

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

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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).

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Abbreviations: GUS, β -D-glucuronidase; X-GlcA, 5-bromo-4-chloro-3-indolyl β -D-glucuronide; pNPG, *p*-nitrophenyl β -D-glucuronide; PFGE, pulsed-field gel electrophoresis; RBS, ribosome-binding site; UAS, upstream activating sequence; YM, yeast-mannitol.

The GenBank accession number for the sequence reported in this paper is M14641.

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 *gusA* 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 *gusA* 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 & Signer, 1990; Feldhaus *et al.*, 1991; Van den Eede *et al.*, 1992; Bardonnat & Blanco, 1992; Metcalf & Warner, 1993; Platteeuw *et al.*, 1994). We had earlier suggested its use as a marker for rhizobial competition studies (Wilson *et al.*, 1991) and the initial GUS transposon developed by us proved useful for studying competition for nodulation of the common bean, *Phaseolus vulgaris* (Streit *et al.*, 1992, 1995). More recently, GUS has been used to look at the physical location of plant-associated bacteria (Christiansen-Weniger & Vanderleyden, 1993; Hurek *et al.*, 1994). However, no comprehensive set of GUS transposons existed that could be used to study the ecology of a wide range of Gram-negative bacteria. In this paper we describe the construction and initial application of a set of *gusA*-expressing transposons for ecological studies. In addition, further tools for the manipulation of the *gusA* gene for gene expression studies in prokaryotes are described.

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 (l⁻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 (l⁻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, 6.6 mg FeCl₃, 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 SØ200 to an *hsdR* genotype. First, strain SØ200 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 *dnaC325* allele with the tetracycline resistance marker from transposon Tn10. One such derivative was infected with a second P1 lysate made 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 *hsdR* genotype from strain K802 by examining the efficiency of transformation with pUC18 DNA prepared from strain DH5α (r_K⁻ m_K⁺) and from strain NM522 (r_K⁻ m_K⁻). The efficiency of transformation of strain SØ200 was three orders of magnitude higher with DNA prepared from strain DH5α than with DNA

Table 1. Bacterial strains

Strain	Relevant characteristics	Source/reference
<i>E. coli</i>		
DH5α	<i>endA1 hsdR17</i> (r _K ⁻ m _K ⁺) <i>supE44 thi-1 recA1 gyrA96</i> (Nal ^r) <i>relA1 Δ(lacZYA-argF) U169 Φ80dlac(lacZ)M15</i>	Woodcock <i>et al.</i> (1989)
SØ200	<i>metB strA purB Δ(add-gus-man)</i>	Jochimsen <i>et al.</i> (1975)
K802	<i>hsdR⁻ hsdM⁺ gal⁻ met⁻ supE mcrA⁻ mcrB⁻</i>	Noreen Murray, University of Edinburgh, Edinburgh, UK
TPC48	<i>dnaC 325 Zjj::Tn10</i>	Millie Masters/Noreen Murray, University of Edinburgh, Edinburgh, UK
NM522	F' <i>lacI^qΔ(lacZ)M15 proA⁺B⁺/supE thi Δ(lac-proAB)</i> <i>Δ(hsdMS-mcrB)5</i> (r _K ⁻ m _K ⁻ McrBC ⁻)	Woodcock <i>et al.</i> (1989)
KW1	<i>metB strA purB Δ(add-gus-man) hsdR⁻ hsdM⁺</i>	This work
S17-1	<i>thi pro hsdR⁻ hsdM⁺ recA RP4 2-</i> Tc::Mu-Km::Tn7(Tp ^f /Sm ^r)	Simon <i>et al.</i> (1983)
S17-1 λ-pir	λ-pir lysogen of S17-1	Victor de Lorenzo, Centro de Investigaciones Biológicas, Madrid, Spain
<i>Rhizobium</i>		
NGR234	<i>Rhizobium</i> sp.; nodulates broad range of tropical legumes	Trinick (1980)
CIAT 899	<i>R. tropici</i> ; nodulates <i>P. vulgaris</i> and <i>Leucaena leucocephala</i>	Martinez-Romero <i>et al.</i> (1991)

Table 2. Plasmids used or constructed during this work (intermediate plasmids are not shown)

Name	Relevant characteristics	Reference/source
pDR540	Ap; plasmid containing hybrid <i>trp-lac</i> (<i>tac</i>) promoter	Russell & Bennett (1982)
pUT/mini-Tn5 Sm/Sp	Ap, Sm, Sp; mini-Tn5 encoding Sm ^r /Sp ^r with a unique <i>NotI</i> site for insertion of cloned fragments on broad-host-range suicide plasmid.	de Lorenzo <i>et al.</i> (1990)
pUC18Not	Ap; pUC18 derivative with <i>NotI</i> sites flanking the polylinker	Herrero <i>et al.</i> (1990)
pWM74	Ap; 1.2 kb <i>lacI^a</i> gene as an <i>EcoRI</i> fragment	W. Margolin, Stanford University, CA, USA
pCQ15	<i>nifH</i> from <i>R. etli</i> strain CFN42	Quinto <i>et al.</i> (1985)
pBN370	Ap; 2.8 kb <i>HindIII</i> fragment in pBR322 containing <i>nifH</i> from <i>Bradyrhizobium</i> sp. (<i>Parasponia</i>) strain Rp501	B. Tracy Nixon, Pennsylvania State University, PA, USA
pBKuidA	Ap, Tc; 6.5 kb <i>EcoRI-HindIII</i> insert in pBR325 containing <i>gusABC</i> and downstream convergently transcribed ORF	Jefferson <i>et al.</i> (1986)
pTTQ18	Ap; high copy vector with the <i>tac</i> promoter and <i>lacI^a</i> enabling regulated expression of cloned genes	Stark (1987)
pBI101.1	Km; <i>gusA</i> plus <i>nos</i> polyA site in pBIN19; reading frame 1	Jefferson <i>et al.</i> (1987)
pBI101.2	Km; <i>gusA</i> plus <i>nos</i> polyA site in pBIN19; reading frame 2	Jefferson (1987)
pBI101.3	Km; <i>gusA</i> plus <i>nos</i> polyA site in pBIN19; reading frame 3	Jefferson (1987)
pRAJ289	Ap; 6.2 kb insert in pTTQ18 containing promoterless <i>gusABC</i> and downstream convergently transcribed ORF	This work
pRAJ294	Ap; promoterless <i>gusA</i> gene with bacterial Shine-Dalgarno sequence in pTTQ18	This work
pTacter	Ap; <i>tac</i> promoter and <i>trpA</i> terminator flanking unique cloning sites in pUC8	This work
pKW28	Ap, Km; Tn5-containing <i>EcoRI</i> fragment from a <i>Bradyrhizobium</i> mutant in pUC13	Wilson (1987)
pKW106	Ap; 2.3 kb <i>Papb-gusA-ter HindIII</i> cassette in pUC13	This work
pKW117	Ap; <i>gusA</i> plus <i>trpA</i> ter for translational fusions: reading frame 1 in pTacter	
pKW118	Ap; <i>gusA</i> plus <i>trpA</i> ter for translational fusions: reading frame 2 in pTacter	
pKW119	Ap; <i>gusA</i> plus <i>trpA</i> ter for translational fusions: reading frame 3 in pTacter	
pKW120	Ap; promoterless <i>gusA</i> fragment from pRAJ294 in pUC18Not	
pKW121	Ap; 2.4 kb <i>XhoI-SstI</i> fragment from pKW111 with <i>Papb-gusA-ter</i> cassette and adjacent <i>SpeI</i> site in pUC18Not	
pJC63	Ap; 1.2 kb <i>EcoRI lacI^a</i> fragment in pUC18Not	
pJC64	Ap; 2 kb <i>Ptac-gusA-ter</i> fragment plus 1.2 kb <i>EcoRI lacI</i> fragment in pUC18Not	
pJC66	Ap; 2 kb <i>Ptac-gusA-ter</i> fragment in pUC18Not	
pJC67	Ap; 2.3 kb <i>Papb-gusA-ter</i> cassette from pKW106 in pUC18Not	
pAS12	Ap; <i>R. etli nifH-gusA</i> translational fusion in pUC18Not with ~ 1 kb of upstream sequence including the UAS	
pAS22	Ap; <i>Bradyrhizobium nifH-gusA</i> translational fusion in pUC18Not with the <i>nifH</i> promoter but no UAS	
pCAM110	Sm/Sp, Ap; mTn5SS <i>gusA10</i> (<i>Ptac-gusA-trpA</i> ter transcriptional fusion and <i>lacI^a</i> gene) in pUT/mini-Tn5 Sm/Sp	
pCAM111	Sm/Sp, Ap; mTn5SS <i>gusA11</i> (<i>Ptac-gusA-trpA</i> ter transcriptional fusion) in pUT/mini-Tn5 Sm/Sp	
pCAM120	Sm/Sp, Ap; mTn5SS <i>gusA20</i> (<i>Papb-gusA-trpA</i> ter translational fusion) in pUT/mini-Tn5 Sm/Sp	
pCAM121	Sm/Sp, Ap; mTn5SS <i>gusA21</i> (<i>Papb-gusA-trpA</i> ter translational fusion with adjacent unique <i>SpeI</i> site) in pUT/mini-Tn5 Sm/Sp	
pCAM130	Sm/Sp, Ap; mTn5SS <i>gusA30</i> (<i>R. etli nifH-gusA-trpA</i> ter translational fusion) in pUT/mini-Tn5 Sm/Sp	
pCAM131	Sm/Sp, Ap; mTn5SS <i>gusA31</i> (<i>Bradyrhizobium nifH-gusA-trpA</i> ter translational fusion) in pUT/mini-Tn5 Sm/Sp	
pCAM140	Sm/Sp, Ap; mTn5SS <i>gusA40</i> (promoterless <i>gusA</i> for transcriptional fusions) in pUT/mini-Tn5 Sm/Sp	

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 *hdsR* 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 presence of a *gusA* insert could be unambiguously detected by formation of blue colonies on media containing 50 $\mu\text{g ml}^{-1}$ X-GlcA.

Introduction of transposons into rhizobial recipients.

Rhizobium sp. strain NGR234 and *R. tropici* strain CIAT899 were used as recipients. Plate matings were carried out as described by Wilson *et al.* (1994) on YM plates at 30 °C using *E. coli* S17-1 λ -*pir* containing the relevant GUS transposon as the donor strain. Transconjugants were selected on modified BD minimal medium (using the optimal carbon source for the recipient strain) supplemented with spectinomycin (50 $\mu\text{g ml}^{-1}$) to select for insertion of the transposon. Recipients were counted using the Miles & Misra (Collins & Lyne, 1985) drop count method on modified BD medium. S17-1 λ -*pir* cannot grow on this medium because it is auxotrophic for proline. Transfer frequencies of the order of 10^{-6} were obtained with both *Rhizobium* strains.

DNA manipulations. Routine DNA manipulations were carried out as described by Ausubel *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 MgCl_2 , 1 μM primers. *Taq* polymerase was from Perkin Elmer. The amplification programme used was: (95 °C, 1 min) \times 1, (95 °C, 10 s; 55 °C, 20 s; 72 °C, 1 min) \times 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 of downstream sequence was isolated from pBK*uidA* on two fragments, a 539 bp *HincII*–*BamHI* fragment lacking any promoter sequences but containing the Shine–Dalgarno sequence and part of the *gusA* coding sequence, and a 5.5 kb *BamHI*–*HindIII* fragment containing the rest of the operon and downstream sequences. These two fragments were combined in *SmaI*/*HindIII*-digested pTTQ18 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-closed, 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 *gusBC* and downstream sequences were then eliminated by digestion with *HindIII* and ligation of the linker and 3' polylinker *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 *NsiI* linkers (AGATGCATCT, New England Biolabs) to the *trpA* transcription terminator GenBlock (AGCCCGCTAATGAGCGGGCTTTTTTTTTT, Pharmacia), cleaving with *NsiI* and then inserting this fragment into the *PstI* site of pUCTac to create pTacter.

(iv) pKW117, pKW118 and pKW119. The upstream polylinker 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*–*XhoI* 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, blunt-ended, and subsequently digested with *SstI* prior to inserting a 1.9 kb *SmaI*–*SstI* *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) mTn5SSgusA20. The 2.3 kb *aph-gusA-ter HindIII* cassette from pKW106 was inserted into pUC18Not to create pJC67, and the resulting 2.4 kb *NotI* cassette was inserted into pUT/mini-Tn5 Sm/Sp to create pCAM120 containing mTn5SSgusA20.

(iii) mTn5SSgusA21. This transposon was created to contain a unique *SpeI* site. The 2.3 kb *HindIII* cassette from pKW106 was inserted into the pBluescript SKII(+) (Stratagene) polylinker, which contains an adjacent *SpeI* site, to create pKW111. This was digested with *XhoI* and *SstI* and the 2.4 kb *Paph-gus-ter* cassette plus adjacent *SpeI* site was inserted into *SalI*–*SstI* digested pUC18Not, creating pKW121. Finally, pCAM121 containing mTn5SSgusA21 was constructed by cloning the *NotI* cassette from pKW121 into *NotI*-digested pUT/mini-Tn5 Sm/Sp. It should be noted that there are three *NotI* sites in pKW121, and that one of the sites bounding the *NotI* cassette in mTn5SSgusA21 therefore derives from the pBluescript SKII(+) polylinker.

Construction of gusA transposons using the regulatable tac promoter

(i) mTn5SSgusA10. The promoterless *gusA* gene from pRAJ294 was inserted as a 1.9 kb blunt-ended *EcoRI*–*HindIII* fragment into the *HincII* 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 *lacIⁿ* gene from pWM74 as a 1.2 kb *EcoRI* fragment in pUC18Not, to create pJC64. *Ptac-gusA-ter* plus *lacIⁿ* was then moved as a 3.3 kb *NotI* fragment into pUT/mini-Tn5 Sm/Sp to create pCAM110 containing mTn5SSgusA10.

(ii) mTn5SSgusA11. A transposon with *gusA* driven consti-

tutively from *Ptac* was also created by deleting the *lacI^R*-containing *EcoRI* cassette from pJC64 to create pJC66, and inserting the *Ptac-gusA-ter* cassette without *lacI^R* as a *NotI* fragment into pUT/mini-Tn5 Sm/Sp, forming mTn5SS*gusA11*.

Construction of symbiotically active *gusA* transposons

(i) mTn5SS*gusA30*. To create a *Rhizobium nifH-gusA* fusion, the *Rhizobium etli* CFN42 *nifH* gene and about 1 kb of flanking sequences on either side was first isolated as a 2.7 kb *EcoRI-HindIII* fragment from pCQ15 and inserted into pBlue-script SKII(+) to create pKW112. About 1.1 kb of upstream sequence and the first 29 codons (i.e. 87 bp) of the *nifH* coding sequence was then subcloned as a 1.2 kb *EcoRI-SalI* fragment into pUC18Not to create pAS11. To make a translational fusion to *gusA*, pAS11 was digested with *HincII* and a 2.0 kb *SmaI*, *HindIII*-digested, blunt-ended fragment from pKW119 containing *gusA* with the *trpA* terminator was inserted, creating pAS12. This *nifH-gusA* fusion was cloned as a 3.3 kb *NotI* fragment into pUT/mini-Tn5 Sm/Sp to create pCAM130 containing mTn5SS*gusA30*.

(ii) mTn5SS*gusA31*. To create a *Bradyrhizobium nifH-gusA* fusion, the upstream 190 bp and the first 22 codons (66 bp) of *Bradyrhizobium* sp. (*Parasponia nifH*) 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 2.0 kb *SmaI-HindIII*-digested, blunt-ended fragment from pKW118 was inserted, creating pAS22. The resulting *nifH-gusA* fusion was cloned as a 2.3 kb *NotI* fragment into pUT/mini-Tn5 Sm/Sp to create pCAM131 containing mTn5SS*gusA31*.

Construction of a promoter-probe transposon

pUT/mTn5SS*gusA40*. The promoterless *gusA* gene from pRAJ294 was inserted as a 1.9 kb *EcoRI-HindIII* fragment into pUC18Not forming pKW120. The resulting 2 kb *NotI* fragment was inserted into pUT/mini-Tn5 Sm/Sp. A clone in which the *gusA* gene was oriented so that it would be transcribed from the outside end of pUT/mini-Tn5 Sm/Sp was designated pCAM140, containing mTn5SS*gusA40*.

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' CTGACTCTTATACACAAGTGC 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' GAATGCCACAGGCCGTCGAG 3'. A DNA amplification product with WIL1 + WIL3 indicated that the *gusA* gene was orientated such that *gusA* was transcribed into the transposon from the O-end, as in mTn5SS*gusA40*. 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 mTn5SS*gusA10*), 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 10⁶–10⁷ 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-GlcA (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, SØ200 (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-GlcA.

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).

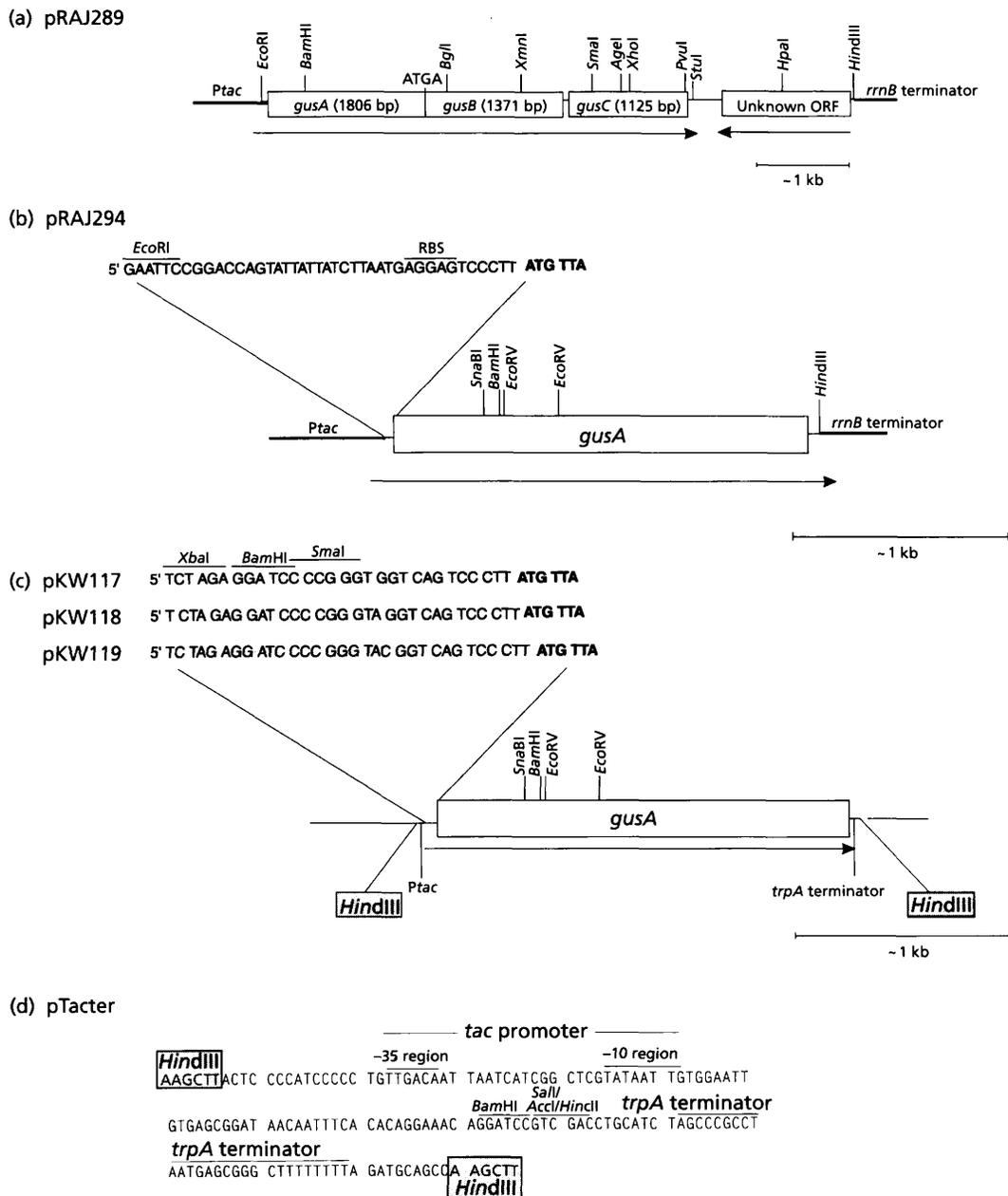


Fig. 1. Plasmids useful for genetic manipulation of *gusA* in bacteria. (a) pRAJ289 contains the *gus* operon from *E. coli* K12 under the control of the *tac* promoter in pTTQ18. *gusA* encodes β -glucuronidase, *gusB* encodes the glucuronide permease and *gusC* is a membrane-associated protein of unknown function (Jefferson *et al.*, 1986; 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 pTTQ18. 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 *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 *aph* gene within IS50L (Rothstein & Reznikoff, 1981). Thus, a region of Tn5 encompassing these upstream sequences and 33 bp of *aph* coding sequence was used to make a translational fusion of the *aph* gene with *gusA*. This fusion was inserted into pUT/mini-Tn5 Sm/Sp to create mTn5SS*gusA20* (Fig. 2a) which gives high-level constitutive expression of GUS in *Rhizobium* (Table 3). mTn5SS*gusA21* is similar to mTn5SS*gusA20*, except that a unique *SpeI* site was incorporated adjacent to the *gusA* gene (Fig. 2b).

Transposons with regulated *gusA* expression. mTn5SS*gusA10* was constructed to enable regulation of *gusA* so that it is only expressed at high levels at the time of assay. This should reduce any metabolic load imposed by GUS expression. mTn5SS*gusA10* contains the *lacI^q* gene and *gusA* under control of the *tac* promoter (Russell & Bennett, 1982), and therefore expression is repressed until the gratuitous inducer of the *lac* operon, IPTG, is added (Fig. 2c). In liquid culture, on addition of IPTG, expression of *gusA* from mTn5SS*gusA10* was induced approximately 30-fold in *Rhizobium* sp. NGR234, and about 20-fold in *R. tropici* CIAT899 (Table 3). Derivatives of strains NGR234 and CIAT899 marked with mTn5SS*gusA11*, which contains the same *Ptac-gusA*-*ter* cassette, but without the *lacI^q* gene, showed constitutive GUS activity slightly higher than that of induced mTn5SS*gusA10* (Fig. 2d, Table 3).

Construction of symbiotically expressed *gusA* cassettes. To ensure strong expression of *gusA* under symbiotic conditions, translational fusions of *gusA* to *nifH* genes from both a *Rhizobium* and a *Bradyrhizobium* strain were made: *nifH* encodes the Fe-component of nitrogenase, and is expressed only in symbiotic or microaerobic conditions (Fischer, 1994). mTn5SS*gusA30* contains more than 1 kb of upstream sequence from the *nifH* promoter of *R. etli* strain CFN42 (Fig. 2e), and thus includes both the RNA polymerase σ^{54} -dependent promoter sequences, and the upstream activating sequence (UAS) that is typically located about 80–150 bp upstream of *nifH* start sites (Fischer, 1994). By contrast, the *nifH-gusA* fusion in mTn5SS*gusA31* contains only about 50 bp of sequence upstream from the deduced *nifH* start site of *Bradyrhizobium* sp. (*Parasponia*) strain Rp501, and does not contain the UAS (Fig. 2f).

Promoter-probe transposon. Finally, a promoter-probe transposon, mTn5SS*gusA40*, was constructed (Fig. 2g). In this transposon *gusA* lacks a promoter and is orientated such that it can be transcribed off adjacent promoters in the genomic DNA. In a test mating of mTn5SS*gusA40* into *Rhizobium* sp. strain NGR234, expression of the *gusA* gene was found to vary widely from no activity, to activity as high as 190 nmol pNPG hydrolysed per min per 10^9 cells. This was reflected in the appearance of

transconjugant colonies on plates containing 50 μ g X-GlcA ml⁻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, *Rhizobium* sp. strain NGR234, marked with all the GUS transposons (except for mTn5SS*gusA21* and mTn5SS*gusA40*), was inoculated onto siratro and pigeonpea; *R. tropici* strain CIAT899 marked with the same set of transposons was inoculated onto common bean. A basic histochemical GUS assay consists of immersing tissue in buffer containing an appropriate 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 μ g X-GlcA ml⁻¹ to stain nodules induced by strain NGR234::*gusA10* on 41-d-old siratro plants. After overnight incubation, nodules incubated in 500 μ g ml⁻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 μ g X-GlcA ml⁻¹ were only slightly blue, those in 100 μ g X-GlcA ml⁻¹ were mid-blue and those in 250 μ g X-GlcA ml⁻¹ were mid- to 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 μ g X-GlcA ml⁻¹, as in those incubated in 500 μ g X-GlcA ml⁻¹. It was only in the treatments that used 50 μ g X-GlcA ml⁻¹ that the nodule staining was less pronounced. We therefore concluded that 100 μ g ml⁻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 μ g X-GlcA ml⁻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 *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 *et al.*, 1992; Wilson *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 500 μ g

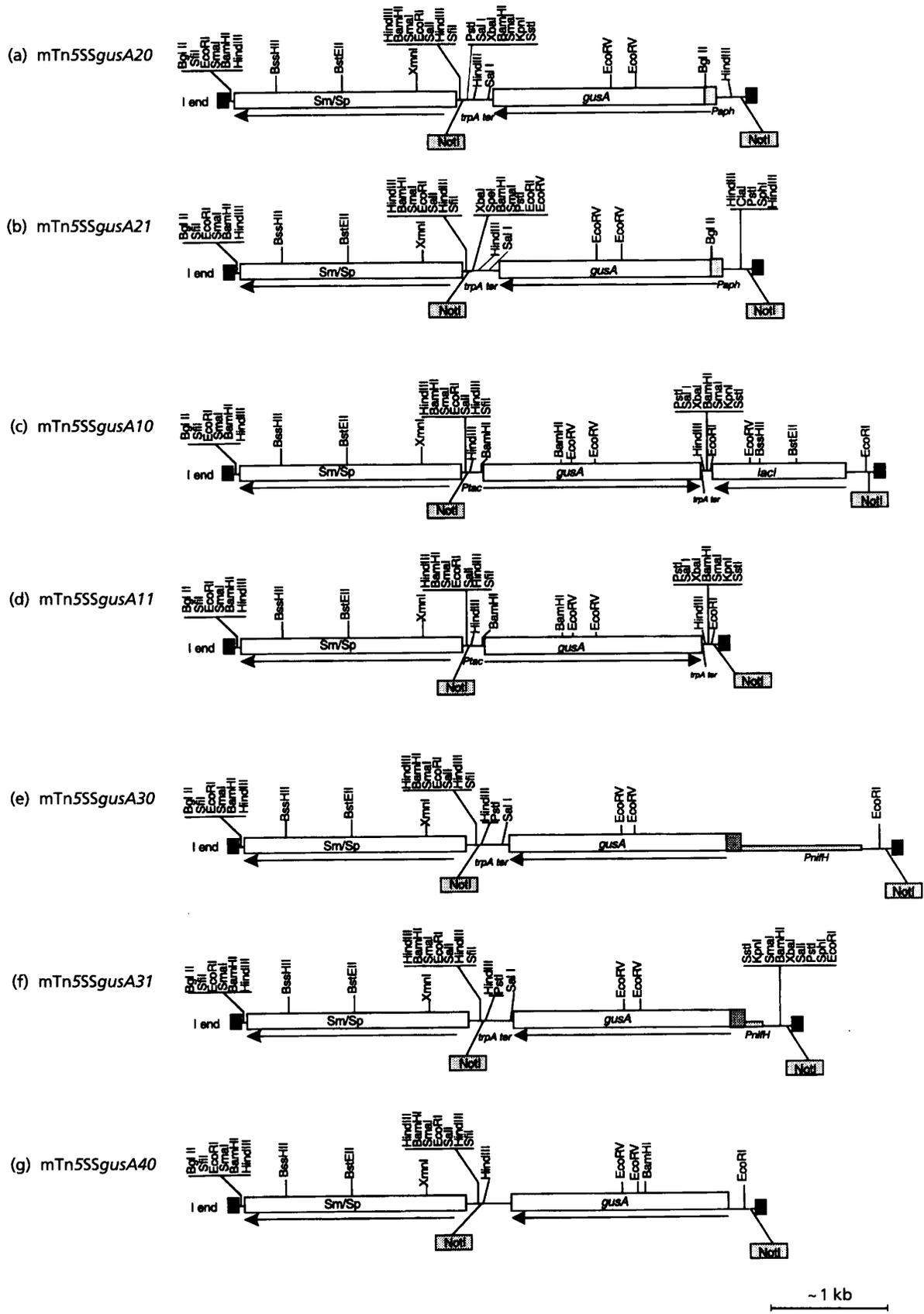


Fig. 2. For legend see facing page.

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

Values represent the means from three independent marked isolates (\pm SEM).

Transposon inserted	GUS activity (nmol pNPG hydrolysed per min per 10^9 viable cells) in:		
	IPTG	Strain NGR234	Strain CIAT899
None	—	0	0
mTn5SSgus.A10	—	4.45 \pm 0.8	6.06 \pm 0.8
mTn5SSgus.A10	+	149 \pm 20	119 \pm 26
mTn5SSgus.A11	—	182 \pm 30	214 \pm 20
mTn5SSgus.A20	—	121 \pm 8	139 \pm 50

X-GlcA ml⁻¹ was prepared and divided into four aliquots with the addition of respectively: nothing; 100 μ g chloramphenicol ml⁻¹; 100 μ g cycloheximide ml⁻¹; 100 μ g chloramphenicol ml⁻¹ plus 100 μ g cycloheximide ml⁻¹. Siratro plants (64-d-old, nodulated by parental strain NGR234, and by strain NGR234::gus.A10) were harvested and incubated in each of these buffers.

Good staining was observed in the nodules of all treatments nodulated by strain NGR234::gus.A10, 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 oxidative dimerization of the colourless 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 μ M to less than 1 μ M 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, mTn5SSgus.A10, mTn5SSgus.A11, mTn5SSgus.A20 and mTn5SSgus.A21 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 μ g 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 mTn5SSgus.A11 or mTn5SSgus.A20, 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 mTn5SSgus.A10, in which gus.A expression is regulated by the product of the lacI^a gene, differs from that of the transposons with constitutive gus.A 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-

Fig. 2. Restriction maps of GUS transposons. mTn5SSgus.A10 expresses GUS in a regulated manner, dependent on induction by IPTG. mTn5SSgus.A11, mTn5SSgus.A20 and mTn5SSgus.A21 express GUS constitutively. mTn5SSgus.A21 is designed to be useful for mapping the site of transposon insertions using PFGE. mTn5SSgus.A30 and mTn5SSgus.A31 both have GUS expressed from a symbiotically active promoter. mTn5SSgus.A40 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 gus.A 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, pUT.

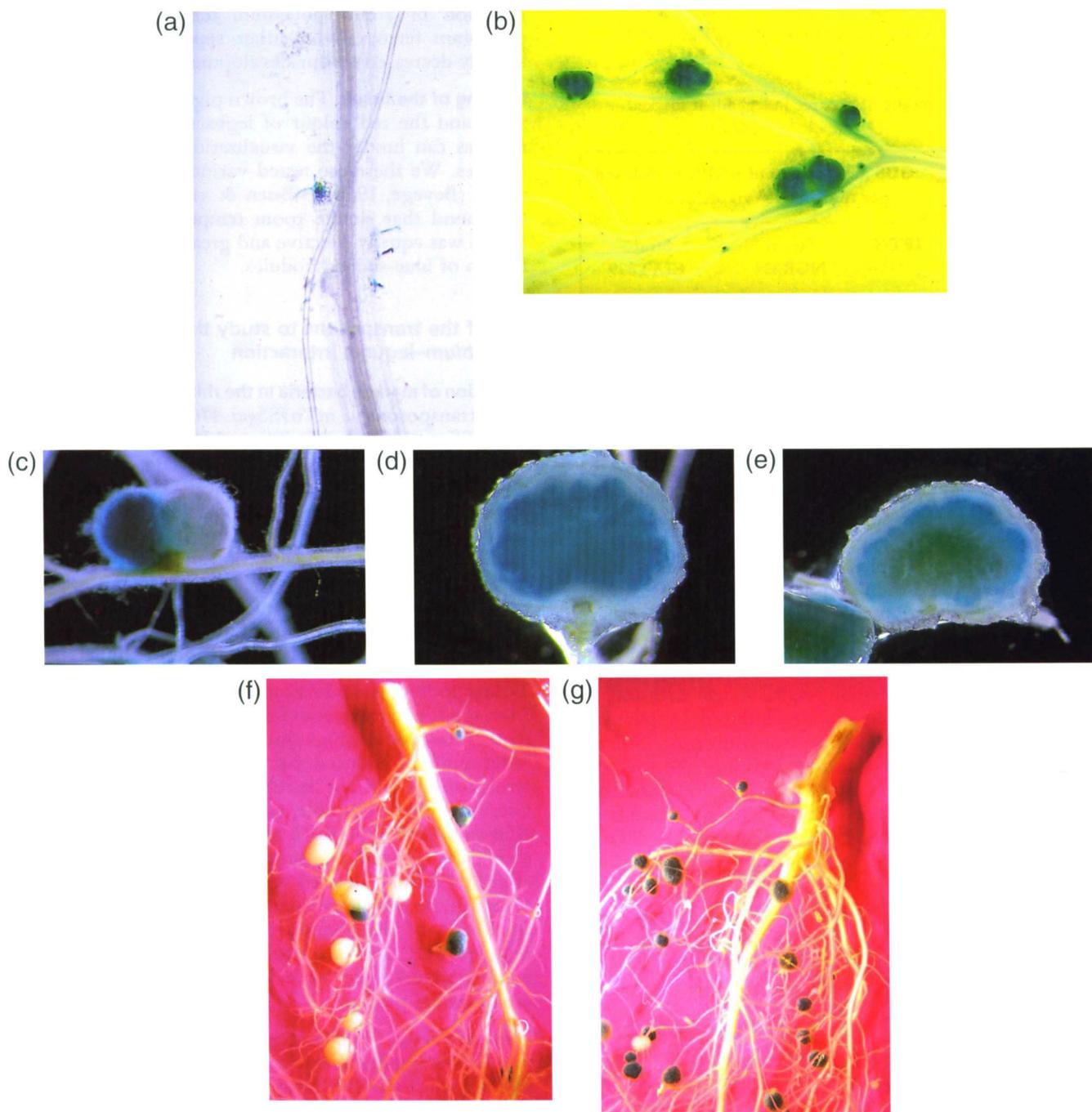


Fig. 3. Examples of assays carried out using GUS-marked strains. (a) Early stages of infection of a siratro root infected with *Rhizobium* sp. NGR234 marked with mTn5SSgusA20. The photograph shows a root harvested 20 d after inoculation. For this type of localization, the roots were vacuum-infiltrated in buffer containing $150 \mu\text{g X-GlcA ml}^{-1}$ 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 mTn5SSgusA11. 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 mTn5SSgusA11 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 mTn5SSgusA31 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 mTn5SS*gusA10*, 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 mTn5SS*gusA11* or mTn5SS*gusA20*; 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 mTn5SS*gusA11* or mTn5SS*gusA20*.

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 (mTn5SS*gusA30*) and to that of *Bradyrhizobium* sp. (*Parasponia*) strain Rp501 (mTn5SS*gusA31*) were used. In separate experiments, *Rhizobium* sp. strain NGR234 marked with mTn5SS*gusA31* 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 mTn5SS*gusA10* and mTn5SS*gusA20*.

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. mTn5SS*gusA10* 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 *lacI* product varies in different Gram-negative bacteria (Fürste *et al.*, 1986), and the extent of induction observed in *Rhizobium* sp. NGR234 and *R. tropici* 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. mTn5SS*gus.A10* 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 mTn5SS*gus.A11* and mTn5SS*gus.A20* 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 longer-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 mTn5SS*gus.A20* to infect subterranean clover (de Boer & Djordjevic, 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.

mTn5SS*gus.A21* is similar to mTn5SS*gus.A20*, 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 mTn5SS*gus.A21* (and all the other mini-Tn5-based GUS transposons) also contains *NotI* 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, mTn5SS*gus.A30* or mTn5SS*gus.A31*, 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, mTn5SS*gus.A40*. The utility of this transposon has not been compared directly to earlier promoter–probe *gusA* transposons described by Sharma & Signer (1990). mTn5SS*gus.A40*, 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

spatial localization is required, as for example in detecting infection threads (Fig. 3a), it is recommended that higher concentrations of X-GlcA be used, with the inclusion of the oxidation catalysts potassium ferricyanide and potassium ferrocyanide.

Another important consideration is the presence of root-associated microbes with endogenous GUS activity. By testing buffers containing prokaryotic and/or eukaryotic protein synthesis inhibitors, we were able to demonstrate that inclusion of chloramphenicol can be used to differentiate between GUS activity due to deliberately marked strains and any endogenous activity. These results indicated that the endogenous activity was prokaryotic, as chloramphenicol inhibits prokaryotic protein synthesis and would therefore inhibit *de novo* induction of GUS in any endogenous root-associated bacteria. By contrast, as GUS expression in the marked rhizobia occurs prior to the addition of assay buffer, the inclusion of chloramphenicol did not inhibit staining of GUS-marked nodules (although the reaction was slowed in relation to the treatments that omitted chloramphenicol). We also found that inclusion of chloramphenicol is not necessary if concentrations of X-GlcA of 100 $\mu\text{g ml}^{-1}$ or lower are used, presumably because this is below the threshold concentration of substrate required for induction of GUS in these bacteria (Stoeber 1961; Wilson *et al.*, 1992).

This set of transposons is designed to be of use in a diverse range of Gram-negative bacteria. Although their use in species other than *Rhizobium* is not described here, Tn5 is known to have an extremely broad host-range (de Bruijn & Lupski, 1984), and there is no reason to suppose that inclusion of the *gusA* gene will affect the host range of these transposons. Indeed, Tn5KW107 (Wilson *et al.*, 1991) and mTn5SSgusA40 have both been used in *Pseudomonas* spp. (Wilson *et al.*, 1994; L. de Weger, personal communication), and mTn5SSgusA11 and mTn5SSgusA20 have both been used in *Azospirillum* spp. (C. Christiansen-Weniger, C. Nirmala & S. Katupitya, personal communications).

The transposons described here all confer streptomycin/spectinomycin resistance and we have found that spectinomycin is an excellent marker in all the strains that we have tested (unpublished data). This is true even in strains with endogenous streptomycin resistance because, although both streptomycin and spectinomycin can be inactivated by the same enzyme, the two antibiotics actually target different components of the ribosome. Streptomycin affects the S12 protein of the 30S ribosomal subunit, whereas spectinomycin acts on the S5 protein of the same subunit (Bryan, 1982). Thus, a strain with endogenous or acquired resistance to streptomycin will often still be sensitive to spectinomycin. Additionally, as mini-transposons are available with a wide range of selectable markers, and with unique *NotI* sites for cloning, it is a matter of a single-step cloning using precursor plasmids described in this paper to construct GUS transposons with the same expression cassettes but with different selectable markers, conferring either antibiotic (de Lorenzo *et al.*, 1990) or 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.

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