC18Not to create pAS12. This mTn5SSgusA fusion was cloned as a 2.3 kb NotI fragment into pUT mini-Tn Sm/Sp to create pCAM131 containing mTnSSgusA30. mTnSSgusA31. To create a Bradyrhizobiun mTn5SSgusA fusion, the upstream 190 bp and the first 22 codons (66 bp) of Bradyrhizobium sp. (Parasponia) mTn5 were cloned from pBN370 into pUC18Not as a 256 bp HindIII-SalI fragment to create pAS21. To make a translational fusion to gusA, pAS21 was digested with SalI, blunt-ended, and a 20 kb SalI-HindIII-digested, blunt-ended fragment from pKW119 containing gusA with the trpA terminator was inserted, creating pAS12. The resulting mTn5SSgusA fusion was cloned as a 2.3 kb NotI fragment into pUT mini-Tn Sm/Sp to create pCAM131 containing mTnSSgusA31.

Determination of orientation of gusA in mini-transposons. The orientation of gusA inserts in the transposons was determined by PCR using the following primers: WIL1 (homologous to the right hand, outside (O) end of mini-Tn5, plus an additional 2 bp from the adjacent NotI site) 5` CTGACTTCTATACACCGATGC 3`; WIL2 (homologous to the region between the BamHI and HindIII sites flanking the interposon) 5` GCTCAATCAATCACCGGATCC 3`; and WIL3 (homologous to the non-coding strand of gusA, 40 nt downstream of the ATG codon) 5` GAATGCCCACAG-GCCCTGAG 3`. A DNA amplification product with WIL1 + WIL3 indicated that the gusA gene was oriented such that gusA was transcribed into the transposon from the O-end, as in mTnSSgusA40. Conversely, an amplification product with WIL2 + WIL3 indicated the reverse orientation. The WIL3 primer was also used to confirm junctions of translational fusions by DNA sequencing.

Quantitative GUS assays. GUS-marked bacteria were grown to mid-exponential phase in YM. Where gusA expression was inducible by IPTG (strains marked with mTnSSgusA10), duplicate cultures were set up, one containing 2 mM IPTG. For the assays, 1-5 ml of a mid-exponential phase culture was centrifuged and the pellet resuspended in 1 ml 50 mM NaPO4, pH 7.0, 1 mM EDTA. Serial dilutions were made for viable cell counts using the Miles & Misra (Collins & Lyne, 1985) drop count method, prior to carrying out quantitative GUS assays using p-nitrophenyl glucuronide (pNPG) as described by Wilson et al. (1992).

Growth and inoculation of plants. Frozen rhizobial inoculum was prepared, and plants were grown in 1:1 sand:vermiculite and watered with nitrogen-free Bergersen's medium as described by Wilson et al. (1987). Siratro (Macroptilium atropurpureum) seedlings were surface-sterilized and scarified by treatment in concentrated H2SO4 for 12 min prior to extensive rinsing in sterile water; pigeonpea (Cajanus cajan, cultivar Quantum) and common bean (P. vulgaris) were surface-sterilized by immersion in 0.1% HgCl2 for 3 min, prior to rinsing in sterile water. Seeds were inoculated with approximately 105-106 rhizobial cells per seed.

Staining for GUS activity. GUS assay buffers were based on the standard phosphate buffer (Jefferson, 1987) containing 50 mM NaPO4, pH 7.0, 1 mM EDTA, 0.1% Sarkosyl, 0.1% Triton X-100. X-GlC A (generally used at 100 μg ml-1) was from Biosynth. Following staining, roots were cleared using 50% (v/v) household bleach (2.5% final concentration hypochlorite) for 30 min, followed by extensive washing with deionized water.

Results

A restriction GUS E. coli K12 host strain

The gusA gene is derived from E. coli, and E. coli K12 isolates routinely used in the laboratory exhibit low-level GUS activity. This can cause problems when screening for GUS-expressing plasmids. An E. coli K12 strain deleted for the entire gus operon, SO200 (Jochimsen et al., 1975), was available, but it retained the EcoK restriction and modification activities (hsdR*, hsdM*). We therefore converted it to a restriction genotype by phage P1 transduction (Miller, 1972), generating strain KW1. Plasmids expressing even low-level GUS activity can be unambiguously detected in strain KW1 by formation of blue colonies on medium containing 50 μg ml-1 X-GlC A.

Plasmids useful for genetic manipulation of GUS in bacteria

Promoterless GUS constructs. For ease of construction of gus cassettes under the control of different promoters, two plasmids containing gusABC (pRAJ289) and gusA only (pRAJ294) were constructed (Fig. 1a, b). In these plasmids the inserts contain the original strong Shine-Dalgarno sequence from the gus operon, but are under control of the vector promoter. pRAJ294 is therefore an excellent plasmid for high-level expression of GUS.

Translational fusion vectors. pKW117, pKW118 and pKW119 allow construction of translational fusions to gusA in all three reading frames (Fig. 1c), with a strong bacterial transcriptional terminator downstream, followed by a convenient HindIII site. They parallel the widely-used translational fusion vectors pBI101.1, pBI101.2 and pBI101.3, which contain the eukaryotic nos polyadenylation site 3` to gusA.

pTacter. pTacter enables cloning of promoterless genes under the control of the tac promoter and with a strong transcriptional terminator downstream, and the subsequent removal of the entire expression cassette as a single HindIII fragment (Fig. 1d).
Fig. 1. Plasmids useful for genetic manipulation of gusA in bacteria. (a) pRAJ289 contains the gus operon from E. coli K12 under the control of the tac promoter in pTQ18. gusA encodes β-glucuronidase, gusB encodes the glucuronide permease and gusC is a membrane-associated protein of unknown function (Jefferson et al., 1987; Liang, 1992; Wilson et al., 1992). The construct also includes an ORF of unknown function which is oriented in the opposite direction (Liang, 1992). The GenBank accession number for the entire insert is M14641. (b) pRAJ294 contains gusA under the control of the tac promoter in pTQ18. The upstream sequence containing the original strong Shine-Dalgarno ribosome-binding site (RBS) from the gus operon is indicated on pRAJ294. (c) Translational fusion vectors. pKW117, pKW118 and pKW119 allow construction of translational fusions to gusA with the trpA bacterial transcriptional terminator downstream. All three sites indicated can be used to construct translational fusions to gusA by choosing the vector with the appropriate reading frame as indicated. (d) pTacter. The sequence of the cassette containing the tac promoter and the trpA transcriptional terminator is shown. The entire cassette is bounded by HindIII sites as indicated and is in a pUC8 backbone.

Construction of gusA-expressing transposons

Transposons with constitutive GUS activity. A key aim was to construct a set of transposons that would be active in as wide a variety of Gram-negative bacteria as possible. It was therefore necessary to identify promoters that could be used to direct transcription of gusA in diverse bacterial species. The first promoter selected was the aph promoter that drives transcription of the kanamycin resistance gene in Tn5. Tn5 mutagenesis has been shown to work in a
diversity of Gram-negative bacteria using kanamycin selection (de Bruijn & Lupski, 1984), and therefore the \( \text{aph} \) promoter must be active in all these bacterial species. This promoter has not been precisely mapped, but evidence indicates that it is influenced by sequences which lie 110 bp upstream of the translational start of the \( \text{aph} \) gene within IS\text{SO}L (Rothstein & Reznikoff, 1981). Thus, a region of Tn5 encompassing these upstream sequences and 33 bp of \( \text{aph} \) coding sequence was used to make a translational fusion of the \( \text{aph} \) gene with \( \text{gus} \). This fusion was inserted into \text{pUT1}/mini-Tn5 Sm\text{&Sp} to create \text{mTnSSgusA20} (Fig. 2a) which gives high-level constitutive expression of GUS in \text{Rhizobium} (Table 3). \text{mTnSSgusA21} is similar to \text{mTnSSgusA20}, except that a unique \( \text{SacI} \) site was incorporated adjacent to the \( \text{gus} \) gene (Fig. 2b).

Transposons with regulated \text{gus}A expression. \text{mTnSSgusA10} was constructed to enable regulation of \( \text{gus} \)A so that it is only expressed at high levels at the time of assay. This should reduce any metabolic load imposed by GUS expression. \text{mTnSSgusA10} contains the \( \text{lacI} \) gene and \( \text{gus} \)A under control of the \( \text{lac} \) promoter (Russell & Bennett, 1982), and therefore expression is repressed until the gratuitous inducer of the \( \text{lac} \) operon, IPTG, is added (Fig. 2c). In liquid culture, on addition of IPTG, expression of \( \text{gus} \)A from \text{mTnSSgusA10} was induced approximately 30-fold in \text{Rhizobium} sp. NGR234, and about 20-fold in \text{R. tropici} CIAT899 (Table 3). Derivatives of strains NGR234 and CIAT899 marked with \text{mTnSSgusA11}, which contains the same \( \text{Ptac-gusA} \)–ter cassette, but without the \( \text{lacI} \) gene, showed constitutive GUS activity slightly higher than that of induced \text{mTnSSgusA10} (Fig. 2d, Table 3).

Construction of symbiotically expressed \text{gus}A cassettes. To ensure strong expression of \( \text{gus} \)A under symbiotic conditions, translational fusions of \( \text{gus} \)A to \( \text{nifH} \) genes from both a \text{Rhizobium} and a \text{Bradyrhizobium} strain were made: \( \text{nifH} \) encodes the Fe-component of nitrogenase, and is expressed only in symbiotic or microaerobic conditions (Fischer, 1994). \text{mTnSSgusA30} contains more than 1 kb of upstream sequence from the \( \text{nifH} \) promoter of \text{R. etli} strain CFN42 (Fig. 2c), and thus includes both the RNA polymerase \( \sigma^4 \)-dependent promoter sequences, and the upstream activating sequence (UAS) that is typically located about 80–150 bp upstream of \( \text{nifH} \) start sites (Fischer, 1994). By contrast, the \( \text{nifH–gusA} \) fusion in \text{mTnSSgusA31} contains only about 50 bp of sequence upstream from the deduced \( \text{nifH} \) start site of \text{Bradyrhizobium} sp. (\text{Parasponia}) strain Rp501, and does not contain the UAS (Fig. 2f).

Promoter–probe transposon. Finally, a promoter–probe transposon, \text{mTnSSgusA40}, was constructed (Fig. 2g). In this transposon \( \text{gus} \)A lacks a promoter and is orientated such that it can be transcribed off adjacent promoters in the genomic DNA. In a test mating of \text{mTnSSgusA40} into \text{Rhizobium} sp. strain NGR234, expression of the \( \text{gus} \)A gene was found to vary widely from no activity, to activity as high as 190 nmol pNPG hydrolysed per min per 10\text{9} cells. This was reflected in the appearance of transconjugant colonies on plates containing 50 \( \mu \)g X-GlcA ml\textsuperscript{-1} which varied from white to deep blue.

Optimization of assay conditions for studying rhizobial infection and nodule occupancy

To determine the optimal staining conditions for studying root colonization and nodule occupancy with regard to sensitivity and cost, \text{Rhizobium} sp. strain NGR234, marked with all the GUS transposons (except for \text{mTnSSgusA21} and \text{mTnSSgusA40}), was inoculated onto siratro and pigeonpea; \text{R. tropici} CIAT899 marked with the same set of transposons was inoculated onto common bean. A basic histochemical GUS assay consists of immersing tissue in a substrate, generally X-GlcA, and looking for spatially restricted colour development (Jefferson, 1987). Factors that can be varied include the concentration of substrate, strategies to eliminate possible background activity from endogenous microbes, and the addition of oxidation catalysts.

Concentration of substrate. The efficiency of detection of nodule occupancy was investigated by using 50, 100, 250 and 500 \( \mu \)g X-GlcA ml\textsuperscript{-1} to stain nodules induced by strain NGR234::\text{gusA10} on 41-d-old siratro plants. After overnight incubation, nodules incubated in 500 \( \mu \)g ml\textsuperscript{-1} X-GlcA were well stained. Colour development was apparent in the other treatments as well, but concentration had a pronounced effect: nodules incubated in 50 \( \mu \)g X-GlcA ml\textsuperscript{-1} were only slightly blue, those in 100 \( \mu \)g X-GlcA ml\textsuperscript{-1} were mid-blue and those in 250 \( \mu \)g X-GlcA ml\textsuperscript{-1} were dark-blue. The plants were then left at room temperature in the substrate. It was apparent that colour development was continuing over several days, and after 1 week colour development was as strong in nodules that had been incubated in 100 \( \mu \)g X-GlcA ml\textsuperscript{-1}, as in those incubated in 500 \( \mu \)g X-GlcA ml\textsuperscript{-1}. It was only in the treatments that used 50 \( \mu \)g X-GlcA ml\textsuperscript{-1} that the nodule staining was less pronounced. We therefore concluded that 100 \( \mu \)g ml\textsuperscript{-1} was a suitable concentration for these assays. No blue colour was observed in the nodules of plants nodulated by the parental strain NGR234 even after 1 week’s incubation in the buffer.

GUS activity from other microbes. Another difference between treatments was the appearance of staining either on the surface or within the root. This staining occurred particularly where the shoot had been cut from the root and at sites of lateral root emergence, and was observed only in the 250 and 500 \( \mu \)g X-GlcA ml\textsuperscript{-1} treatments. Significantly, this staining was also observed on the roots of plants that were inoculated with the unmarked, parental strain NGR234 in which the nodules remained unstained.

In \text{E. coli}, and at least some other bacteria that possess GUS activity, GUS is induced only in the presence of particular concentrations of glucuronide substrates (Stoeber, 1961; Tör \textit{et al.}, 1992; Wilson \textit{et al.}, 1992). A possible explanation, therefore, was the presence of additional microbes with inducible GUS activity. To test this possibility, basic phosphate buffer containing 50 \( \mu \)g
Fig. 2. For legend see facing page.
X-GlcA ml⁻¹ was prepared and divided into four aliquots with the addition of respectively: nothing; 100 μg chloramphenicol ml⁻¹; 100 μg cycloheximide ml⁻¹; 100 μg chloramphenicol ml⁻¹ plus 100 μg cycloheximide ml⁻¹. Siratro plants (64-d-old, nodulated by parental strain NGR234, and by strain NGR234::gusA10) were harvested and incubated in each of these buffers.

Good staining was observed in the nodules of all treatments nodulated by strain NGR234::gusA10, and no staining was observed in nodules induced by parental strain NGR234. In chloramphenicol-containing buffers, no staining was observed on the surface of the roots of any plants. By contrast, after overnight incubation at 37 °C, there was substantial staining on the root surface of all plants incubated in the two sets of buffers that did not contain chloramphenicol (no addition, or plus cycloheximide only), including those nodulated by the unmarked parental strain NGR234.

**Oxidation of substrate.** One of the factors affecting the rate of development of blue product is the oxidative potential. The reaction that produces the indigo precipitate from X-GlcA occurs in two steps, the first step being hydrolysis of the substrate by GUS and the second step being oxidation of the product indoxyl that is released following GUS cleavage (Wilson, 1995). This could be a particular problem in nodules since the ambient oxygen concentration in an active nodule can drop from about 250 μM to less than 1 μM from the outer cortex to the inner bacteroid zone (Witty et al., 1987). We therefore examined the effect of adding oxidation catalysts to the buffer to see whether this would aid the development of blue colour. In fact the opposite effect was observed: the inclusion of 1 mM potassium ferricyanide or 1 mM potassium ferrocyanide, either separately or together, slightly decreased colour development in intact nodules.

**Clearing of the tissue.** The brown pigmentation present in roots and the red colour of leghaemoglobin in mature nodules can hinder the visualization of blue colour in nodules. We therefore tested various root-clearing protocols (Bevege, 1968; O'Brien & von Teichman, 1974), but found that simple room temperature treatment in bleach was equally effective and greatly facilitated visualization of blue-stained nodules.

**Use of the transposons to study the Rhizobium-legume interaction**

**Detection of marked bacteria in the rhizosphere.** A subset of the transposons, mTnSSgusA10, mTnSSgusA11, mTnSSgusA20 and mTnSSgusA21 enable expression of GUS in rhizobia in the free-living state, as well as in nodules. Following a histochemical GUS assay, dense areas of colonizing bacteria were visible as blue patches on the root surface and early stages of infection, including root hair colonization and infection and penetration of the root cortex could be readily visualized (Fig. 3a). To examine these early stages of infection, higher concentrations (150 μg ml⁻¹) of substrate were used and 1 mM potassium ferricyanide was included in the buffer.

Young nodules, including incipient nodules just emerging from the root cortex, could also be readily detected using these transposons (Fig. 3b). However, we found that older nodules induced by strains marked with these transposons could not be reliably identified using the X-GlcA assay. For example, siratro plants inoculated with Rhizobium strain NGR234 marked with mTnSSgusA11 or mTnSSgusA20, examined for nodule occupancy 42 d after inoculation, showed very variable results. On some plants all the nodules stained blue, whereas in others less than 50% of the nodules stained blue. The unstained nodules were almost certainly not due to cross-contamination as there were no nodules on any of the uninoculated plants.

The behaviour of mTnSSgusA10, in which gusA expression is regulated by the product of the lacI gene, differs from that of the transposons with constitutive gusA expression in free-living bacteria. On solid medium containing X-GlcA, blue colonies were formed by Rhizobium strains containing this transposon only in the presence of IPTG (1 mM), in contrast to strains marked with the other transposons in this group which did not require any inducer to form blue colonies on solid medium. When used to infect plants, minimal surface-

---

**Table 3. Expression of GUS transposons used for study of free-living bacteria in Rhizobium sp. NGR234 and R. tropici CIAT899**

<table>
<thead>
<tr>
<th>Transposon inserted</th>
<th>GUS activity (nmol pNPG hydolysed per min per 10⁹ viable cells) in:</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>IPTG</td>
</tr>
<tr>
<td>None</td>
<td>0</td>
</tr>
<tr>
<td>mTnSSgusA10</td>
<td>4.45±0.8</td>
</tr>
<tr>
<td>mTnSSgusA10</td>
<td>149±20</td>
</tr>
<tr>
<td>mTnSSgusA11</td>
<td>182±30</td>
</tr>
<tr>
<td>mTnSSgusA20</td>
<td>121±8</td>
</tr>
</tbody>
</table>

---

**Fig. 2. Restriction maps of GUS transposons.** mTnSSgusA10 expresses GUS in a regulated manner, dependent on induction by IPTG. mTnSSgusA11, mTnSSgusA20 and mTnSSgusA21 express GUS constitutively. mTnSSgusA21 is designed to be useful for mapping the site of transposon insertions using PFGE. mTnSSgusA30 and mTnSSgusA40 both have GUS expressed from a symbiotically active promoter. mTnSSgusA40 is a promoter–probe transposon. The antibiotic resistance cassette in each transposon is flanked by transcriptional and translational terminators. Note that there is an internal BamHI site as indicated in the gusA gene in a number of the transposons. The restriction maps refer specifically to the transposons, and do not take into account restriction sites present in the delivery plasmid, pU7.
Fig. 3. Examples of assays carried out using GUS-marked strains. (a) Early stages of infection of a siratro root infected with *Rhizobium* sp. NGR234 marked with mTnSSSgusA20. The photograph shows a root harvested 20 d after inoculation. For this type of localization, the roots were vacuum-infiltrated in buffer containing 150 μg X-GlCIA ml⁻¹ and 1 mM potassium ferricyanide, and then incubated at 37°C overnight. Roots were cleared using 2.5% hypochlorite prior to photography. (b) Nodules induced on a siratro root (20 d after inoculation) infected with *Rhizobium* sp. NGR234 marked with mTnSSSgusA11. Note surface staining and detection of very young nodules. (c) Adjacent pigeonpea nodules induced by *Rhizobium* sp. NGR234 and *Rhizobium* sp. NGR234: gusA31. Note the precise discrimination between nodules induced by a marked and an unmarked strain, and the absence of surface staining on the root. (d) Hand section through a pigeonpea nodule induced by *Rhizobium* sp. NGR234 marked with mTnSSSgusA11 showing expression is limited to the outer regions of the nodule. (e) Hand section through a pigeonpea nodule induced by *Rhizobium* sp. NGR234 marked with mTnSSSgusA31 showing expression is maximal in the central regions of the nodule. (f) Pigeonpea plant inoculated with *Rhizobium* sp. NGR234 and *Rhizobium* sp. NGR234: gusA31 in a ratio of 7:1. (g) Pigeonpea plant inoculated with *Rhizobium* sp. NGR234 and *Rhizobium* sp. NGR234: gusA31 in a ratio of 1:15.
staining was observed on roots inoculated with strains marked with mTnSSgusA10, and incubation in 1 mM IPTG for 5 h at 30 °C prior to the GUS assay had no effect on the degree of staining using this transposon. Surprisingly, siratro nodules occupied by strains marked with this transposon could be reliably detected at a later stage than those induced by strains marked with either mTnSSgusA11 or mTnSSgusA20; on siratro plants 42-d post-inoculation, all nodules induced by two independent isolates of strain NGR234::gusA10 stained blue in contrast to the results discussed above for mTnSSgusA11 or mTnSSgusA20.

**Use of transposons to determine nodule occupancy**

To obtain efficient staining in mature nitrogen-fixing nodules, two transposons with translational fusions of gusA to the nifH gene of R. etli strain CFN42 (mTnSSgusA30) and to that of Bradyrhizobium sp. (Parasponia) strain Rp501 (mTnSSgusA31) were used. In separate experiments, Bradyrhizobium sp. strain NGR234 marked with mTnSSgusA30 was inoculated onto siratro and pigeonpea, and R. tropici strain CIAT899 marked with both transposons was inoculated onto Phaseolus plants. Very deep blue staining was observed in all nodules induced by CIAT899::gusA30 and CIAT899::gusA31 on P. vulgaris up to 37 d after inoculation (date of final harvest). No difference in intensity of staining was observed between strains marked with the two transposons. Likewise, pigeonpea and siratro nodules induced by NGR234::gusA31 stained deeply even at a harvest date of 70 d after planting. No staining was observed on the root surface when using these transposons (Fig. 3c). Fig. 3(c) also indicates how precise the discrimination is between adjacent nodules occupied by a marked and an unmarked strain, even using X-GlcA buffer without inclusion of potassium ferricyanide or potassium ferrocyanide, and leaving the nodules in the staining buffer for 3 d prior to photography.

**Pattern of expression of transposons within nodules.** The different promoters used to drive gusA expression in the transposons might be expected to give different spatial patterns of activity in the nodule. This was examined by hand-sectioning pigeonpea nodules from roots harvested 26 d after planting and inoculation. Nodules induced by **Rhizobium** sp. NGR234::gusA31 showed strong GUS activity in the central, nitrogen-fixing zone of the nodule (Fig. 3d), as did nodules induced on **P. vulgaris** by CIAT899::gusA30. By contrast, nodules induced by **Rhizobium** sp. NGR234::gusA11 showed maximal expression in the peripheral area of the nodule, presumably where new infections are taking place (pigeonpea forms indeterminate nodules) (Fig. 3e). The latter pattern of expression is also observed in nodules induced by isolates marked with transposons mTnSSgusA10 and mTnSSgusA20.

**Time delay between harvest and assay.** If this assay is to be of practical use in field analysis it is important that initiation of the GUS assay can be delayed for several hours post-harvest to allow transport of nodulated root systems from the field to the laboratory. To examine this, staining of 42-d-old siratro plants inoculated with strains NGR234::gusA10, NGR234::gusA11 and NGR234::gusA20 was commenced at three different times after harvest. The first group were stained within 1 h of harvest, the second set 6 h after harvest, having been kept at ambient temperature (about 20 °C), and the final set was stained the following day after being kept at ambient temperature for 6 h, and then stored at 4 °C overnight. No correlation was observed between the time delay before staining and the percentage of nodules stained.

**Effect on symbiotic properties.** Dry shoot weights were measured at all harvest dates and no significant differences were observed between plants inoculated with the parental strains NGR234 or CIAT899 and the GUS-marked derivatives. This included pigeonpea and siratro plants harvested 70 d after planting. By contrast, non-nodulated plants showed yellow leaves and significantly reduced shoot weight compared to plants inoculated with the wild-type strains.

**Use of GUS as a marker in nodule occupancy competition assays.** A key aim is to use GUS as a marker in rhizobial competition studies. An experiment was set up in which the parental strain NGR234 was co-inoculated with NGR234::gusA31 in about 10:1, 1:1 or 1:10 ratios to demonstrate the principle of using these markers in competition assays. Independent nodules induced by marked versus unmarked strains could be discriminated very precisely (Fig. 3c), and the proportion of blue nodules increased with an increasing proportion of the GUS-marked strain in the inoculum. Following viable cell number counts at the time of inoculation, the actual ratios of inoculation of wild-type to GUS+ strains were found to be closer to 7:1, 1:1.5, and 1:15, and these gave rise respectively to 19, 80 and 86% blue nodules on pigeonpea, and to 0, 82 and 91% blue nodules on siratro (average of two plants assayed at final harvest) (Fig. 3f, g).

**DISCUSSION**

We describe here additional vectors useful for the molecular manipulation of the **gusA** gene (Fig. 1), and a series of transposons that express **gusA** from different promoters (Fig. 2). The transposons are designed primarily for use by microbial ecologists for measuring population changes in soil and in the rhizosphere and, in particular, as a tool for determining nodule occupancy in rhizobial competition studies.

Each of the different transposons is designed for a specific purpose. mTnSSgusA10 is primarily for studying populations of free-living bacteria, as **gusA** expression remains at a basal level until the addition of IPTG, when it shows strong induction both in liquid culture (Table 3) and as colonies on agar plates. The efficiency of regulation of the tac promoter by the lacZ product varies in different Gram-negative bacteria (Furste et al., 1986), and the extent of induction observed in **Rhizobium** sp. NGR234 and R. **tropic** CIAT899 (about 30-fold and 20-fold, respectively)
falls within the range observed in other species (10-200-fold induction; Fürste et al., 1986). This regulation should reduce possible effects on ecological fitness as high-level expression of the marker gene is not induced until the experimental assay is initiated. mTnSSgusA10 can also be used in nodule occupancy studies, but this may be due to basal expression as there is no obvious effect of addition of IPTG on the development of blue colour in nodules. This is unlikely to be a problem of IPTG penetration, as galactosides are not charged, in contrast to glucuronides, and therefore should pass through membranes more readily than the accompanying GUS substrates. The lack of apparent induction could be because the bacteria are not multiplying as rapidly as in free-living culture in rich medium, and are therefore unable to initiate high-level synthesis of new proteins as efficiently. Quantitative assays on nodule tissue using pNPG as substrate would be required to analyse this further.

The transposons mTnSSgusA11 and mTnSSgusA20 both give strong constitutive GUS expression in the free-living state (Table 3), and are optimal for studies of rhizosphere colonization (Fig. 3a). They can also be used for nodule occupancy studies in young plants (Fig. 3b). However, expression from these transposons declines in older nodules, and thus they are not optimal for long-term nodule occupancy experiments. This decline in expression in older nodules may be due in part to the temporal and spatial patterns of GUS expression conferred by these promoters, which appears strongest in the outer zones of pigeonpea nodules where undifferentiated bacteria may still be present, and reduced in the central nitrogen-fixing zone of the nodule (Fig. 3d). Similar spatial restriction of GUS expression has been obtained using an *R. leguminosarum* bv. *trifolii* strain marked with mTnSSgusA20 to infect subterranean clover (de Boer et al., 1995). It is known that expression of some genes is specifically repressed in bacteroids (e.g. de Maagd et al., 1994). However, further work would be needed to clarify whether this is the case here.

mTnSSgusA21 is similar to mTnSSgusA20, except that a unique *SpeI* site was incorporated adjacent to the *gusA* gene, to facilitate chromosomal mapping of insertions as *SpeI* is a rare-cutting enzyme in bacteria with high G+C contents, including rhizobia (Sobral et al., 1991). For example, *SpeI* was used in PFGE to analyse the symbiotic plasmid and facilitate ordering of an overlapping cosmid library in *Rhizobium* strain NGR234 (Perret et al., 1991). Since mTnSSgusA21 (and all the other mini-Tn5-based GUS transposons) also contains *NolI* sites, which are rare cutters in other species, the transposons could alternatively be used for chromosomal mapping of insertions with PFGE using this enzyme.

For longer-term nodule occupancy experiments, either of the two transposons, mTnSSgusA30 or mTnSSgusA31, containing *gusA* expressed from a symbiotic promoter, are recommended. Although these two transposons differ both in the origin of the *nifH* promoter and in the presence versus the absence of the NifA-dependent upstream activating sequence, no differences in GUS expression were apparent in symbiotic conditions using the histochemical assay on nodulated *P. vulgaris* plants. Hand sections revealed maximal expression of GUS in the central, nitrogen-fixing zone of active nodules as expected. The importance of the UASs on symbiotic, as opposed to microaerobic, activity of *nif* promoters is uncertain. In *B. japonicum*, deletion of UAS sequences from the *nifD* promoter reduced its activity to about 10% of that of the wild-type promoter in nodules (Alvarez-Morales et al., 1986), whereas in *R. meliloti*, a *nifH* promoter lacking the UAS still retained 50% of the wild-type symbiotic activity (Wang et al., 1991). Quantitative GUS assays on individual nodules are necessary to examine this further.

Finally we describe a *gusA* promoter–probe transposon, mTnSSgusA40. The utility of this transposon has not been compared directly to earlier promoter–probe *gusA* transposons described by Sharma & Signer (1990). mTnSSgusA40, like the transposons described by Sharma & Signer (1990) should be of use both for molecular genetic studies, and for screening bacteria for promoters which respond to specific environmental signals, such as components of root exudate, or for promoters which are of utility in other experimental situations. For example, to optimize rhizobial competition assays it would be possible to screen individual isolates of a *Rhizobium* strain marked with this transposon for isolates which gave the maximal longevity of expression in mature legume nodules.

The assay conditions developed here for studying nodule occupancy differ in a number of parameters from those routinely used in plant molecular biology (Jefferson, 1987; De Block & Debrouwer, 1992). In plant molecular biology, where absolutely precise cellular or sub-cellular localization of GUS activity is required, recommended conditions are 1–3 mM X-GlcA, with the inclusion of agents to promote the oxidative dimerization of the colourless product of X-GlcA cleavage, to give the blue precipitate. Unfortunately, X-GlcA is an expensive substrate, and while such conditions would be perfectly suitable for nodule occupancy studies, they would lead to the assay being very costly. By keeping the substrate concentration low (100 μg ml⁻¹), the cost of the assay is reduced by at least 10-fold.

To maximize sensitivity of the assay with a low substrate concentration, potassium ferricyanide and potassium ferrocyanide are omitted from the buffer as these compounds, while enhancing the precision of spatial localization of GUS activity, also reduce the degree of blue staining (our results, and De Block & Debrouwer, 1992). Finally, as GUS is stable over several days, it is possible to allow reactions to proceed for much longer time periods if necessary. As long as controls inoculated with unmarked bacteria are included, these reaction conditions work well to distinguish nodules occupied by GUS-marked bacteria from those occupied by unmarked strains. For example Fig. 3(c) shows the precision of discrimination between two adjacent nodules following incubation of the roots in buffer without any oxidation catalyst over a period of 3 d. However, if more precise
plasmids described in this paper to construct GUS different selectable markers, conferring either antibiotic it is a matter of a single-step cloning using precursor mini-transposons are available with a wide range of transposons with the same expression cassettes but with often still be sensitive to spectinomycin. Additionally, as selectable markers, and with unique Not1 sites for cloning, (de Lorenzo

nomycin is an excellent marker in all the strains that we although both streptomycin and spectinomycin can be inactivated by the same enzyme, the two antibiotics Streptomycin affects the S12 protein of the 30s ribosomal S. Katupitya, 1990). and as non-antibiotic resistance (Herrero et al., 1990).

The key advantage of using GUS-marked strains for rhizobial competition studies is that whole root systems – and hence extremely high numbers of nodules – can be analysed for nodule occupancy in a one-step assay. It is also practical for future field use as initiation of the assay can be delayed for at least 24 h after harvest to allow transport of harvested roots from the field to the laboratory. It is relatively inexpensive, despite the high cost of the substrate, costing no more than one or two dollars to assay nodule occupancy on a whole root system and hence comparing favourably with the costs of labour involved in the alternative methods available. Additionally, there is the strong advantage that this assay makes it easy to record types of information that are usually too laborious to gather. For example, information regarding the position of nodules induced by the inoculum strain down the root is preserved.

As GUS-marked bacteria can be localized on the root surface or in infection threads, this opens the possibility of studying the early stages of infection and relating this to ultimate success in competition for nodule occupancy. For instance, if relationships between rhizosphere colonization, root hair infection and nodulation could be assessed, this would provide a means of studying the interaction between different strains and the plant root at a resolution not previously possible. These transposons have already been used to study the point at which nodule development fails in strain–cultivar specific interactions between different R. leguminosarum bv. trifolii strains and subterranean clover cultivars (de Boer & Djordjevic, 1995).

The question of the effect on symbiotic or other ecological properties is also an important one. It is clear that some preliminary screening of marked strains is necessary to ensure that there are no major changes in these properties. However, our initial results (unpublished data) and those of others (Streit et al., 1995) indicate that it is easy to identify marked derivatives which do not differ from the parent in competitive ability for use in ecological studies. The dramatic increase in throughput of nodule typing, and the consequent increase in statistical accuracy (see Wilson, 1995), are more than sufficient to compensate for the work involved in the initial screening step. The throughput of analysis is also far greater, and the iterative cost far lower, than with DNA-based methods which are currently under development (e.g. Richardson et al., 1995), although the latter have a significant role to play in assessment of general population structure (see Wilson, 1995).

The efficacy of these marker genes now needs to be rigorously tested in greenhouse and field experiments (under authority of the appropriate regulatory bodies). For example, questions that need to be answered include (i) over what period of the plant’s lifespan will the GUS assay be efficient, and (ii) how successful will the assay be in field-grown plants? If successful in field situations, such methods which allow rapid, cost-effective screening of the field performance of beneficial microbes could become standard tools for analysing rhizobial competition and many other aspects of microbial ecology.
ACKNOWLEDGEMENTS

K. J. W. was in receipt of a fellowship from the British Royal Society for much of the work carried out in this manuscript. Further support was provided by BioSynth AG, Switzerland. We thank Glynn Bowen, Gudni Hardarson, Wilma Akkermans, Mark Peoples, Wei-Jun Liang, Bob Gaul, Stuart Craig, Roland Henderson, Gayle Williams, Emma Hely and Narelle Dryden for their assistance in the work reported here. The transposons and other vectors described in this manuscript are available from the CAMBIA Molecular Genetic Resource Service (MGRS), supported by the Directorate General for International Cooperation, Ministry of Foreign Affairs, The Netherlands.

REFERENCES


GUS transposons for microbial ecology


Received 16 February 1995; revised 10 March 1995; accepted 27 March 1995.