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FEMS Microbiology Letters 145 (1996) 87–94

FEMS MICROBIOLOGY LETTERS

Construction of GFP vectors for use in Gram-negative bacteria other than *Escherichia coli*

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Received 14 August 1996; accepted 12 September 1996

Abstract

A set of vectors containing a mutated *gfp* gene was constructed for use with Gram-negative bacteria other than *Escherichia coli*. These constructs were: pTn*3gfp* for making random promoter probe *gfp* insertions into cloned DNA in *E. coli* for subsequent introduction into host strains; pUTmini-Tn*5gfp* for making random promoter probe *gfp* insertions directly into host strains; p519*gfp* and p519*ngfp*, broad host range *mob*$^+$ plasmids containing *gfp* expressed from a *lac* and an *npt2* promoter, respectively.

Keywords: Green fluorescent protein; Bacterial promoter probe; Transposon; Plant pathogenic bacteria

1. Introduction

The gene encoding green fluorescent protein (GFP), cloned from the jellyfish *Aquoria victoria* [1] has been used as a visual marker of gene expression and cell structure in studies of eukaryotic organisms [1–3]. Its use in prokaryotic systems has not, however, paralleled its use in eukaryotes, even though the gene has been available commercially for the past few years in *Escherichia coli* vectors (Clontech). Indeed, many initial attempts to use the commercially available clones in bacteria other than *E. coli* generally yielded disappointing results in that fluorescence was poor, and not detectable in single cells. Cormack et al. [4] found that poor fluorescence in bacteria was caused by GFP folding incorrectly and precipitating in the cells. They mutated *gfp* so that resulting mutant proteins folded correctly and remained soluble in the cells. In addition, the excitation optimum was shifted to between 481 and 501 nm [4]. These mutations resulted in GFP fluorescence enhanced about 100 times compared to the wild-type protein when expressed in *E. coli*. In addition, Cormack et al. [4] cloned each mutated gene into an *E. coli* vector such that GFP was expressed from a *tac* promoter with the T7 (gene10) ribosome binding site optimally placed.

Work from our laboratories is focussed on gaining a better understanding of microbial behaviour and

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PII S0378-1097(96)00392-8

activity in situ, generally in complex communities [5–9]. Since a microorganism's activity is controlled at the genetic level, a way to achieve this aim is to investigate the genetic regulation of microbial activity, preferably in single cells in situ. The use of transposon mutagenesis, combined with reporter gene fusion, is a powerful method with which to pursue such studies [9–13].

lacZ and *lux* reporter gene fusions have been used to investigate gene expression in single cells in cell smears [14], as well as in live cells in situ [15–17]. These systems, however, require that the substrates/cofactors for enzyme action penetrate each cell. In addition, enzymatic activity of luciferase requires oxygen and is dependent on cellular ATP levels. GFP requires no substrates or cofactors for activity [4], thus GFP reporter fusions have the potential to be a simpler system for visualisation by microscopy of specific gene expression in single bacterial cells in situ. Our aim in this work was to construct several vectors containing the GFPmut2 mutant gene [4] which will be useful for studies of Gram-negative bacteria other than *E. coli*. These vectors can be used to generate reporter gene fusions or to tag bacteria with a simple phenotypic marker for visualisation and detection in environmental samples.

2. Materials and methods

2.1. Bacterial strains and plasmids

The bacterial strains and plasmids used in this study are described in Table 1. Plasmid constructs

Table 1
Strains and plasmids used in this study

Strain	Genotype	Reference/source
E. coli		
DH5α	*sup*E, Δ*lac* (ϕ80 *lac*ZΔM15), *hsd*R, *rec*A *end*A, *gyr*A, *thi*, *rel*A	[20]
C2110R	Rp^R spontaneous mutant of C2110, Nx^R, *pol*A	this study and [24]
S17-1 λ*pir*	Sm^R, *pro*, *thi*, *hsd*R^-M^+, RP4-2-Tc:Mu-Km:Tn*7*, λ*pir*	[27]
C600	*sup*E, *hsd*R, *thi*, *thr*, *leu*, *lac*Y, *ton*A	[20]
A. tumefaciens		
C58	wild type, virulent	[25]
A. rhizogenes		
K1347	K84 derivative, plasmid free	[28]
P. putida		
UWC1	Rp^R spontaneous mutant, plasmid free	[29]
Plasmid		
GFPmut2	Ap^R, *gfp*$^+$	[4]
pBC SK+	Cm^R	Stratagene
pNJ5000	Tc^R, *tra*$^+$	[30]
pRK2013	Nm^R, *tra*$^+$	[31]
pTn*3*HoHo1	Ap^R (Tn*3*, Δ*tnp* with *lacZ* reporter)	[24]
pSShe	Cm^R (contains *tnp* for Tn*3*)	[24]
pUTmini-Tn*5luxAB*	Ap^R; Tc^R and *lux* reporter on mini-Tn*5*; *mob*+	[12]
pDSK519	RSF1010 derivative, Km^R, *lac* promoter, *mob*$^+$	[32]
pCP13.101	Tc^R, *A. tumefaciens cel* operon, *mob*+	[25]
pBC*gfp*	Cm^R, *gfp*$^+$	this study
pTn*3gfp*	as for pTn3HoHo1, with *gfp* replacing *lacZ*	this study
pUTmini-Tn*5gfp*	as for pUTmini-Tn*5luxAB*, with *gfp* replacing *luxAB*	this study
p519*gfp*	pDSK519 with *gfp* cloned behind *lac* promoter	this study
p519*ngfp*	p519*gfp* with *pnpt2* cloned directly in front of *gfp*	this study

made during this study are shown in Fig. 1. Each plasmid construct, in *E. coli* DH5α, has been lodged with the American Type Culture Collection.

2.2. Growth conditions and media

E. coli strains were grown in Luria broth (LB, [18]) at 37°C, *Pseudomonas putida* UWC1 in LB at 28°C, *Agrobacterium tumefaciens* C58 in LB or in RK minimal medium [19] at 26°C, A. *rhizogenes* strains K1347 in RK medium or in Nutrient Broth (Difco) at 26°C. Agar plates contained 15 g l^{-1} Bitek agar (Sigma). The following antibiotics (Sigma) and concentrations were used when appropriate (μg ml^{-1}): *E. coli*, ampicillin/carbenicillin (Ap/Cb, 50), chloramphenicol (Cm, 10) kanamycin (Km, 50), naladixic acid (Nx, 20), rifampicin (Rp, 100) and tetracycline (Tc, 10); *P. putida*, Rp (50) and Tc (50); Agrobacteria, Cb (50), neomycin (Nm, 60 in plates and 20 in liquid) and Tc (5).

2.3. DNA cloning

Plasmid extractions, restriction enzyme digests, gel-isolated DNA fragment purifications, ligations, transformations and agarose gel electrophoresis were carried out using standard methods [20], and following the manufacturers' instructions where appropriate. Restriction and other enzymes were from New England BioLabs Inc.

2.4. Polymerase chain reaction (PCR)

PCR amplification was achieved using an FTS-320 thermal sequencer (Corbett Research, Australia) with Tth+ DNA polymerase (Biotech International, Australia). The PCR solution contained 1×reaction buffer (67 mM Tris-HCl pH 8.8, 16.6 mM $(NH_4)_2SO_4$, 0.45% Triton X-100, 0.2 mg ml^{-1} gelatin), 2 mM $MgCl_2$, 200 mM dNTPs, 0.05 mM of each primer, 1 unit Tth+ and 10 ng template DNA. A total of 30 cycles were run using the following programme; denaturation at 94°C for 30 s, annealing at appropriate temperature for 30 s and extension at 72°C for 2 min, followed by 1 cycle at 72°C for 10 min. Mineral oil was removed from the PCR product which was extracted once with chloroform/isoamyl alcohol and purified by standard methods. Oligonucleotide primers were purchased from AMRAD Pharmacia Biotech.

The 740 bp *gfp* fragment, including the RBS, was amplified from pBC*gfp* at an annealing temperature of 47°C, using the following primers: *gfpHin*dIII-F (5′-CTCAAGCTTGATTTCTAGATTTAAGAAGG), and *gfpEco*RI-R (5′-CTCGAATTCTCATTATTTGTATAGTTCATCCATGCC). The *npt*2 promoter was amplified as a 158 bp fragment from Tn*5* (Genbank accession no. L19385) at an annealing temperature of 60°C, using the following primers: *pnpt2Hin*dIII-F (5′-CTCAAGCTTGCAGGTAGCTTGCAGTGGG), and *pnpt2Xba*I-R (5′-CTCTCTAGAGCGCCATCAGATCCTTGGCG).

2.5. Conjugation

Conjugations between *A. tumefaciens* or *A. rhizogenes* and *E. coli* using plate matings were carried out as previously described [21–23], using either pRK2013 or pNJ5000 (Table 1) as a helper plasmid where appropriate.

2.6. Microscopy

An epifluorescence Olympus BX50 microscope, fitted with a halogen lamp, a 100 W mercury burner, and a PM-30 automatic photomicrographic system, was used. Photomicrographs were generated using either Nomarski (differential interference contrast) or epifluorescence (excitation 488 nm, emission 520 nm) optics, using a 40× objective with a numerical aperture of 1.0. Living bacterial cells were suspended in 100% glycerol on gelatin coated slides to prevent motility. Cut roots of 1–8-week-old *Arabidopsis thaliana* plants were incubated with 10^6–10^7 bacteria ml^{-1} in a 1 in 10 dilution of Murashige and Skoog salts medium (MS, Gibco BRL) containing 0.4% sucrose.

3. Results and discussion

3.1. pBCgfp with varying restriction enzyme sites for convenient subcloning of gfp

gfp with its ribosome binding site and most of the surrounding restriction enzyme sites was excised

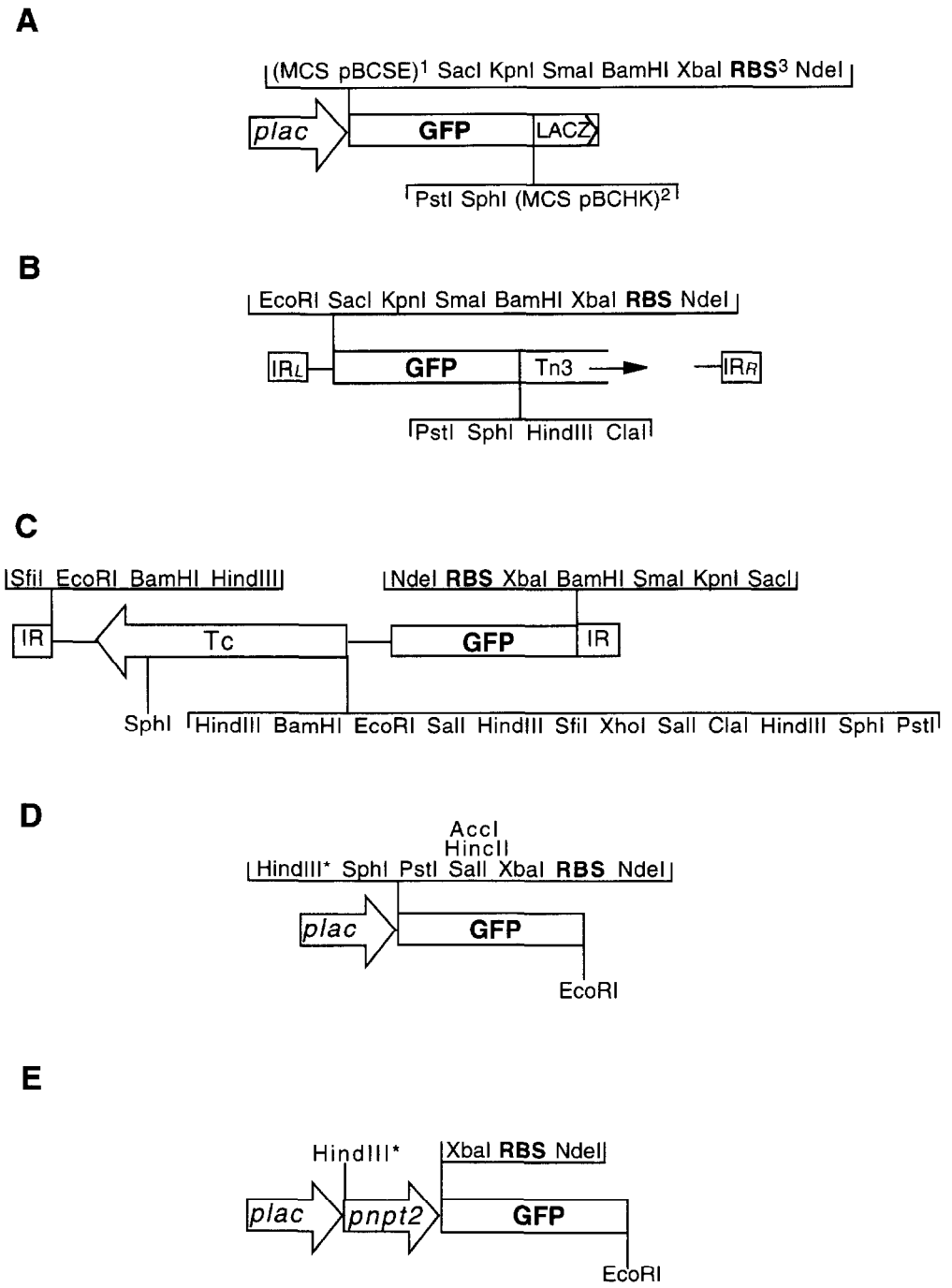

Fig. 1. Restriction enzyme maps of *gfp* constructs. (A) pBC*gfp*; [1]multiple cloning site (MCS) from pBC SK+ from *Sac*I to *Eco*RI inclusive, [2]multiple cloning site from pBC SK+ from *Hin*dIII to *Kpn*I inclusive, [3]ribosome binding site (RBS). (B) Tn*3gfp*; the right end of pTn*3*HoHoI from the *Cla*I site to the right inverted repeat (IR_R) is unchanged, and includes the *bla* gene and a *Sac*Isite. (C) pUTmini-Tn*5gfp*; *lux* in pUTmini-Tn*5luxAB* was replaced by *gfp*. (D) p519*gfp*, *gfp* replaced part of the polylinker downstream of *plac* in pDSK519; *there is a second *Hin*dIII site in the Nm^R gene in the vector. (E) p519*ngfp*, *pnpt2* was inserted between the *Hin*dIII and *Xba*I sites in front of *gfp* in p519*gfp*. Diagrams are not to scale and only show altered parts of the vectors.

from GFPmut2 using *Eco*RI and *Hin*dIII. The resulting approx. 750 bp *gfp* fragment was isolated by agarose gel electrophoresis and ligated into a Cm^R bluescript vector, pBC SK+, which had also been digested with *Eco*RI and *Hin*dIII. The resulting plasmid, pBC*gfp* (Fig. 1A), was transformed into *E. coli* DH5α, selecting for Cm^R transformants. DH5α(pBC*gfp*) cells from liquid cultures were brightly fluorescent under epifluorescence microscopy (Fig. 2A,B). Bacteria grown on agar plates and resuspended in water showed variable fluorescence, but all cells were fluorescent to some degree (data not shown).

3.2. *pTn3gfp for transposition to cloned DNA in E. coli*

The transposon mutagenesis system developed by Stachel et al. [24] using pSShe and pTn*3*HoHo1 has been used to introduce a promoterless β-galactosidase gene into random sites in cloned DNA in *E. coli*. We have modified this system for use with a promoterless *gfp* reporter gene. The plasmids pSShe and pTn*3*HoHo1 were prepared from *E. coli* and separated by gel electrophoresis. pSShe was excised from the gel and used without modification to transform *E. coli* DH5α. Isolated pTn*3*HoHo1 was digested with *Eco*RI and *Cla*1 and fragments separated by gel electrophoresis. The 7 kb vector/Tn*3* fragment was excised, purified, and ligated to the approx. 800 bp fragment resulting from the digestion of pBC*gfp* with *Eco*RI and *Cla*I. The ligation mixture was transformed into *E. coli* DH5α(pSShe) and transconjugants selected for resistance to both Cb and Cm. The resulting plasmid construct was named pTn*3gfp* (Fig. 1B).

To test the ability of *E. coli* DH5α(pSShe, pTn*3gfp*) to introduce the transposon into cloned DNA, a library clone from *A. tumefaciens* containing *cel* genes (pCP13.101 [25]) was transformed into the *E. coli* strain, selecting for resistance to Cm (pSShe), Cb (pTn*3gfp*), and Tc (pCP13.101). The procedure described by Stachel et al. [24] was used to obtain *E. coli* C2110R carrying pCP13.101 with Tn*3gfp* transposon insertions. Cell smears of transconjugant colonies were examined using epifluorescence microscopy and 4 fluorescent isolates were retained. The transposon had inserted into pCP13.101 at a single site in each of the 4 isolates, as determined by restriction mapping. These 4 plasmids were introduced into *A. tumefaciens* C58 by conjugation (using helper plasmid pRK2013), with transconjugants being selected by growth on minimal RK medium containing Cb and Tc. Wild-type *A. tumefaciens* C58(pCP13.101) is not fluorescent at an excitation wavelength of 488 nm. *A. tumefaciens* C58 transconjugants containing each of the 4 pCP13.101::Tn*3gfp* plasmids were fluorescent when incubated with *A. thaliana* root segments (data not shown).

3.3. *pUTmini-Tn5gfp for transposition to random genomic sites in non-E. coli bacteria*

pUTmini-Tn*5luxAB* was digested with *Not*I, blunt ended with DNA polymerase I (Klenow) and phosphatased with calf intestinal alkaline phosphatase. An approx. 800 bp fragment encoding the promoterless *gfp* was isolated from pBC*gfp* by cutting with *Eco*RI and *Xho*I and blunt ending with DNA polymerase I (Klenow). Vector and insert fragments were isolated after separation in an agarose gel, ligated and transformed into *E. coli* S17.1(λpir). Plasmid preparations from Ap and Tc resistant transformants were digested with *Xba*I to determine the orientation of *gfp* in relation to the Tc resistance marker in the mini-Tn*5gfp*. One plasmid containing *gfp* in the correct orientation was named pUTmini-Tn*5gfp* (Fig. 1C).

E. coli S17.1(λpir)(pUTmini-Tn*5gfp*) was conjugated with recipient *A. tumefaciens* C58, *A. rhizogenes* K1347 or *P. putida* UWC1. From plating 200 μl of each mating mixture, >300 transconjugants were recovered on each selection plate. Fifty transconjugants from each plate were patched onto the same selective media and incubated at 28°C overnight. Colonies were resuspended in a drop of water on microscope slides and viewed under epifluorescence microscopy at an excitation of 488 nm. Cells which showed a high level of fluorescence were noted, and their corresponding colonies were restreaked on the selective media. From the 50 colonies tested from each mating, approx. 4–5 containing strongly fluorescent cells were isolated (Fig. 2C–F). In order to examine the potential use of these bacteria, *A. tumefaciens* C58::mini-Tn*5gfp* tagged cells were incubated with roots of *A. thaliana*. Fluorescent

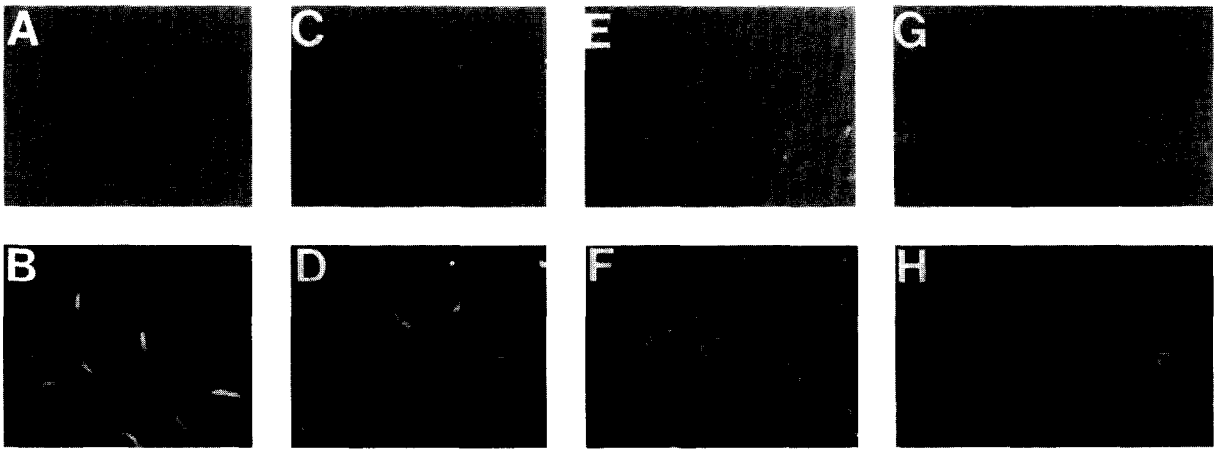

Fig. 2. Photomicrographs of bacteria containing *gfp* contsructs. (A,C,E,G) Taken with Nomarski optics; (B,D,F,H) show the same images, respectively, under epifluorescence microscopy. (A,B) *E. coli*(pBC*gfp*); (C,D) *P. putida*::mini-Tn*5gfp*; (E–H) *A. tumefaciens*::mini-Tn*5gfp*; (E,F) free bacteria; (G,H) bacteria attached to the surface of roots of *Arabidopsis thaliana*. Note in H the bacterial fluorescence is bright in comparison to root autofluorescence.

bacteria adhering to the root surface were clearly visible (Fig. 2G,H).

3.4. *p519gfp and p519ngfp, broad host range mob*$^+$ *plasmids*

The *gfp* and *pnpt2* PCR products were amplified with both primers containing restriction enzyme sites introduced at the 5 prime end to facilitate cloning. In order to construct a broad host range *mob*+ plasmid containing an expressed *gfp*, the approx. 740 bp *gfp* PCR fragment was digested with *Eco*RI and *Xba*I and ligated to pDSK519, also digested with the same restriction enzymes. The ligation mixture was transformed into *E. coli* DH5α cells and transformants were selected for Nm^R. Plasmid DNA was isolated from one transformant that showed green fluoresence under epifluorescence microscopy. This plasmid, p519*gfp* (Fig. 1D), was shown to contain a single copy of the *gfp* fragment in the correct position by restriction enzyme analysis. GFP is expressed in pDSK*gfp* from the *lac* promoter which is known to have poor activity in other Gram-negative bacteria [26]. Therefore, another construct was made in which the *npt2* constitutive promoter was used to express GFP. p519*gfp* DNA was digested with *Xba*I and partially digested with *Hin*dIII. The approx. 9 kb vector fragment was separated by agarose gel electrophoresis, excised, purified and ligated to the 158 bp *npt2* promoter PCR product, also digested with *Hin*dIII and *Xba*I. *Pst*I (the unique site which had been eliminated in the new construct, compare Fig. 1D and E) was added to the ligation mixture immediately prior to transformation into *E. coli* DH5α, to prevent transformation by p519*gfp*. Transformants were selected for Nm^R on LB containing glucose (0.2%) to inhibit *lac* promoter activity. One transformant that showed green fluoresence under epifluorescence microscopy was checked by PCR amplification with the primers *pnpt2Hin*dIII-F and *gfpEco*RI-R at an annealing temperature of 50°C and was shown to contain the correctly sized, approx. 900 bp, fragment containing *pnpt2-gfp*. This plasmid was named p519*ngfp* (Fig. 1E). *E. coli* DH5α(p519*gfp*) grown on LB containing glucose (0.2%) to reduce expression from the *lac* promoter, fluoresced less brightly than when grown without glucose. The addition of glucose to the medium had no affect on the fluorescence of *E. coli* DH5α(p519*ngfp*) cells indicating that the *npt2* promoter in p519*ngfp* was functional.

E. coli DH5α carrying either pDSK519, p519*gfp*, or p519*ngfp*, were conjugated to *A. tumefaciens* C58 using helper plasmid pNJ5000. Transconjugants were selected for Nm^R on minimal medium without glucose and were assessed under epifluorescence microscopy. *A. tumefaciens* cells containing pDSK519 (no *gfp*) did not fluoresce. Cells containing

p519*ngfp* fluoresced more brightly than cells containing p519*gfp*. This is consistent with the work of Labes et al. [26] who showed that the *npt*2 promoter was approximately twice as strong as the *lac* promoter in Rhizobia.

Acknowledgments

We thank Brendan Cormack for the generous gift of plasmid GFPmut2, and Corbett Research Australia for supplying the thermal cycler. A.G.M. thanks the University of North Carolina for a Keenan leave which allowed her to undertake this work at the Flinders University. Part of this work was supported by NSF grant number MCB-9405844 to A.G.M., by a grant from the Patawalonga and Torrens Catchment Water Management Boards to N.C.M., and by a grant from the Flinders University to A.E.G. S.S. was supported by a Flinders University postgraduate scholarship.

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