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Constructs for insertional mutagenesis, transcriptional signal localization and gene regulation studies in root nodule and other bacteria

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Cassettes have been developed that contain an antibiotic resistance marker with and without a promoterless *gusA* reporter gene. The *nptII* (encoding kanamycin resistance) or *aacCI* (encoding gentamicin resistance) genes were equipped with the *tac* promoter (P_{tac}) and the *trpA* terminator (T_{trpA}) and then cloned between *Not*I sites to construct the CAS-Nm (P_{tac}-*nptII*-T_{trpA}) and CAS-Gm (P_{tac}/P_{aacCI}-*aacCI*-T_{trpA}) cassettes. The markers were also cloned downstream to a modified promoterless *Escherichia coli gusA* gene (containing TGA stop codons in all three reading frames prior to its RBS and start codon) to construct the CAS-GNm (*gusA*-P_{tac}-*nptII*-T_{trpA}) or CAS-GGm (*gusA*- P_{tac}/P_{aacCI}-*aacCI*-T_{trpA}) cassettes. Cassettes containing the promoterless *gusA* create type I fusions with a target DNA sequence to detect transcriptional activity. The promoterless *gusA* gene has also been cloned into a broad-host-range IncP1 plasmid. This construct will enable transcriptional activity to be monitored in different genetic backgrounds. Each cassette was cloned as a *Not*I fragment into the *Not*I site of a pUT derivative to construct four minitransposons. The mTn*5*-Nm (containing P_{tac}-*nptII*-T_{trpA}) and mTn*5*-Gm (containing P_{tac}/P_{aacCI}-*aacCI*-T_{trpA}) minitransposons have been constructed specifically for insertional inactivation studies. The minitransposons mTn*5*-GNm (containing *gusA*-P_{tac}-*nptII*-T_{trpA}) and mTn*5*-GGm (containing *gusA*-P_{tac}/P_{aacCI}-*aacCI*-T_{trpA}) can be used for transcription signal localization or insertional inactivation. The TAC-31R and TAC-105F primers can be used to sequence DNA flanking both sides of CAS-Nm, CAS-Gm, mTn*5*-Nm and mTn*5*-Gm. The WIL3 and TAC-105F primers can be used to sequence DNA flanking both sides of CAS-GNm, CAS-GGm, mTn*5*-GNm and mTn*5*-GGm. The specific application of these constructs to generate acid- or nodule-inducible fusions is presented. The new constructs provide useful tools for insertional mutagenesis, transcriptional signal localization and gene regulation studies in the root nodule bacteria and possibly other Gram-negative bacteria.

Keywords: *gusA*, induction, *phr* genes, reporter, *Rhizobium*

INTRODUCTION

Transposon Tn*5* has become a valuable tool for genetic analysis, primarily because it can transpose with little specificity in a wide variety of Gram-negative bacteria (Berg & Howe, 1989; Reznikoff, 1993). The inclusion of a promoterless reporter gene into the transposon provides a powerful tool to follow gene expression and regulation in these prokaryotes (Silhavy & Beckwith, 1985; Simons *et al.*, 1987).

The *Escherichia coli gusA* gene has gained widespread

Abbreviations: mTn*5*, minitransposon Tn*5*; X-GlcA, 5-bromo-4-chloro-3-indolyl β-D-glucuronide.

The GenBank accession numbers for the sequences of the minitransposons mTn*5*-Nm, mTn*5*-GNm, mTn*5*-Gm and mTn*5*-GGm are AF080389, AF080390, AF080391 and AF080392, respectively.

acceptance as a reporter since its product (β-glucuronidase) can be quantified by a simple and sensitive assay (Jefferson *et al.*, 1986). β-Glucuronidase-positive (coloured) colonies can be easily differentiated from those that are negative (colourless) on solid media containing a substrate such as X-GlcA (5-bromo-4-chloro-3-indolyl β-D-glucuronide, cyclohexyl-ammonium salt).

A promoterless *gusA* gene has been cloned into the transposon Tn5 and successfully applied to detect genes whose transcriptional pattern is altered by a specific signal (Sharma & Signer, 1990). However, the integration of the Tn5 transposon into a bacterial cell inhibits secondary Tn5 mutagenesis due to expression of a transposition inhibitor encoded by the *inh* gene present within the transposon (Reznikoff, 1993). The expression of transposase from the *tnpA* gene within the transposon may also result in further transpositional events (Reznikoff, 1993). The minitransposon Tn5s (mTn5s) developed by de Lorenzo *et al.* (1990) and Herrero *et al.* (1990) provide a set of derivatives that overcome the problems discussed above. The *gusA* reporter has been cloned into the mTn5 to provide a set of derivatives valuable for ecological studies (Wilson *et al.*, 1995). However, these *gusA*-containing mTn5 derivatives all contain the Ω-Sm/Sp interposon that provides resistance to both streptomycin and spectinomycin, thus restricting host range to those bacteria that are sensitive to at least one of these antibiotics.

This paper describes alternative minitransposons that overcome the problems described above and may be used to complement the Tn5 derivatives already in existence. The number of restriction sites in the mTn5s described in this paper has been significantly reduced to increase the range of endonucleases that can be used to cut mutated target DNA in preparation for cloning. The promoterless *gusA*-containing constructs have been specifically designed to detect transcriptional activity and have been used to generate fusions that are activated by a nodule specific or low pH signal in the agriculturally important root nodule bacterium *Sinorhizobium meliloti*.

METHODS

Bacterial strains, plasmids and media. Bacterial strains and plasmids used in this study are listed in Table 1. *E. coli* strains were grown at 37 °C on LB medium (Miller, 1972) or Antibiotic Medium No. 3 (Oxoid) when using gentamicin. Root nodule bacterial strains were grown in either TY medium or JMM minimal medium (O'Hara *et al.*, 1989). Media were supplemented with the following concentrations of antibiotics (μg ml^{-1}): ampicillin (100), chloramphenicol (20), gentamicin (7·5 for *E. coli*, 40 for *S. meliloti*), kanamycin (50), streptomycin (30 for *E coli*, 200 for *S. meliloti* or *Rhizobium leguminosarum*), tetracycline (20). The β-glucuronidase substrate X-GlcA was incorporated in solid medium at 50 μg ml^{-1} and in root staining buffer at 300 μg ml^{-1}.

Plasmid transfer and mutagenesis. Plasmid DNA was mobilized from an *E. coli* strain such as DH5α (not containing RP4 DNA) into a rhizobial strain by performing a triparental mating with HB101(pRK2013). Plasmids containing the R6K origin of replication were mobilized directly from BW20767 (an RP4 integrant containing the *pir* gene) into a rhizobial strain using a biparental mating. Cells were prepared for conjugation as described by O'Hara *et al.* (1989). Prior to conjugation, cultures were concentrated by centrifugation for 30 s in a microfuge. The cell pellets were resuspended in 100 μl antibiotic-free TY broth. This mating mixture was incubated on a TY plate overnight at 28 °C and then resuspended in 1 ml normal saline. For long-term storage at −80 °C, glycerol was added to the mating mixture to give a final concentration of 10%. Transconjugants of *S. meliloti* were selected by plating samples of the mating mixture onto TY plates containing chloramphenicol (to prevent growth of *E. coli*) and an additional antibiotic (dependent on the cassette or plasmid transferred). For the selection of *R. leguminosarum* PW711 transconjugants, streptomycin was used instead of chloramphenicol. Low-pH-inducible mutants of *S. meliloti*, or mutants displaying very low level β-glucuronidase expression, were isolated by replica-patching kanamycin-resistant colonies onto JMM (pH 7·0 and 5·7) plates containing X-GlcA and kanamycin.

DNA preparation and manipulation. Competent *E. coli* cells were prepared and transformed with plasmid DNA as described by Inoue *et al.* (1990). Plasmid or genomic DNA isolation and manipulation techniques were as described by Sambrook *et al.* (1989) and Reeve *et al.* (1997). Restriction and modification enzymes were purchased from either Life Technology or Boehringer Mannheim. When required, the 'sticky ends' generated from restriction enzyme digestion were blunted using the Klenow fragment of DNA polymerase I. Reverse-phase cartridge-purified oligonucleotide primers were synthesized by Bresatec. Probe preparation, labelling and hybridization were carried out as described by Tiwari *et al.* (1996a).

Construction of a promoterless *gusA* gene suitable for transcriptional analysis. The ΩKm interposon (Fellay *et al.*, 1987) was cloned as an *Eco*RI fragment into the *Eco*RI site of p18Not (Herrero *et al.*, 1990) to construct pCRS400. This plasmid was digested with *Hin*dIII and re-ligated to form pCRS410. The construct pCRS410 was digested with *Eco*RI and *Bam*HI, the sites blunted and then re-ligated to construct pCRS416. The *Not*I insert in this vector contains stop codons in three different frames followed by a unique *Hin*dIII site. The promoterless *gusA* gene from pCAM140 (Wilson *et al.*, 1995) was excised as a blunted *Eco*RI–*Not*I fragment and cloned into a blunted *Hin*dIII site of pCRS416 to construct pCRS426. The *Not*I cassette in pCRS426 containing the promoterless *gusA* was named CAS-G.

Construction of antibiotic cartridges. A truncated *nptII* gene was developed in the following manner. A promoterless *nptII* gene was excised as a blunted *Bcl*I–*Hin*dIII fragment from pCRS399 and cloned into a blunted *Bam*HI site present within the plasmid pTACTER (Wilson *et al.*, 1995). The resulting plasmid was digested with *Bam*HI and partially digested with *Eco*RI, the sites blunted and then the DNA was re-ligated to construct pCRS447. A *Hin*dIII fragment from this plasmid containing *nptII* was blunted and cloned into blunted *Eco*RI- and *Hin*dIII-digested p18Not to construct pCRS563. The *Not*I cassette containing P_{tac} (*tac* promoter)-*nptII*-T_{trpA} (*trpA* terminator) in the latter plasmid was named CAS-Nm.

The *aacCI* gene (encoding gentamicin resistance) from pMS272 (Becker *et al.*, 1995) was excised as a blunted *Bam*HI

Table 1. List of strains and plasmids used

Strain/plasmid	Relevant characteristics*	Source/reference
Strains		
E. coli		
BW20767	RP4-2-*tet*::Mu-1*kan*::Tn7 integrant *leu-63*::IS*10 recA1 creC510 hsdR17 endA1 zbf-5 uidA*(ΔMluI)::*pir*$^+$ *thi*	Metcalf *et al.* (1996)
DH5α	F$^-$ *ϕ*80d*lacZ*ΔM15 *recA1 endA1 gyrA96 thi-1 hsdR17* ($r_K^- m_K^+$) *supE44 relA1 deoR* Δ(*lacZYA–argF*)U169	Bethesda Research Laboratory (1986)
HB101	F$^-$ *thi-1 hsdS20* ($r_B^- m_B^-$) *supE44 recA13 ara-14 leuB6 proA2 lacY1 rpsL20* (Sm^r) *xyl-5 mtl-1*	Boyer & Roulland-Dussoix (1969)
Bradyrhizobium sp. (Lupinus)		
WU425	Commercial inoculant for Lupinus	This study
R. leguminosarum bv. *viciae*		
PW711	Spontaneous Sm^r mutant of WSM710	This study
WSM710	Strain isolated from *Vicia* spp. in Japan	This study
S. meliloti		
WR101	Acid^t mTn5-GNm derivative of WSM419; Cm^r Km^r	This study
WR1A	Acid^t mTn5-GNm derivative of WSM419; Cm^r Km^r	This study
WR4A	Acid^t mTn5-GNm derivative of WSM419; Cm^r Km^r	This study
WR5A	Acid^t mTn5-GNm derivative of WSM419; Cm^r Km^r	This study
WSM419	Acid^t Sardinian isolate; Cm^r	This study
Plasmids		
p18Not	pUC18 derivative with *Not*I-*Eco*RI-*Sal*I-*Hin*dIII-*Not*I as MCS; Ap^r	Herrero *et al.* (1990)
pAB2001	pUC6S derivative containing *lacZ*-Gm^r cassette; Ap^r Gm^r	Becker *et al.* (1995)
pCAM140	pUT::mTn*5SSgusA40*; Ap^r Sm^r Sp^r	Wilson *et al.* (1995)
pCRS399	pUC18 derivative containing a blunted *Eco*RI ΩTc interposon cloned into a blunted *Sfu*I site downstream from the stop codon of *nptII*; Ap^r Km^r Tc^r	This study
pCRS400	p18Not containing ΩKm cloned into the *Eco*RI site; Ap^r Km^r	This study
pCRS410	pCRS400 digested with *Hin*dIII and re-ligated to remove ΩKm	This study
pCRS416	pCRS410 derivative with *Eco*RI–*Bam*HI restriction sites removed	This study
pCRS426	pCRS416 derivative containing the CAS-G cassette; Ap^r	This study
pCRS433	*Xho*I deletion derivative of pMP220 devoid of *lacZ*; Tc^r	This study
pCRS447	pTACTER containing *nptII* gene; Ap^r Km^r	This study
pCRS472	p18Not containing CAS-GNm cassette; Ap^r Km^r	This study

Table 1 *(cont.)*

Strain/plasmid	Relevant characteristics*	Source/reference
pCRS482	pUT::mTn*5*-Sm/Sp derivative containing *Not*I CAS-GNm cloned at *Not*I; Ap^r Km^r Sm^r Sp^r	This study
pCRS487	pUT::mTn*5*-GNm; Ap^r Km^r	This study
pCRS518	pTACTER containing *aacCI* gene; Ap^r Gm^r	This study
pCRS520	pUK21 derivative containing *aacCI* from pCRS518; Km^r Gm^r	This study
pCRS529	p18Not derivative containing CAS-Gm; Ap^r Gm^r	This study
pCRS530	pAB2001 derivative containing the CAS-GNm cassette flanked by *Not*I-*Hin*dIII-*Eco*RI-*Sma*I sites for excision; Ap^r Km^r	This study
pCRS536	pCRS433 derivative with a *Hin*dIII fragment from WR101 containing *lpiA*::mTn*5*-GNm; Km^r Tc^r	This study
pCRS538	pUT::mTn*5*-Gm; Ap^r Gm^r	This study
pCRS542	pCRS426 derivative containing the CAS-GGm cassette; Ap^r Gm^r	This study
pCRS543	pUT::mTn*5*-Sm/Sp derivative containing *Not*I CAS-GGm cloned at *Not*I; Ap^r Gm^r Sm^r Sp^r	This study
pCRS548	pUT::mTn*5*-GGm; Ap^r Gm^r	This study
pCRS549	pAB2001 derivative containing the CAS-GGm cassette flanked by *Not*I-*Hin*dIII-*Eco*RI-*Sma*I sites for excision; Ap^r Gm^r	This study
pCRS563	p18Not derivative containing CAS-Nm; Ap^r Km^r	This study
pCRS589	pUT::mTn*5*-Nm; Ap^r Km^r	This study
pFUS1	pMP220 derivative containing a promoterless *gusA*; Tc^r	This study
pFUS1-1	pFUS1 containing *phrR* promoter region; Tc^r	This study
pHP45ΩKm	pHP45Ω derivative with Km^r interposon; Ap^r Km^r	Fellay *et al.* (1987)
pMP220	Broad-host-range *lacZ* fusion vector; Tc^r	Spaink *et al.* (1987)
pMS272	pK18 derivative containing Gm^r cassette; Km^r Gm^r	Becker *et al.* (1995)
pRK2013	Helper plasmid; Km^r	Figurski & Helinski (1979)
pTACTER	pUC8 derivative containing *tac* promoter and *trpA* terminator; Ap^r	Wilson *et al.* (1995)
pTG2-6S	pUC18 containing Km^r *Sal*I fragment of TG2-6; Ap^r Km^r	Goss *et al.* (1990)
pUC18	Cloning vector; Ap^r	Yanisch-Perron *et al.* (1985)
pUK21	Cloning vector; Km^r	Vieira & Messing (1991)

* Acid^t, acid-tolerant; Ap^r, Cm^r, Gm^r, Km^r, Sm^r, Sp^r, Tc^r, resistant to ampicillin, chloramphenicol, gentamicin, kanamycin, streptomycin, spectinomycin, tetracycline; MCS, multiple cloning site.

fragment and cloned into a blunted *Bam*HI site of pTACTER to form pCRS518. The plasmid pCRS518 was digested with *Hin*dIII, blunted and the fragment containing *aacCI* was cloned into blunted *Eco*RI- and *Hin*dIII-digested p18Not to construct pCRS529. The *Not*I cassette containing P_{tac}/P_{aacCI}-*aacCI*-T_{trpA} was named CAS-Gm.

Promoterless *gusA-nptII* or *gusA-aacCI* linked cartridges. The plasmids pCRS447 and pCRS518 were digested with *Hin*dIII, blunted and the fragment containing either the *nptII* gene or the *aacCI* gene was cloned into a blunted *Hin*dIII site of pCRS426 to construct pCRS472 (the *Sal*I site present within the cassette was destroyed) or pCRS542, respectively. The *Not*I cassette from either pCRS472 or pCRS542 was named CAS-GNm (*gusA*-P_{tac}-*nptII*-T_{trpA}) or CAS-GGm (*gusA*-P_{tac}/P_{aacCI}-*aacCI*-T_{trpA}), respectively. The *Not*I cassette was blunted and cloned into the blunted *Asp*718 sites of the plasmid pAB2001 (Becker *et al.*, 1995) to construct pCRS530 or pCRS549 containing CAS-GNm or CAS-GGm, respectively.

Minitransposons. The plasmids pCRS563 or pCRS529 were digested with *Not*I and the fragments containing either P_{tac}-*nptII*-T_{trpA} or P_{tac}/P_{aacCI}-*aacCI*-T_{trpA} cloned into the *Not*I site of pUT::mTn5-Sm/Sp (Herrero *et al.*, 1990). The ΩSm/Sp interposon was then removed by re-ligating *Sfi*I-digested DNA to construct pCRS589 or pCRS538 containing mTn5-Nm or mTn5-Gm, respectively. To construct promoterless *gusA*-antibiotic cartridges, the plasmids pCRS472 and pCRS542 were digested with *Not*I and the fragment containing the cartridge was cloned into the *Not*I site of mTn5-Sm/Sp to construct pCRS482 and pCRS543, respectively. *Sfi*I digestion and re-ligation removed the ΩSm/Sp interposon producing pCRS487 or pCRS548 containing mTn5-GNm (containing *gusA*-P_{tac}-*nptII*-T_{trpA}) or mTn5-GGm (containing *gusA*-P_{tac}/P_{aacCI}-*aacCI*-T_{trpA}), respectively.

DNA sequencing and analysis. DNA sequencing and analysis were as reported by Tiwari *et al.* (1996a). The primer TAC-105F (5′-GCA TCT AGC CCG CCT AAT G-3′) was used to generate sequence downstream from the antibiotic marker present within the CAS-GNm, CAS-GGm, CAS-Gm and CAS-Nm cassettes or mTn5-GNm, mTn5-GGm, mTn5-Gm and mTn5-Nm minitransposons.

Sequence upstream of *gusA* was generated by using the primer WIL3 (5′-GAA TGC CCA CAG GCC GTC GAG-3′). The primer TAC-31R (5′-AAT TGT CAA CAG GGG GAT GGG GAG-3′) was used to sequence DNA upstream of the antibiotic marker present within CAS-Nm, CAS-Gm, mTn5-Nm or mTn5-Gm.

Nodulation. Seeds of *Medicago murex* were surface-sterilized, germinated and sown as described by Reeve *et al.* (1997). Immediately after planting, *M. murex* seedlings were inoculated with either the wild-type WSM419, or the mutants WR101, WR1A, WR4A and WR5A. Pots were covered with sterile plastic beads and watered via a side tube to maintain axenic culture. Plants were watered with sterile nutrient solution (Howieson *et al.*, 1995). Nodules were surface-sterilized, crushed in saline and replica-patched onto TY plates in the absence and presence of kanamycin as described by Reeve *et al.* (1997).

Root nodules were stained for β-glucuronidase activity using the procedure described by Wilson *et al.* (1995).

Expression studies. An *S. meliloti* culture was grown to late exponential phase in JMM broth at pH 7·0 containing kanamycin (for WR101) or tetracycline [WSM419(pFUS1 or 1-1)], the cells harvested by centrifugation (3024 ***g***, 5 min; Beckman Avanti J25I centrifuge) and the cell pellet resuspended in saline (0·89% NaCl, w/v). An aliquot of cells was used to inoculate flasks containing JMM (pH 7·0 and 5·7) to an OD_{600} of 0·05 or 0·2, respectively. Cultures were incubated overnight at 28 °C on a gyratory shaker set at 250 r.p.m. until the OD_{600} reached 0·2–0·5.

β-Glucuronidase activity was quantified and the specific activity expressed [in nmol *p*-nitrophenol min^{-1} (mg protein)$^{-1}$ at 28 °C] as described by Reeve *et al.* (1998).

RESULTS AND DISCUSSION

Construction of promoterless *gusA* to facilitate transcription analysis

The *E. coli gusA* gene is particularly suited for use as a reporter in the agriculturally important root nodule bacteria since they have no β-glucuronidase activity (Wilson *et al.*, 1995) and their legume hosts also lack this enzyme activity. To use the *gusA* gene as an effective reporter gene for transcriptional (type I) fusions, the nucleotide sequence upstream of the RBS and ATG start codon was modified. Stop codons in all three reading frames (Fig. 1) were incorporated in the sequence (see Methods) to ensure termination of translation originating upstream. To facilitate cloning work, the *Eco*RI site upstream to the RBS of *gusA* was destroyed, whilst the unique *Hin*dIII site positioned after the stop codon of *gusA* was retained. To facilitate cloning, the cassette CAS-G was constructed, in which the promoterless *gusA* gene was flanked by the sequence for the rare base cutter *Not*I.

Construction of a broad-host-range vector

The CAS-G cassette (containing the promoterless *gusA* gene) was cloned from pCRS426 into the *Not*I site of the multiple cloning region of pUK21. A blunted *Spe*I fragment from the latter construct was then introduced into a blunted *Xho*I site of the broad-host-range plasmid pCRS433 (Fig. 2a; a *lacZ*-deleted derivative of pMP220) to construct a promoter probe vector pFUS1 (Fig. 2a). This cloning generated *Spe*I sites in pFUS1. The restriction sites (Fig. 2b) flanking CAS-G in pFUS1 have been verified by a combination of DNA sequence and restriction pattern analysis. The plasmid pFUS1 can be used to study DNA regions for promoter activity in different genetic backgrounds and to generate a promoter library utilizing the unique *Xho*I site and a partial end-fill strategy developed by Promega.

The application of the modified *gusA* was tested and confirmed by cloning the low-pH-inducible *phrR* promoter of *S. meliloti* WSM419 (Reeve *et al.*, 1998) into pFUS1 (to create pFUS1-1) and quantifying β-glucuronidase activity. A fivefold induction of β-glucuronidase activity occurred when WSM419(pFUS1-1) was cultured at pH 5·7 compared to cells grown at pH 7·0 in JMM broth. The same level of induction has been observed in cells of WSM419 containing a *phrR* promoter region fused to the promoterless *lacZ* gene present within the broad-host-range vector pMP220 (Reeve *et al.*, 1998).

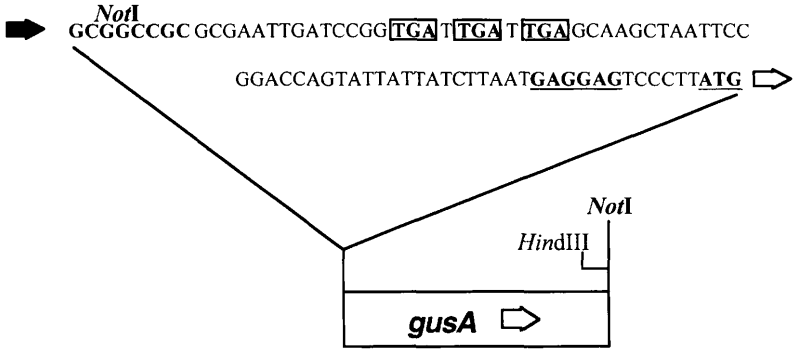

Fig. 1. The promoterless cartridge used for constructing all the cassettes containing *gusA* described in this paper. The DNA sequence containing the modified region is presented showing stop codons (boxed) in the three reading frames. The RBS (GAGGAG) and start codon (ATG) are underlined. The filled arrow represents the direction of transcription from a promoter whilst the open arrow represents the orientation of *gusA*.

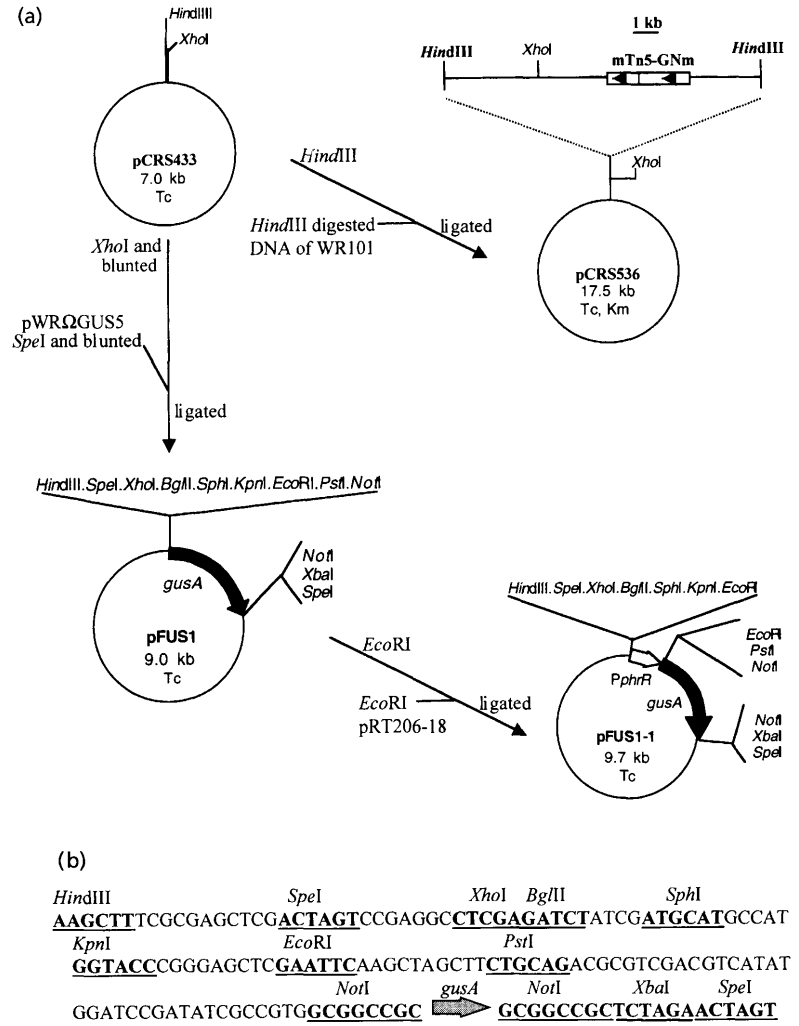

Fig. 2. (a) Construction of the broad-host-range promoter probe vector pFUS1 from a derivative of the IncP1 plasmid pMP220. A *gusA* fusion can be cloned into the intermediate plasmid pCRS433 (as demonstrated by cloning a *Hin*dIII fragment from WR101) to follow β-glucuronidase expression in different genetic backgrounds. The plasmid pFUS1 can be used as a broad-host-range vector to analyse transcription from a putative promoter located on a specific restriction fragment (such as the *Eco*RI fragment containing the promoter of the incomplete *phrR* gene). (b) Nucleotide sequence of the multiple cloning region of the plasmid pFUS1 showing restriction sites (underlined).

Cassettes containing the promoterless *gusA* linked with an antibiotic marker

Either a promoterless *nptII* gene (Tn*5*-derived; devoid of the I end) or the *aacCI* gene (R1033-derived; Becker *et al.*, 1995) was cloned in between the *tac* promoter (P_{tac}) and a *trpA* terminator (T_{trpA}) present within the plasmid pTACTER (Wilson *et al.*, 1995) to construct the fusions P_{tac}-*nptII*-T_{trpA} and P_{tac}/P_{aacCI}-*aacCI*-T_{trpA}, respectively. The pTACTER sequence permits the same primers to be used to sequence DNA flanking both constructs (see Methods). Cloning P_{tac}-*nptII*-T_{trpA} or P_{tac}/P_{aacCI}-*aacCI*-T_{trpA} between *Not*I sites generated the CAS-Nm and CAS-Gm cassettes, respectively (Fig. 3a). The fusion P_{tac}/P_{aacCI}-*aacCI*-T_{trpA} or P_{tac}-*nptII*-T_{trpA} was cloned into the unique *Hin*dIII site downstream of the *gusA*

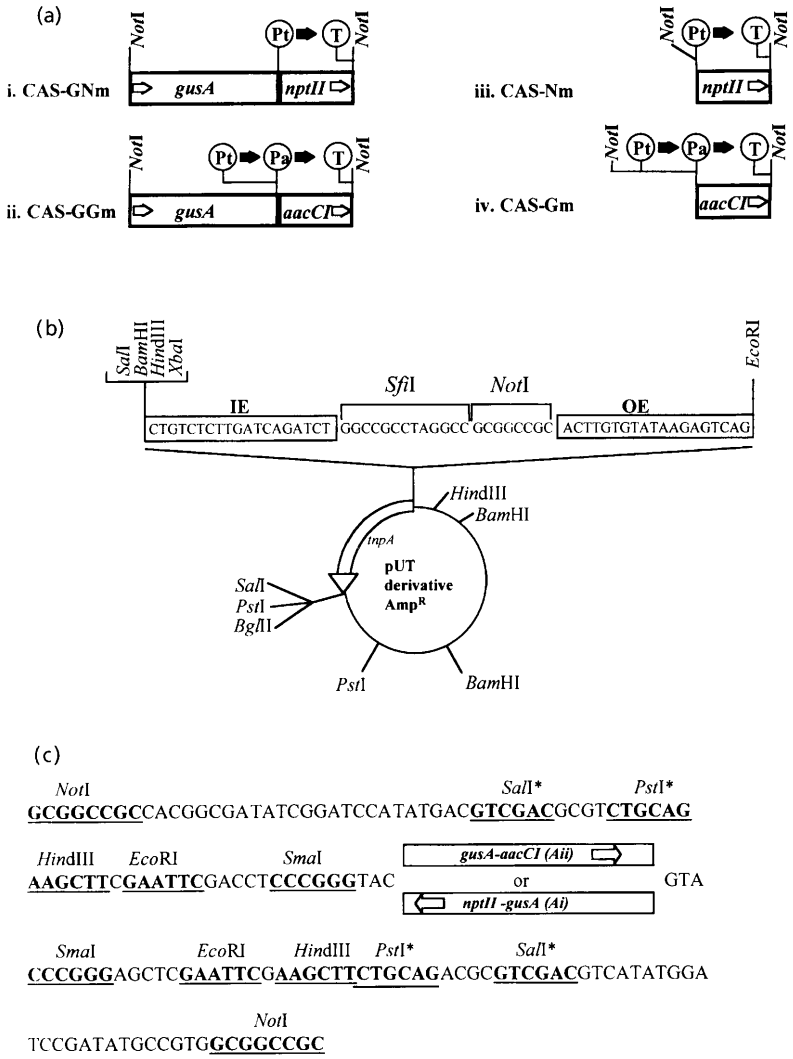

Fig. 3. Cassettes for insertional gene inactivation (a). For random mutagenesis each cassette has been cloned into the *Not*I site of a pUT derivative (b) to construct their mTn*5* derivatives (CAS prefix changes to mTn*5*). Constructs are available where the delivery vehicle containing the minitransposon has been marked with streptomycin and spectinomycin markers. Orientation of transcription of *gusA*, *nptII* or *aacCI* is in the same direction as the *tnpA* gene in the pUT derivatives. The filled arrow represents the direction of transcription from a promoter whilst the open arrow represents the orientation of the respective genes. IE, 19 bp inside end from Tn*5*; OE, 19 bp outside end from Tn*5*; Pa, *aacCI* promoter; Pt, *tac* promoter; T, terminator; *tnpA*, transposase gene from Tn*5*. (c) The sequence containing restriction sites that can be used to excise either the CAS-GNm or the CAS-GGm cassettes from the plasmids pCRS530 and pCRS549, respectively. Sites marked with an asterisk can only be used to excise the entire cassette from one plasmid. The restriction enzyme *Pst*I can only be used to excise the CAS-GGm cassette from pCRS549. The restriction enzyme *Sal*I can only be used to excise the CAS-GNm cassette from pCRS530. Restriction sites were verified by sequencing the DNA flanking the cassette.

gene in CAS-G (Fig. 1) to construct the CAS-GGm and CAS-GNm cassettes, respectively (Fig. 3a). To create a more versatile system for cloning work, the CAS-GNm and CAS-GGm cassettes were positioned into a multiple cloning region such that the cassettes can be excised using *Sma*I, *Eco*RI, *Hin*dIII and *Not*I (Fig. 3c) from the plasmids pCRS530 or pCRS549, respectively.

Minitransposons

A promoterless *gusA*-antibiotic resistance cassette, or an antibiotic resistance cassette, was cloned into the *Not*I site in a pUT derivative (Fig. 3b) containing the minitransposon mTn*5* (de Lorenzo *et al.*, 1990; Herrero *et al.*, 1990) to construct mTn*5*-GNm, mTn*5*-GGm, mTn*5*-Nm and mTn*5*-Gm. The elements were designed such that the orientation of transcription of *gusA*, *nptII* or *aacCI* is in the same direction as the *tnpA* (transposase) gene in the pUT derivative. All the minitransposons constructed in this paper contain only one Tn*5* inside end (IE) and one Tn*5* outside end (OE) which flank an internal cassette. This ensures that only transposition of the entire minitransposon can occur. The first two minitransposons can be used for insertional or transcriptional analysis, whereas the other two (containing only an antibiotic marker) can be used only for insertional mutagenesis. There are a number of special features in the design of these minitransposons. First, restriction sites for frequently used enzymes have been eliminated to facilitate cloning work. Restriction enzymes that can be used for this purpose can be determined from the sequence data deposited in GenBank. Secondly, *Not*I flanks all the cassettes, which facilitates the exchange of one cassette for another by utilizing a different antibiotic resistance marker. Thirdly, the *Not*I cassette present within the minitransposon can be reversed to enable transcriptional activity to be determined from the opposite orientation as well.

The minitransposable elements have been used successfully to mutate *R. leguminosarum* bv. *viciae* WSM710 and *S. meliloti* WSM419 with a transposition rate of 1×10^{-6}. This is equivalent to the transposition rate of mTn*5*-Sm/Sp in *Pseudomonas* (de Lorenzo *et al.*, 1990). Antibiotic resistance levels have been tested up to

1000 μg ml^{-1} for kanamycin or 60 μg ml^{-1} for gentamicin in TY plates with no reduction in the viable cell count of an appropriate mTn5-marked strain of *Sinorhizobium*. The constructs described in this paper have also been successfully used to mutate *Bradyrhizobium* sp. (Lupinus) WU425. They will also be useful to mutate other bacteria that lack the π protein (required for replication of plasmids containing the R6K origin) and where Tn5 transposition is effective.

Mutagenesis of *S. meliloti*

As part of a broad investigation into the response of *M. murex*-nodulating strains of rhizobia to acidity, we have been studying the physiology and genetics of a moderately acid-tolerant strain of *S. meliloti* (Glenn *et al.*, 1997).

In *S. meliloti* WSM419 certain genes are essential for acid tolerance and are expressed at the same rate independent of the external pH. These genes include *actA* (possibly involved in lipid metabolism; Tiwari *et al.*, 1996a) and *actR*/*actS* (a two-component signal transduction system; Tiwari *et al.*, 1996b). In contrast to this, the expression of *phrR* (encoding a putative repressor) is up-regulated by low pH but is not essential for growth in acidic conditions (Reeve *et al.*, 1998). The regulator ActR does not control the expression of *phrR* (Reeve *et al.*, 1998). Structural genes regulated by ActR or PhrR have yet to be identified. The construction and analysis of low-pH-regulated fusions will contribute to our understanding of the role and regulation of these genes and their products.

The kanamycin resistance marker has proven to be extremely useful for genetic studies of WSM419 in neutral and low-pH conditions (Goss *et al.*, 1990) and the mTn5-GNm element was therefore used to mutagenize WSM419. A total of 2000 transconjugants were patched onto JMM plates (containing X-GlcA) at pH 7·0 and 5·7. Of these transconjugants, 35% were white on X-GlcA plates at both pH values after 1 week of incubation. Extended incubation reduces this frequency. Potential pH-regulated fusions were first selected on these plates as colonies that displayed pH-dependent colour development. Quantification of β-glucuronidase activities from the selected colonies showed that 0·55% and 0·15% of the transconjugants generated from mutagenesis contained fusions that were either up- or down-regulated, respectively, by exposure to low pH.

A total of 25 kanamycin-resistant transconjugants were selected from 15 independent conjugations of *S. meliloti* with BW20767(pCRS487). Genomic DNA was extracted from each mutant, digested with either *Eco*RI or *Hin*dIII and then hybridized to DIG-labelled pCRS487 (pUT::mTn5-GNm). Southern analyses established that the minitransposon had inserted into a single *Eco*RI or *Hin*dIII fragment that hybridized with the probe. If vector DNA had integrated into any of these mutants, the presence of an *Eco*RI and *Hin*dIII site in pCRS487 would have resulted in more than one restriction fragment hybridizing to the probe; in each mutant the vector DNA must therefore have been lost after the transposition event. The minitransposon was cloned from each mutant; the rhizobial DNA sequence generated using TAC-105F and WIL3 primers identified a single point of insertion that was unique in each mutant. This analysis indicates that the occurrence of vector integration or multiple minitransposon insertions should be less than 4%. Four of the 25 transconjugants (WR1A, WR4A, WR5A and WR101) were selected for further characterization.

S. meliloti WR1A, WR4A and WR5A were white on X-GlcA plates at pH 7·0 and 5·7 (even after incubation for 2 weeks). These mutants nodulated *M. murex* and fixed nitrogen in association with this host. No nodulation was observed on uninoculated controls. Plants inoculated with WR1A (Fig. 4a) or WR4A (Fig. 4b) had nodules that were very pale blue after staining the root system, whereas plants inoculated with WR5A (Fig. 4c) had nodules that stained blue more intensely. There was no β-glucuronidase activity expressed in the nodules of plants inoculated with *S. meliloti* WSM419 since there was no colour development even after overnight staining in buffer containing X-GlcA (Fig. 4d). It is interesting to note that although the fusion in WR5A appears to be up-regulated in the nodule environment, it does not compromise the ability of this strain to form an effective symbiosis.

Characterization of the low-pH-responsive fusion in WR101

One of the transconjugants that contained a pH-regulated fusion was selected for further study. The pH induction profile of *S. meliloti* WR101 is shown in Fig. 5; significant expression of the fusion occurred below pH 6·0. The specific activity of β-glucuronidase increased from 41 ± 4 (at pH 7·0) to 906 ± 39 (at pH 5·7), representing a 22-fold increase in expression. The kinetics of this profile closely resemble those observed for the expression of the *phrR* gene (Reeve *et al.*, 1998). The gene inactivated by mTn5-GNm has been designated as *lpiA* (*l*ow *p*H *i*nducible). Although both *lpiA*–*gusA* and *phrR*–*gusA* are both up-regulated in response to low pH, inactivation of either gene does not result in acid sensitivity.

A 10·5 kb *Hin*dIII fragment containing mTn5-GNm was cloned from WR101 into pCRS433 to construct pCRS536 (see Fig. 2). Cells of WSM419(pCRS536) showed an increase in β-glucuronidase activity from 49 ± 5 to 1205 ± 62 when shifted from pH 7·0 to 5·7. This indicates that the pH-regulated promoter resides within a 2·8 kb region upstream of *gusA* (see Fig. 2). Mobilization of a plasmid containing an acid-inducible fusion into TG5-46 (*actR*::Tn5) or RT10 (*phrR*-ΩKm) should enable us to determine whether pH-regulated genes are controlled by ActR or PhrR.

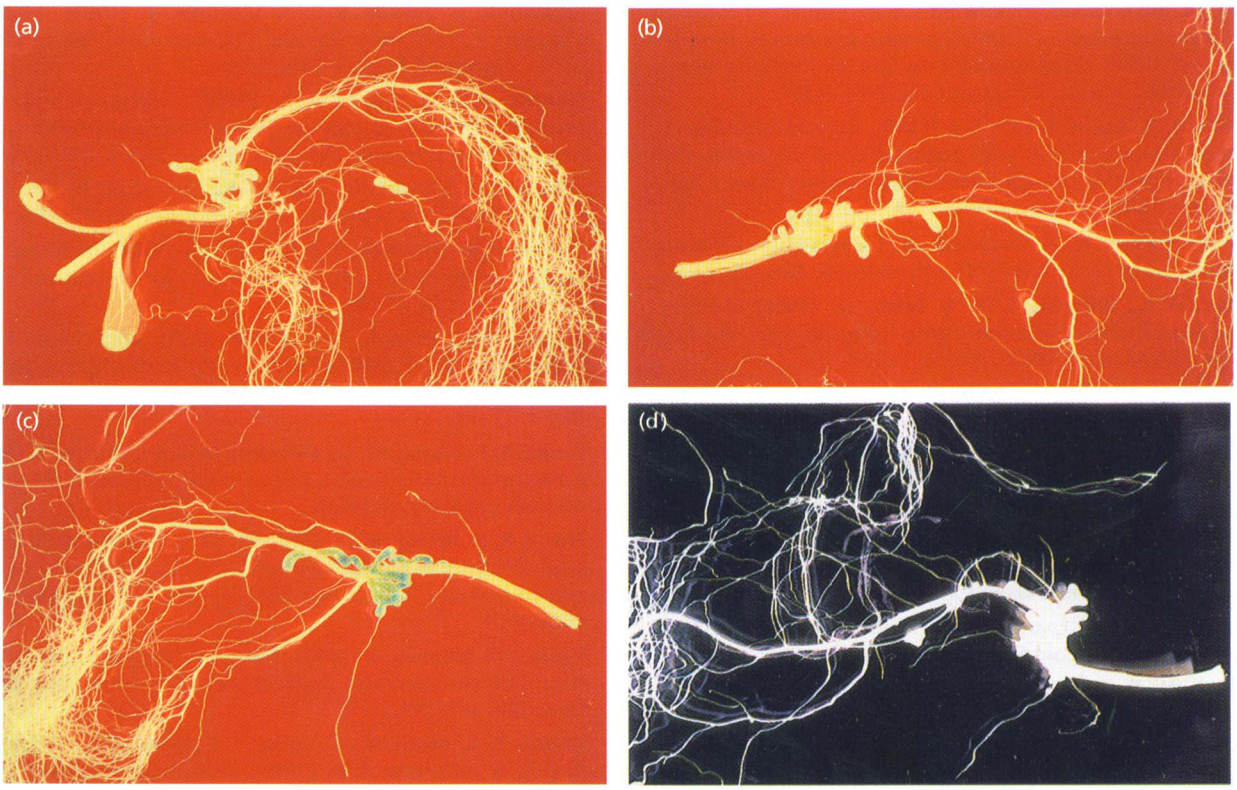

Fig. 4. *M. murex* root nodules stained with X-GlcA. Plants were inoculated with the mTn*5*-induced mutants WR1A (a), WR4A (b) and WR5A (c) and the wild-type *S. meliloti* WSM419 (d) and are shown actual size.

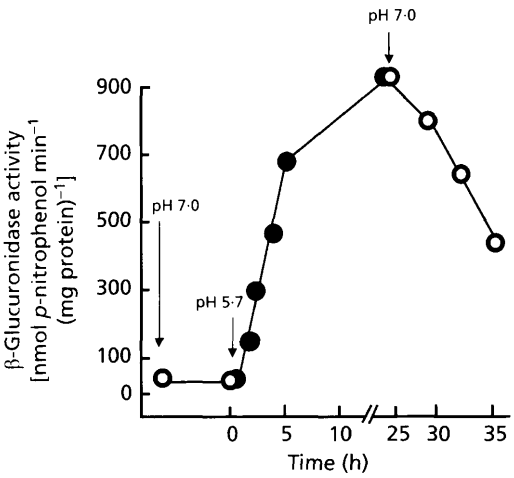

Fig. 5. Expression of β-glucuronidase by *S. meliloti* WR101 cultured in buffered JMM minimal medium. The pH of the medium was changed by resuspension of the cell pellet following centrifugation.

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