

# A family of promoter probe vectors incorporating autofluorescent and chromogenic reporter proteins for studying gene expression in Gram-negative bacteria

R. Karunakaran, T. H. Mauchline, A. H. F. Hosiet and P. S. Poole

School of Biological Sciences, University of Reading, Whiteknights, PO Box 228, Reading RG6 6AJ, UK

## Correspondence

P. S. Poole

p.s.poole@reading.ac.uk

Received 29 June 2005

Accepted 11 July 2005

A series of promoter probe vectors for use in Gram-negative bacteria has been made in two broad-host-range vectors, pOT (pBBR replicon) and pJP2 (incP replicon). Reporter fusions can be made to *gfpUV*, *gfpmut3.1*, unstable *gfpmut3.1* variants (LAA, LVA, AAV and ASV), *gfp+*, *dsRed2*, *dsRedT.3*, *dsRedT.4*, *mRFP1*, *gusA* or *lacZ*. The two vector families, pOT and pJP2, are compatible with one another and share the same polylinker for facile interchange of promoter regions. Vectors based on pJP2 have the advantage of being ultra-stable in the environment due to the presence of the *parABCDE* genes. As a confirmation of their usefulness, the dicarboxylic acid transport system promoter (*dctA<sub>p</sub>*) was cloned into a pOT (pRU1097)- and a pJP2 (pRU1156)-based vector and shown to be expressed by *Rhizobium leguminosarum* in infection threads of vetch. This indicates the presence of dicarboxylates at the earliest stages of nodule formation.

## INTRODUCTION

The fusing of promoters of interest to a reporter gene has greatly enhanced our ability to study gene expression both in the laboratory and in natural environments. Various reporter gene systems, including *lacZ* (Labele *et al.*, 1990), *gusA* (Prell *et al.*, 2002; Reeve *et al.*, 1999), *luc* and *lux* (Prosser *et al.*, 1996) and *inaZ* (Miller *et al.*, 2000) have all been used for molecular genetic analyses. More recently, autofluorescent proteins (AFPs) have been used widely (Gage, 2002; Stuurman *et al.*, 2000; Xi *et al.*, 2001). The most common of these is green fluorescent protein (GFP), a monomeric 23 kDa protein, which was isolated from luminous coelenterates of the genus *Aequorea* (Chalfie *et al.*, 1994). GFP contains a natural chromophore in an internal hexapeptide, which requires O<sub>2</sub> for cyclization (Chalfie *et al.*, 1994; Inouye & Tsuji, 1994). The three-dimensional structure of GFP has been solved, and this shows that it has 11 antiparallel beta strands forming a cylinder (or beta-can) that surrounds an inner alpha-helix where the chromophore is located (Yang *et al.*, 1996). This structure functions to protect the chromophore and confers the stability of the native GFP protein. The great advantage

of GFP as a reporter protein is that its autofluorescence does not require any cofactors for expression, enabling its detection at the single-cell level via non-destructive sampling. It can also be viewed under a wide range of conditions, such as in agar plates, fluorescent plate readers as well as by fluorescence-activated cell sorting (FACS).

In addition to the wild-type protein, there are many derivatives of GFP, which have increased levels of fluorescence emission, and shifted excitation or emission spectra (Cormack *et al.*, 1996; Cramer *et al.*, 1996; Ellenberg *et al.*, 1998). GFP<sub>UV</sub> has mutations at F99S, M153T and V163A, produced by shuffle mutagenesis, which result in a 16-fold higher emission than wild-type GFP, but it retains the wild-type excitation spectrum (Cramer *et al.*, 1996). GFP<sub>UV</sub> appears to have a higher fluorescence emission because it is more soluble than wild-type GFP. Site-directed mutagenesis of wild-type *gfp* has been used to change F64L and S65T to produce a series of GFP<sub>mut</sub> derivatives that have a red-shifted excitation spectrum (excitation maximum 488 nm) and a 35-fold increase in fluorescence, giving them characteristics close to those of FITC, and therefore making them better suited to FAC sorters (Cormack *et al.*, 1996). Furthermore, the addition of a protease-targeting signal to GFP<sub>mut</sub> has led to the creation of a suite of GFP<sub>mut</sub> proteins with different stabilities (Anderson *et al.*, 1998). A further derivative, GFP<sub>+</sub>, has been produced that incorporates the chromophore change from GFP<sub>mut3.1</sub> into the protein backbone of GFP<sub>UV</sub>, giving up to a 130-fold

†Present address: Department of Microbiology, The Dental Institute, King's College London, Floor 28, Guy's Tower, Guy's Hospital, London SE1 9RT, UK.

Abbreviations: AFP, autofluorescent protein; FAC sorter, fluorescence-activated cell sorter; FACS, fluorescence-activated cell sorting; GFP, green fluorescent protein.

increase in fluorescence emission (Scholz *et al.*, 2000). This is due to the combination of the red-shifted chromophore of GFPmut3.1 with the greater solubility of GFPuv. GFP mutants with blue, cyan and yellowish-green emission spectra are now available, but none of these mutants has emission spectra at wavelengths longer than 529 nm, and as such are limited for dual-labelling experiments with GFP (Baird *et al.*, 2000). However, another fluorescent protein, DsRed, which is 28 kDa in size and originally isolated from corals of the genus *Discoma* (Baird *et al.*, 2000), shares certain structural and chromophore motifs with GFP, but has an emission maximum of 583 nm, and so can be used in conjunction with GFP. A disadvantage of wild-type DsRed is that it is tetrameric and is slow to mature compared to GFP. However, mutant derivatives, DsRedT.3 and DsRedT.4, have recently been isolated, which, while still yielding tetrameric proteins, mature much faster than the wild-type (Bevis & Glick, 2002). In addition, a more rapidly maturing monomeric variant of DsRed has been developed, called monomeric red fluorescent protein (mRFP1) (Campbell *et al.*, 2002).

Due to the advantages of AFPs as reporter proteins, a large number of vectors incorporating them have been made (Allaway *et al.*, 2001; Miller *et al.*, 2000; Stuurman *et al.*, 2000). However, we considered it would be of great use if a suite of these AFPs was available in the same polylinker background in two compatible vectors, enabling the easy switching of promoters between vectors. In many cases it is still desirable to use chromogenic reporter systems (GusA and LacZ), which have increased sensitivity relative to AFPs and have been the 'gold standard' for decades. We therefore developed two families of stable vectors, containing a compatible polylinker upstream of various *gfp* derivatives, *gusA*, *lacZ*, *dsRed* derivatives and *mRFP1*, suitable for use in Gram-negative bacteria in the environment.

## METHODS

**Bacterial strains and growth conditions.** The bacterial strains and plasmids used in this study are listed in Table 1. *Escherichia coli* strains were grown at 37 °C in Luria-Bertani broth (LB) or agar (LA). *Rhizobium leguminosarum* 3841 was grown at 28 °C on either tryptone-yeast extract (TY) (Beringer, 1974), acid minimal salts (AMS), or acid minimal salts agar (AMA) (Poole *et al.*, 1994a) with 10 mM D-glucose or succinate and 10 mM ammonium chloride as sole sources of carbon and nitrogen, respectively. Antibiotics were used at the following concentrations: streptomycin, 500 µg ml<sup>-1</sup>; chloramphenicol, 10 µg ml<sup>-1</sup>; kanamycin 40 µg ml<sup>-1</sup>; tetracycline, 2 µg ml<sup>-1</sup> (in AMS), 5 µg ml<sup>-1</sup> (in TY), 10 µg ml<sup>-1</sup> (in LA); gentamicin, 20 µg ml<sup>-1</sup> (for *E. coli* 10 µg ml<sup>-1</sup>).

**Genetics and molecular biology.** All general DNA cloning and analysis was performed as previously described (Sambrook & Russell, 2001). *gfpmut3.1* and its unstable derivatives (LAA, LVA, AAV and ASV), *gfp+*, *gusA*, *lacZ*, *dsRed2.0* (Clontech), *dsRed T.3* and *T.4* and the monomeric red fluorescent protein *mRFP1* were PCR-amplified from different vectors (see Table 1 for details), using the oligonucleotide primers listed in Table 2. The primers included *SpeI* and *SacI* sites at the 5' ends to allow cloning, except for the primers for *lacZ* (p348 and p349), which contained *SpeI* and *XhoI* sites.

PCR reactions were conducted in 100 µl, using 2.5 units Pfu Turbo (Stratagene), 10–30 ng genomic DNA, 1 × PCR buffer (Stratagene), 0.2 mM dNTPs, 1 µM primers. The cycling conditions were as follows: 1 cycle of 95 °C for 2 min, 30 cycles of 95 °C for 45 s, 57 °C for 45 s, 72 °C for 2 min and a final extension of 72 °C for 10 min. All these PCR products were then cloned into pCR2.1 (Invitrogen) according to the manufacturer's protocol. Once the PCR products were cloned, the plasmids were digested with relevant restriction enzymes and subsequently ligated into pOT2 (a pBBR replicon). Reporter fusions were then transferred from pOT2 to pJP2 (derived from pTR101, an incP replicon), carrying across the entire polylinker. The cloning of *gfp+* was slightly different in that the *SpeI*–*XhoI* fragment of *gfp+*, rather than the *SpeI*–*SacI* fragment that was used for other *gfp* genes, was used to replace the *SpeI*–*XhoI* fragment in the *gfpUV* gene of pOT2. This leaves the *gfpUV* backbone intact but replaces the F64L and S65T mutations in the chromophore. This was done because the *gfpUV* of pOT2 has had the *Sall* site removed by site-directed mutagenesis (Allaway *et al.*, 2001). Brief details of the cloning of *gfp+* have already been described in a parallel study (Hosie *et al.*, 2002), but are included here in greater detail.

All DNA inserts were confirmed by sequencing (MWG Germany). All plasmids were conjugated into rhizobial strains, using pRK2013 as a helper plasmid to provide the transfer genes, as previously described (Poole *et al.*, 1994b).

**Measurement of reporter fusion activity.** GFP fluorescence was measured using a Tecan GENios fluorometer equipped with excitation filters of 390 nm (for GFPuv) and 485 nm (for GFPmut3.1 and all other GFP derivatives), and emission filter 510 nm. Strain 3841, containing various pOT2 derivatives with the *dctA* promoter cloned into them, was grown in AMS supplemented with 10 mM succinate or glucose. When the cells reached an OD<sub>595</sub> of 0.4–0.6, the specific fluorescence was measured by dividing the fluorescence of the sample by the OD.

For measurement of β-D-glucuronidase (GusA) activity on agar plates, AMA was supplemented with 5-bromo-4-chloro-3-indolyl β-D-glucuronic acid (X-Glc) to a final concentration of 40 µg ml<sup>-1</sup> and for LacZ, 5-bromo-4-chloro-3-indolyl β-D-galactopyranoside (X-Gal) was added at a final concentration of 40 µg ml<sup>-1</sup>. In liquid culture, β-glucuronidase activity was measured as previously described for β-galactosidase reactions (Lodwig *et al.*, 2004), except that *p*-nitrophenyl-β-D-glucuronide was substituted as the chromogenic substrate.

**Microscopy.** Microscopy was performed with a Carl Zeiss Axioskop2.0 epifluorescence microscope with appropriate fluorescence sets. Images were captured using an Axiocam digital camera. For GFPmut3.1 and DsRed the FITC filter set (no. 10, 450–470 nm excitation band pass), and the rhodamine filter set (no. 15, 450–490 nm excitation band pass), respectively, were used.

**Plant growth and inoculation.** Vetch (*Vicia sativa*) seeds were surface-sterilized in 95% ethanol for 30 s and then immersed in a solution of 2% sodium hypochlorite for 10 min. The seeds were washed extensively with sterile water and then allowed to germinate on Falcon tube slopes made from 0.75% agarose containing nitrogen-free rooting solution (Poole *et al.*, 1994a) for 3 days in the dark. The plants were then inoculated with 10<sup>3</sup>–10<sup>5</sup> c.f.u. bacteria. The tubes were then placed in a growth chamber (23 °C, 16 h/8 h light/dark period). Three to seven days post-inoculation, the plants were examined for the formation of infection threads.

## RESULTS AND DISCUSSION

In this work we have made a family of vectors which contain either *gfpmut3.1*, the unstable derivatives of *gfpmut3.1*,

**Table 1.** Bacterial strains and plasmids used in this study

Ap, ampicillin; Gm, gentamicin; Tc, tetracycline.

Strain or plasmid	Description	Source/reference
<b><i>E. coli</i></b>		
<i>E. coli</i> TOP10	F <sup>-</sup> <i>mcrA</i> Δ( <i>mrr-hsdRMS-mcrBC</i> )φ80 <i>lacZ</i> ΔM15 Δ <i>lacX74 recA1 ara</i> Δ139Δ( <i>ara-leu</i> )7697 <i>galU galK rpsL</i> (Str <sup>R</sup> ) <i>endA1 nupG</i>	Invitrogen
<i>E. coli</i> DH5α T-1	F <sup>-</sup> φ80 <i>lacZ</i> ΔM15 Δ( <i>lacZYA-argF</i> ) U169 <i>recA1 endA1 hsdR17</i> (r <sub>k</sub> <sup>-</sup> , m <sub>k</sub> <sup>+</sup> ) <i>phoA supE44 thi-1 gyrA96 relA1 tonA</i>	Invitrogen
<b><i>R. leguminosarum</i></b>		
3841	Str derivative of <i>R. leguminosarum</i> biovar <i>viciae</i> strain 300	Johnston & Beringer (1975)
RU1416	3841 containing pJP2	This work
RU1683–RU1692	3841 with plasmids pRU1119–pRU1128 containing <i>dctA<sub>p</sub></i>	This work
RU1708	3841 containing pRU1140	This work
RU1709	3841 containing pRU1141	This work
RU1712	3841 containing pRU1147	This work
RU1713	3841 containing pRU1148	This work
RU1715	3841 containing pRU1156	This work
RU1716	3841 containing pRU1157	This work
RU1724	3841 containing pRU1164	This work
RU1725	3841 containing pRU1161	This work
RU1728	3841 containing pRU1167	This work
<b>Plasmids</b>		
pCR2.1TOPO	Ap <sup>r</sup> , Km <sup>r</sup> ; PCR product cloning vector	Invitrogen
pOT2	Gm <sup>r</sup> ; promoter probe vector with promoterless <i>gfpUV</i>	Allaway <i>et al.</i> (2001)
pJP2	Tc <sup>r</sup> ; stable broad-host-range cloning vector	Prell <i>et al.</i> (2002)
pBJA27	Ap <sup>r</sup> ; <i>gfpmut3.1</i>	Anderson <i>et al.</i> (1998)
pBJA110	Ap <sup>r</sup> ; <i>gfpmut3.1</i> (LAA)	
pBJA111	Ap <sup>r</sup> ; <i>gfpmut3.1</i> (LVA)	
pBJA112	Ap <sup>r</sup> ; <i>gfpmut3.1</i> (AAV)	
pBJA113	Ap <sup>r</sup> ; <i>gfpmut3.1</i> (ASV)	
DsRed2.0	Ap <sup>r</sup> ; <i>dsRed2.0</i> from <i>Discosoma</i>	Clontech
DsRedT.3, DsRedT.4	Ap <sup>r</sup> ; fast-maturing versions of <i>dsRed2.0</i>	Bevis & Glick (2002)
mRFP1	Ap <sup>r</sup> ; mRFP1 from <i>Discosoma</i> in pRSET <sub>B</sub>	Campbell <i>et al.</i> (2002)
pMN402	Hygromycin <sup>r</sup> ; <i>gfp</i> +	Scholz <i>et al.</i> (2000)
pMP220	Tc <sup>r</sup> ; IncP broad-host-range mobilizable promoter probe vector employing <i>E. coli lacZ</i> as reporter gene	Spaink <i>et al.</i> (1987)
pRK2013	Km <sup>r</sup> ; ColEI replicon with RK2 <i>tra</i> genes, helper plasmid used for mobilizing plasmids	Figurski & Helinski (1979)
pRSET <sub>B</sub>	Ap <sup>r</sup> ; T7 expression vector	Invitrogen
pRU491	Gm <sup>r</sup> ; <i>SpeI</i> – <i>HindIII</i> fragment containing the <i>dpp</i> promoter from <i>R.</i> <i>leguminosarum</i> strain 3841	This work
pRU604	Gm <sup>r</sup> ; <i>PmeI</i> – <i>HindIII</i> fragment containing the xylose kinase promoter	This work
pRU977	pRU1701 containing the <i>dctA</i> promoter	This work
pRU1064	Tc <sup>r</sup> ; <i>HindIII</i> – <i>SacI</i> fragment containing <i>gfpUV</i> from pOT2 cloned into pJP2	This work
pRU1097	Gm <sup>r</sup> ; p318/p319 PCR product ( <i>gfpmut3.1</i> ) from pBJA27 cloned in pOT2 as <i>SpeI</i> – <i>SacI</i>	This work
pRU1098	Gm <sup>r</sup> ; p318/p320 PCR product; ( <i>gfpmut3.1</i> LAA) from pBJA110 cloned in pOT2 as <i>SpeI</i> – <i>SacI</i>	This work
pRU1099	Gm <sup>r</sup> ; p318/p321 PCR product ( <i>gfpmut3.1</i> LVA) from pBJA111 cloned in pOT2 as <i>SpeI</i> – <i>SacI</i>	This work
pRU1100	Gm <sup>r</sup> ; p318/p322 PCR product ( <i>gfpmut3.1</i> AAV) from pBJA112 cloned in pOT2 as <i>SpeI</i> – <i>SacI</i>	This work
pRU1101	Gm <sup>r</sup> ; p318/p323 PCR product ( <i>gfpmut3.1</i> ASV) from pBJA113 cloned in pOT2 as <i>SpeI</i> – <i>SacI</i>	This work
pRU1102	Gm <sup>r</sup> ; p201/p203 PCR product ( <i>gusA</i> ) from pJP2 cloned in pOT2 as <i>SpeI</i> – <i>SacI</i>	This work

**Table 1.** cont.

Strain or plasmid	Description	Source/reference
pRU1103	Gm <sup>r</sup> ; p348/p349 PCR product ( <i>lacZ</i> ) from pMP220 cloned in pOT2 as <i>SpeI</i> - <i>XhoI</i>	This work
pRU1104	Gm <sup>r</sup> ; p346/p347 PCR product ( <i>DsRed2.0</i> ) from <i>DsRed2.0</i> cloned in pOT2 as <i>SpeI</i> - <i>SacI</i>	This work
pRU1105	Gm <sup>r</sup> ; p385/p386 PCR product ( <i>DsRedT.3</i> ) from <i>DsRedT.3</i> cloned in pOT2 as <i>SpeI</i> - <i>SacI</i>	This work
pRU1106	Gm <sup>r</sup> ; p385/p386 PCR product ( <i>DsRedT.4</i> ) from <i>DsRedT.4</i> cloned in pOT2 as <i>SpeI</i> - <i>SacI</i>	This work
pRU1119–pRU1128	Gm <sup>r</sup> ; <i>dctA</i> promoter from pRU977 cloned in pRU1097–pRU1106, respectively, using <i>SphI</i> - <i>SstII</i> sites	This work
pRU1140	Gm <sup>r</sup> ; <i>dpp</i> promoter from pRU491 cloned in pRU1104 as <i>SpeI</i> - <i>HindIII</i> sites	This work
pRU1141	Gm <sup>r</sup> ; <i>dpp</i> promoter from pRU491 cloned in pRU1105 as <i>SpeI</i> - <i>HindIII</i> sites	This work
pRU1144	Gm <sup>r</sup> ; p408/p409 PCR product ( <i>mRFP1</i> ) from pRSET <sub>B</sub> cloned in pOT2 as <i>SpeI</i> - <i>SacI</i>	This work
pRU1147	Tc <sup>r</sup> ; xylose promoter from pRU604 cloned in pRU1144 using <i>PmeI</i> - <i>HindIII</i> sites	This work
pRU1148	Gm <sup>r</sup> ; <i>dpp</i> promoter from pRU491 cloned in pRU1144 as <i>SpeI</i> - <i>HindIII</i> sites	This work
pRU1156	Tc <sup>r</sup> ; <i>HindIII</i> - <i>SacI</i> fragment containing <i>gfpmut3.1</i> from pRU1097 cloned into pJP2	This work
pRU1157	Tc <sup>r</sup> ; xylose promoter from pRU604 cloned in pRU1156 using <i>PmeI</i> - <i>HindIII</i> sites	This work
pRU1161	Tc <sup>r</sup> ; <i>HindIII</i> / <i>SacI</i> fragment containing <i>mRFP1</i> from pRU1144 cloned into pJP2	This work
pRU1164	Tc <sup>r</sup> ; <i>dpp</i> promoter from pRU491 cloned in pRU1161 as <i>SpeI</i> - <i>HindIII</i> sites	This work
pRU1167	Tc <sup>r</sup> ; xylose promoter from pRU604 cloned in pRU1064 using <i>PmeI</i> - <i>HindIII</i> sites	This work
pRU1701	Gm <sup>r</sup> ; promoter probe vector with promoterless <i>gfp+</i>	Hosie <i>et al.</i> (2002)
pRU1716	Gm <sup>r</sup> ; <i>SphI</i> - <i>SstII</i> fragment containing <i>dctA</i> promoter in pOT2	Poole <i>et al.</i> (1994b)

**Table 2.** Primers used in the study

For all unstable GFP derivatives, p318 was used as the forward primer in conjunction with the respective reverse primers. *SacI* and *SpeI* restriction sites are in bold type.

Primer	Sequence	Gene
P201	GAGAGAGA <b>ACTAGT</b> GGAGGAAGAAAAAATGTTACGTCCTGTAGAAAC	<i>gusA</i> forward
P203	GAGAGAGAG <b>AGCTCT</b> CATTGTTTGCCCTCCCTGCT	<i>gusA</i> reverse
P318	GAGAGAGA <b>ACTAGT</b> GGAGGAAGAAAAAATGCGTAAAGGAGAAGA <b>ACTTTTCA</b>	<i>mut3.1</i> forward
p319	CTCTC <b>GAGCTC</b> ATTGTATAGTTTCATCCATGC	<i>mut3.1</i> reverse
p320	CTCTC <b>GAGCTC</b> ATTAAGCTGCTAAAGCGTAG	<i>mut3.1LAA</i> reverse
p321	CTCTC <b>GAGCTC</b> ATTA <b>AACTGCTGC</b> AGCGTAG	<i>mut3.1AAV</i> reverse
p322	CTCTC <b>GAGCTC</b> ATTAAGCTACTAAAGCGTAG	<i>mut3.1LVA</i> reverse
p323	CTCTC <b>GAGCTC</b> ATTA <b>AACTGATGC</b> AGCGTAG	<i>mut3.1ASV</i> reverse
p346	GAGAA <b>CTAGT</b> GGAGGAAGAAAAAATGGCCTCCTCCGAGAACGTCATC	<i>DsRed2.0</i> forward
p347	CT <b>GAGCTC</b> CTACAGGAACAGGTGGTGGCGG	<i>DsRed2.0</i> reverse
p348	CC <b>ACTAGT</b> GGAGGAAGAAAAAATGACCATGATTACGGATTC	<i>lacZ</i> forward
p349	GAG <b>ACTCGAGT</b> TATTTTGGACACCGACCA	<i>lacZ</i> reverse
p385	GAGAGAGA <b>ACTAGT</b> GGAGGAAGAAAAAATGGCCTCCTCCGAGGACGTC	<i>DsRedT.3</i> & <i>T.4</i> forward
p386	AAA <b>AGAGCTC</b> CTACAGGAACAGGTGGTGGCGG	<i>DsRedT.3</i> & <i>T.4</i> reverse
p408	GAGAGAGA <b>ACTAGT</b> GGAGGAAGAAAAAATGGCCTCCTCCGAGGACGTC	<i>mRFP1</i> forward
p409	AA <b>ATTGAGCTC</b> TTAGGCGCCGGTGGAGTGGCG	<i>mRFP1</i> reverse

**Table 3.** Summary of reporter fusions

Plasmid	Replicon	Size (kb)	Accession number	Resistance	Reporter	Excitation maximum (nm)	Emission maximum (nm)	Detection/uses*
pOT2	pBBR	5.3	AJ310442	Gentamicin	GFPUV	395	509	Plates
pRU1701	pBBR	5.3	AJ851277	Gentamicin	GFP +	491	512	Plates/microscopy
						(Shoulder at 396)		
pRU1097	pBBR	5.3	AJ851278	Gentamicin	GFPmut3.1	488	509	Microscopy
pRU1098	pBBR	5.3	AJ851279	Gentamicin	Unstable GFPmut3.1 LAA	488	509	Microscopy
pRU1099	pBBR	5.3	AJ851280	Gentamicin	Unstable GFPmut3.1 AAV	488	509	Microscopy
pRU1100	pBBR	5.3	AJ851281	Gentamicin	Unstable GFPmut3.1 LVA	488	509	Microscopy
pRU1101	pBBR	5.3	AJ851282	Gentamicin	Unstable GFPmut3.1 ASV	488	509	Microscopy
pRU1103	pBBR	7.9	AJ851283	Gentamicin	LacZ			Plates/sectioning
pRU1104	pBBR	5.3	AJ851284	Gentamicin	DsRed2.0	561	586	Microscopy/dual labelling
pRU1105	pBBR	5.3	AJ851285	Gentamicin	DsRedT.3	560	587	Microscopy/dual labelling
pRU1106	pBBR	5.3	AJ851286	Gentamicin	DsRedT.4	555	586	Microscopy/dual labelling
pRU1144	pBBR	5.3	AJ851287	Gentamicin	mRFP1	584	607	Microscopy/dual labelling
pRU1064	IncP	13	AJ851288	Tetracycline	GFPUV–GusA	395	509	Plates/sectioning
pRU1156	IncP	13	AJ851292	Tetracycline	GFPmut3.1–GusA	488	509	Microscopy/sectioning
pRU1161	IncP	13	AJ851291	Tetracycline	mRFP1–GusA	584	607	Microscopy/dual labelling

\*Plates, growth on agar plates with detection using a long-wavelength transilluminator; microscopy, suitable for fluorescence microscopy or FACS; sectioning, chromogenic reporter protein allowing sectioning and histological staining; dual labelling, fluorescent microscopy of a red fluorescent protein in conjunction with GFP.

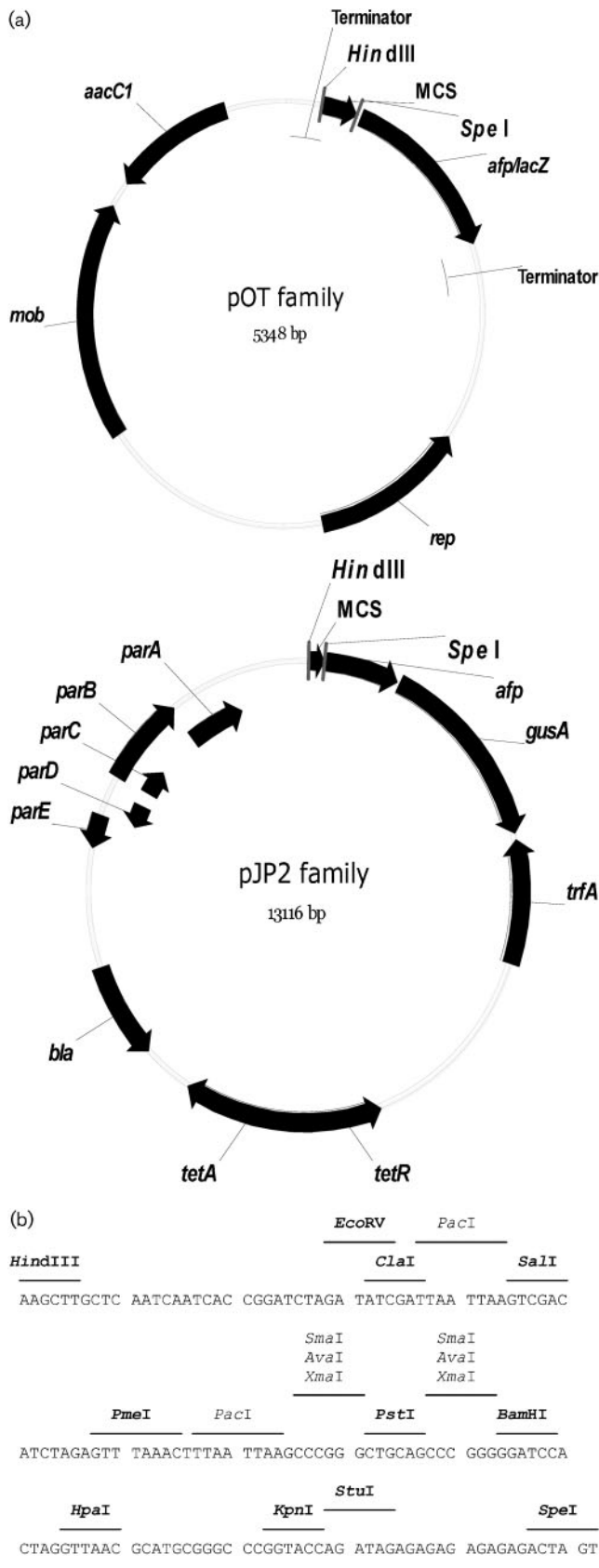
*gfp+*, *dsRed2*, *T.3*, *T.4* or *mRFP1* in two broad-host-range vectors. The first vector is based on pOT (pBBR incompatibility group) and the second, pJP2, is based on the ultra-stable pTR101 (incP incompatibility group). They have gentamicin (pOT) or tetracycline (pJP2) resistance markers, so they can be introduced into the same background. The properties of the vectors are summarized in Table 3 and Fig. 1. The sequences of the vectors were deduced from the known sequences of their components and the accession numbers are shown (Table 3).

### Construction of pOT-based promoter probe vectors

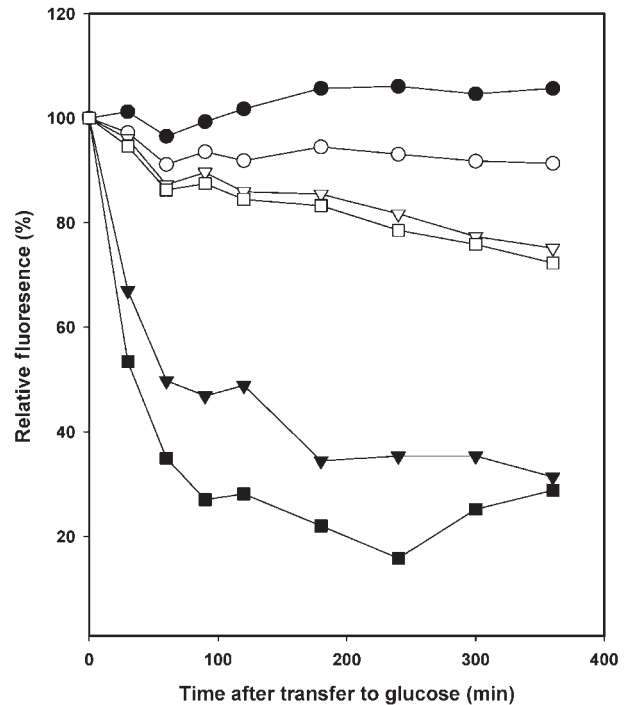
The mutant protein GFPmut3.1 is approximately 35-fold more fluorescent than wild-type GFP when excited at 488 nm, but it is only weakly excited by UV light (Cormack *et al.*, 1996). GFPmut3.1, along with all its unstable derivatives (LAA, AAV, LVA and ASV), was cloned into pOT2 as described in Methods, creating pRU1097, pRU1098, pRU1099, pRU1100 and pRU1101, respectively. To examine the expression of these reporter genes, the dicarboxylate transport system promoter (*dctA<sub>p</sub>*) of *R. leguminosarum* was cloned (Poole *et al.*, 1994b) as a *SphI*–*SstII* fragment (from pRU1716) into all pOT derivatives. *R. leguminosarum* cells containing the plasmids were grown overnight on medium containing succinate to induce the fusions, and then the next day the cells were resuspended in medium containing glucose, to remove the inducer (Fig. 2). The unstable GFP derivatives contain a proteolysis targeting peptide at

their C-termini and this experiment confirmed that *R. leguminosarum* behaves the same as the original *E. coli* strains (Anderson *et al.*, 1998). GFP + is reported to be up to 130-fold brighter than wild-type GFP, so *gfp+* was cloned into pOT2, creating pRU1701. The *dctA* promoter was then cloned into pRU1701, creating pRU977, and *gfp+* was induced specifically by dicarboxylates (Fig. 2). We have previously constructed a pOT2 vector containing GFPUV (Allaway *et al.*, 2001). It should be noted that GFPUV can be used in a plate reader or FAC sorter with excitation at 485 nm, although it gives much lower fluorescence than when excited at 390 nm.

In order to compare expression of GFPmut3.1, GFPUV and GFP + on agar plates, strains containing plasmids with either the inducible *dctA<sub>p</sub>* or the constitutive monocarboxylate permease promoter (*mct<sub>p</sub>*) (Hosie *et al.*, 2002) were grown on AMA with appropriate carbon sources. The expression of GFPUV (excitation  $\lambda_{\max}$  395 nm) and GFP + (excitation  $\lambda_{\max}$  491 nm, but with a shoulder around 390 nm) was visible under the long UV wavelength, but as expected GFPmut3.1 did not fluoresce detectably under this condition (excitation  $\lambda_{\max}$  488 nm). This is a bonus for the use of GFP +, since it can be used both for routine screening of colonies on a long-wavelength transilluminator after growth on agar plates, and in fluorescent plate readers and FAC sorters with excitation at 485 nm. However, it is possible to check the expression of GFPmut3.1 on agar plates with the use of a visi-blue filter (UVB instruments) and this was confirmed with pRU1119.



**Fig. 1.** (a) Maps of pOT- and pJP2-based vectors. (b) Common polylinker of vectors. In (b), unique sites are in bold type.



**Fig. 2.** Stability of Gfp variants in *R. leguminosarum* strain 3841 grown on medium containing succinate then transferred to glucose. All *gfp* variants are fused to the *dctA* promoter. ●, pRU977 (*dctA<sub>p</sub>::gfp+*); ○, pRU1119 [*dctA<sub>p</sub>::gfp(mut3.1)*]; ▼, pRU1120 [*dctA<sub>p</sub>::gfp(mut3.1)-LAA*]; ▽, pRU1121 [*dctA<sub>p</sub>::gfp(mut3.1)-LVA*]; ■, pRU1122 [*dctA<sub>p</sub>::gfp(mut3.1)-AAV*]; □, pRU1123 [*dctA<sub>p</sub>::gfp(mut3.1)-ASV*].

It is often desirable to detect two different AFPs simultaneously. The only other AFP that can be detected at the same time as GFP is DsRed. However, the wild-type DsRed has the disadvantage that it is very slow to mature and is tetrameric. Faster-maturing variants of DsRed have been developed (Bevis & Glick, 2002) and a new monomeric red fluorescent protein, mRFP1, isolated (Campbell *et al.*, 2002). We therefore cloned DsRed2.0 and its fast-maturing derivatives (T.3 and T.4), as well as *mRFP1*, into pOT2, creating pRU1104, pRU1105, pRU1106 and pRU1144, respectively.

### Construction of ultra-stable IncP-based vectors

The environmentally stable IncP plasmid, pTR102, was made by addition of the *parABCDE* genes (Weinstein *et al.*, 1992), and this region was subsequently used to create a stable *gusA* reporter probe vector (pJP2) (Prell *et al.*, 2002). These vectors are very stable upon repeated subculturing and are completely retained in individual bacteroids in legume nodules, as revealed by histological staining (Prell *et al.*, 2002; Weinstein *et al.*, 1992). To construct a vector with tandem *gusA* and AFP, *gfpmut3.1* and its associated multiple cloning site was cloned as a *SacI*-*HindIII* fragment into pJP2, creating pRU1156. In order to check the expression of *gfpmut3.1*, the xylose promoter (*xylA* from

pRU604) of *R. leguminosarum* was cloned as a *PmeI*–*HindIII* fragment into pRU1156, creating pRU1157. The plasmid was conjugated into *R. leguminosarum*, which was grown on AMS medium with glucose or xylose as carbon source, and the specific fluorescence of GFPmut3.1 (764 versus 5890 fluorescence units, respectively) and GusA activity [719 versus 1895 nmol min<sup>-1</sup> (mg prot)<sup>-1</sup>, respectively] was measured.

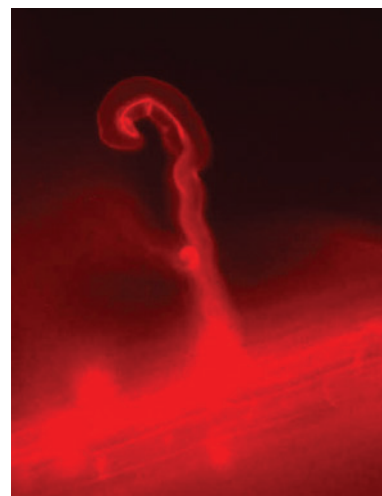
To enable monitoring on agar plates, *gfpUV* from pOT2 was cloned as a *HindIII*–*SacI* fragment into pJP2, deleting the two C-terminal amino acids (YK) and forming pRU1064. The *xylA* promoter was cloned into pRU1064 and this was conjugated into *R. leguminosarum*. After growth on agar plates containing xylose, it was confirmed that expression was inducible and unaffected by the two-amino-acid deletion (data not shown).

To make a DsRed marked vector in the IncP background that is compatible with pOT2, mRFP1 (a monomeric and fast-maturing derivative of DsRed) was cloned into pJP2, creating pRU1161. As a test, the *dpp* promoter from strain 3841 was cloned into pRU1161 as a *SpeI*–*HindIII* fragment to create pRU1164, resulting in strong expression (data not shown).

As a general observation, *R. leguminosarum* strains containing pOT-based plasmids gave higher fluorescence readings than pJP2-based plasmids. This is probably due to plasmid copy number, since RP4-based plasmids such as pJP2 have a modest copy number of around 25 in *E. coli* (Fang & Helinski, 1991) and yields of pOT plasmids isolated from *E. coli* are much higher than those of pJP2. However, this has not been confirmed by measurement of plasmid copy number in *R. leguminosarum*. For environmental work, the pJP2-based plasmids have the advantage of being ultra-stable, with no detectable curing even in single bacteroids stained for GusA activity (Prell *et al.*, 2002). *R. leguminosarum* carrying pOT plasmids retained the plasmid in 48% of cells (78 colonies from 13 nodules) recovered from 4-week-old pea nodules. This result is similar to the value for vetch plants reported previously (Stuurman *et al.*, 2000) and indicates reasonable stability, but clearly pJP2-based vectors are superior for long-term environmental applications.

### Monitoring gene expression *in situ*

AFPs are very useful to monitor gene expression of single cells in the environment. To infect plants, rhizobia must first attach to root hairs before growing down a plant-derived infection thread. This ultimately leads to bacteroid formation, where bacteria are engulfed by plant cortical cells. In order to test the expression of AFPs in infection threads, plasmids pRU1119 (GFPmut3.1) and pRU1127 (DsRedT.3), which are under the control of *dctA<sub>p</sub>*, were inoculated onto vetch seedlings. *dctA<sub>p</sub>* was chosen because its expression in bacteroids is essential for nitrogen fixation (Finan *et al.*, 1981), but it is not known whether it is expressed in infection threads. It can be seen that *dctA<sub>p</sub>::DsRedT.3*



**Fig. 3.** Root hair of vetch containing a fluorescent infection thread (dsRedT.3) formed by *R. leguminosarum* expressing a *dctA* promoter (pRU1127).

was expressed throughout infection threads (Fig. 3), and *dctA<sub>p</sub>::gfpmut3.1* gave a similar result (data not shown). However, dicarboxylates are not the only carbon sources available during nodule development, since dicarboxylate transport mutants develop into bacteroids (Finan *et al.*, 1981). Thus, while dicarboxylates are available from this early stage of contact between the bacteria and the plant, their absolute requirement for nitrogen fixation by mature bacteroids must be related to the final metabolic cycling with the plant (Lodwig *et al.*, 2003). Other compounds, such as sugars, polyols and amino acids, are likely to be available for growth of *R. leguminosarum* in the infection thread. The vector families developed here are powerful tools with which to investigate this problem.

### ACKNOWLEDGEMENTS

We would like to thank the BBSRC for supporting this research and R. Y. Tsien for providing plasmids.

### REFERENCES

- Allaway, D., Schofield, N. A., Leonard, M. E., Gilardoni, L., Finan, T. M. & Poole, P. S. (2001). Use of differential fluorescence induction and optical trapping to isolate environmentally induced genes. *Environ Microbiol* **3**, 397–406.
- Anderson, J. B., Sternberg, C., Poulsen, L. K., Bjorn, S. P., Givskov, M. & Molin, S. (1998). New unstable variants of green fluorescent protein for studies of transient gene expression in bacteria. *Appl Environ Microbiol* **64**, 2240–2246.
- Baird, G. S., Zacharias, D. A. & Tsien, R. Y. (2000). Biochemistry, mutagenesis, and oligomerization of DsRed, a red fluorescent protein from coral. *Proc Natl Acad Sci U S A* **97**, 11984–11989.
- Beringer, J. E. (1974). R factor transfer in *Rhizobium leguminosarum*. *J Gen Microbiol* **84**, 188–198.

- Bevis, B. J. & Glick, B. S. (2002). Rapidly maturing variants of the *Discosoma* red fluorescent protein (DsRed). *Nature Biotechnol* **20**, 83–87.
- Campbell, R. E., Tour, O., Palmer, A. E., Steinbach, P., Baird, G. S., Zacharias, D. A. & Tsien, R. Y. (2002). A monomeric red fluorescent protein. *Proc Natl Acad Sci U S A* **99**, 7877–7882.
- Chalfie, M., Tu, Y., Euskirchen, G., Ward, W. W. & Prasher, D. C. (1994). Green fluorescent protein as a marker for gene expression. *Science* **263**, 802–805.
- Cormack, B. P., Valdivia, R. H. & Falkow, S. (1996). FACS-optimized mutants of the green fluorescent protein (GFP). *Gene* **173**, 33–38.
- Cramer, A., Whitehorn, E. A., Tate, E. & Stemmer, W. P. C. (1996). Improved green fluorescent protein by molecular evolution using DNA shuffling. *Nature Biotechnol* **14**, 315–319.
- Ellenberg, J., Lippincott-Schwartz, J. & Presley, J. F. (1998). Two-color green fluorescent protein time-lapse imaging. *Biotechniques* **25**, 838–846.
- Fang, F. C. & Helinski, D. R. (1991). Broad-host-range properties of plasmid-RK2 – importance of overlapping genes encoding the plasmid replication initiation protein TrfA. *J Bacteriol* **173**, 5861–5868.
- Figurski, D. H. & Helinski, D. R. (1979). Replication of an origin-containing derivative of plasmid RK2 dependent on a plasmid function provided in trans. *Proc Natl Acad Sci U S A* **76**, 1648–1652.
- Finan, T. M., Wood, J. M. & Jordan, C. (1981). Succinate transport in *Rhizobium leguminosarum*. *J Bacteriol* **148**, 193–202.
- Gage, D. J. (2002). Analysis of infection thread development using Gfp- and DsRed- expressing *Sinorhizobium meliloti*. *J Bacteriol* **184**, 7042–7046.
- Hosie, A. H. F., Allaway, D. & Poole, P. S. (2002). A monocarboxylate permease of *Rhizobium leguminosarum* is the first member of a new subfamily of transporters. *J Bacteriol* **184**, 5436–5448.
- Inouye, S. & Tsuji, F. I. (1994). *Aequorea* green fluorescent protein – expression of the gene and fluorescence characteristics of the recombinant protein. *FEBS Lett* **341**, 277–280.
- Johnston, A. W. B. & Beringer, J. E. (1975). Identification of the *Rhizobium* strains in pea root nodules using genetic markers. *J Gen Microbiol* **87**, 343–350.
- Labes, M., Puhler, A. & Simon, R. (1990). A new family of RSF1010-derived expression and *lac*-fusion broad-host-range vectors for Gram-negative bacteria. *Gene* **89**, 37–46.
- Lodwig, E. M., Hosie, A. H. F., Bourdes, A., Findlay, K., Allaway, D., Karunakaran, R., Downie, J. A. & Poole, P. S. (2003). Amino-acid cycling drives nitrogen fixation in the legume-*Rhizobium* symbiosis. *Nature* **422**, 722–726.
- Lodwig, E., Kumar, S., Allaway, D., Bourdès, A., Prell, J., Priefer, U. & Poole, P. (2004). Regulation of L-alanine dehydrogenase in *Rhizobium leguminosarum* bv. *viciae* and its role in pea nodules. *J Bacteriol* **186**, 842–849.
- Miller, W. G., Leveau, J. H. & Lindow, S. E. (2000). Improved *gfp* and *inaZ* broad-host-range promoter-probe vectors. *Mol Plant Microbe Interact* **13**, 1243–1250.
- Poole, P. S., Blyth, A., Reid, C. J. & Walters, K. (1994a). *myo*-Inositol catabolism and catabolite regulation in *Rhizobium leguminosarum* bv. *viciae*. *Microbiology* **140**, 2787–2795.
- Poole, P. S., Schofield, N. A., Reid, C. J., Drew, E. M. & Walshaw, D. L. (1994b). Identification of chromosomal genes located downstream of *dctD* that affect the requirement for calcium and the lipopolysaccharide layer of *Rhizobium leguminosarum*. *Microbiology* **140**, 2797–2809.
- Prell, J., Boesten, B., Poole, P. & Priefer, U. B. (2002). The *Rhizobium leguminosarum* bv. *viciae* VF39 gamma aminobutyrate (GABA) aminotransferase gene (*gabT*) is induced by GABA and highly expressed in bacteroids. *Microbiology* **148**, 615–623.
- Prosser, J. I., Killham, K., Glover, L. A. & Rattray, E. A. S. (1996). Luminescence-based systems for detection of bacteria in the environment. *Crit Rev Biotechnol* **16**, 157–183.
- Reeve, W. G., Tiwari, R. P., Worsley, P. S., Dilworth, M. J., Glenn, A. R. & Howieson, J. G. (1999). Constructs for insertional mutagenesis, transcriptional signal localization and gene regulation studies in root nodule and other bacteria. *Microbiology* **145**, 1307–1316.
- Sambrook, J. & Russell, D. W. (2001). *Molecular Cloning: a Laboratory Manual*, 3rd edn. Cold Spring Harbor, NY: Cold Spring Harbor Laboratory.
- Scholz, Q., Thiel, A., Hillen, W. & Niederweis, M. (2000). Quantitative analysis of gene expression with an improved green fluorescent protein. *Eur J Biochem* **267**, 1565–1570.
- Spaink, H. P., Okker, R. J. H., Wijffelman, C. A., Pees, E. & Lugtenberg, B. J. J. (1987). Promoters in the nodulation region of the *Rhizobium leguminosarum* SYM plasmid pRL1J1. *Plant Mol Biol* **9**, 27–39.
- Stuurman, N., Bras, C. P., Schlaman, H. R. M., Wijffjes, A. H. M., Bloemberg, G. & Spaink, H. P. (2000). Use of green fluorescent protein color variants expressed on stable broad-host-range vectors to visualize rhizobia interacting with plants. *Mol Plant Microbe Interact* **13**, 1163–1169.
- Weinstein, M., Roberts, R. C. & Helinski, D. R. (1992). A region of the broad-host-range plasmid RK2 causes stable in planta inheritance of plasmids in *Rhizobium meliloti* cells isolated from alfalfa root nodules. *J Bacteriol* **174**, 7486–7489.
- Xi, C., Dirix, G., Hofkens, J., De Schryver, F. C., Vanderleyden, J. & Michiels, J. (2001). Use of dual marker transposons to identify new symbiosis genes in *Rhizobium*. *Microb Ecol* **41**, 325–332.
- Yang, F., Moss, L. G. & Phillips, G. N. J. (1996). The molecular structure of green fluorescent protein. *Nature Biotechnol* **14**, 1246–1251.