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NOTES

Simultaneous Detection of Different *Rhizobium* Strains Marked with Either the *Escherichia coli gusA* Gene or the *Pyrococcus furiosus celB* Gene

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A new marker system for gram-negative bacteria was developed on the basis of the *celB* gene from the hyperthermophilic archaeon *Pyrococcus furiosus*, which encodes a thermostable β-glucosidase with a high level of β-galactosidase activity. The *celB* gene is highly suitable as a marker for studying plant-bacterium interaction because endogenous background β-glucosidase and β-galactosidase enzyme activity can readily be inactivated by heat and because inexpensive substrates for detection are commercially available. Two *celB*-expressing transposons were constructed for use in ecological studies of a variety of gram-negative bacteria. The combined use of the *gusA* marker gene and *celB* allowed the simultaneous detection of several *Rhizobium* strains on a plant, and multiple-strain occupancy of individual nodules also could be easily detected.

Many studies of rhizobial ecology require simultaneous detection of several strains in symbiosis with a plant. The *Escherichia coli gusA* gene, encoding the enzyme β-glucuronidase (GUS), has been proven to facilitate competition studies of *Rhizobium* spp. (11–13, 19). Because of the absence of background activity in most plant tissues and bacteria that interact with plants (17), *gusA*-marked strains can be easily detected in nodules by using a histochemical substrate. An additional marker gene would facilitate the identification of two or more *Rhizobium* or *Bradyrhizobium* strains on a single plant and would enable study of the competition of two inoculant strains in natural soils in the presence of indigenous bacteria. Additionally, differentially marked strains could be easily detected within the same nodule.

The *E. coli lacZ* gene, encoding β-galactosidase, has been used to monitor engineered soil bacteria under field conditions (5–8) and has been demonstrated to be particularly suitable for use with Lac$^-$ bacteria. The *lacZ* marker system has been used for *Rhizobium* spp., but the high levels of endogenous enzymes in plants and bacteria require procedures that eliminate background activity (1, 9, 10). We have developed a marker gene system based on a thermostable β-galactosidase that allows simpler detection of rhizobial strains on plants. The *celB* gene from the hyperthermophilic archaeon *Pyrococcus furiosus* has been expressed in *E. coli* and encodes a thermostable and thermoactive β-glucosidase that has a high level of β-galactosidase activity and whose half time is 85 h at 100°C (16). In addition, background activity of thermostable and thermoactive β-galactosidase is not expected in soils containing rhizobia. Moreover, cheap histochemical substrates for determining β-galactosidase activity are available. We describe here the construction and expression in *E. coli* and other gram-negative bacteria of *celB*-expressing transposons for use in ecological studies. Furthermore, we report the combined use of *Rhizobium* strains marked with the *gusA* and *celB* genes to study competition between two *Rhizobium* strains.

Two *celB*-containing transposons were constructed in *E. coli* on the basis of the existing *gusA* transposons (19). One, mTn*5*SS*celB10*, contains the *celB* gene under the control of the *tac* promoter, which is regulated by the product of the adjacent *lacI*^{q} gene. This transposon should be suitable for studies of free-living bacteria. In the second transposon, mTn*5*SS*celB31*, the *celB* gene is expressed from the *Bradyrhizobium* (*Parasponia*) sp. *nifH* promoter, which is active in nitrogen-fixing legume nodules. To construct mTn*5*SS*celB10*, a 1.9-kb *Ssp*I-*Sma*I fragment carrying the promoterless *celB* gene was isolated from pLUW503 (16) and inserted into the *Hin*cII site of pTacter (19) to create pAS71. Subsequently, the blunt-ended 2.7-kb *Eco*RI-*Pvu*II fragment from pAS71 containing P*tac-celB*-ter was cloned into the *Sma*I site of pJC63 (19), resulting in pAS72. Finally, the P*tac-celB*-ter cassette and the *lacI*^{q} gene from pAS72 were inserted as a 3.9-kb *Not*I fragment into the *Not*I site of pUT/mini-Tn*5* Sm-Sp (4). The resulting plasmid, pAS110, contains the mTn*5*SS*celB10* element (Fig. 1). For the symbiotically active *celB* transposon, mTn*5*SS*celB31*, the promoterless *celB* gene was isolated as a 2.5-kb *Bam*HI-*Pvu*II fragment from pAS71 and inserted into the *Bam*HI and *Sma*I sites of pAS21 (19). The resulting plasmid, pAS73, was digested with *Not*I, and the resulting 2.8-kb P*nifH-celB* fragment was cloned into pUT/mini-Tn*5* Sm-Sp to create pAS131 containing the mTn*5*SS*celB31* element (Fig. 1).

Rhizobium tropici CIAT899 was marked with mTn*5*SS*celB10* and mTn*5*SS*celB31* by using *E. coli* S17-1 λ-*pir* as the donor strain in a biparental mating as described previously (18, 19). Four individual transconjugants from each mating, designated CIAT899::*celB10* A through D and CIAT899::*celB31* A through D, were used for further characterization. CIAT899

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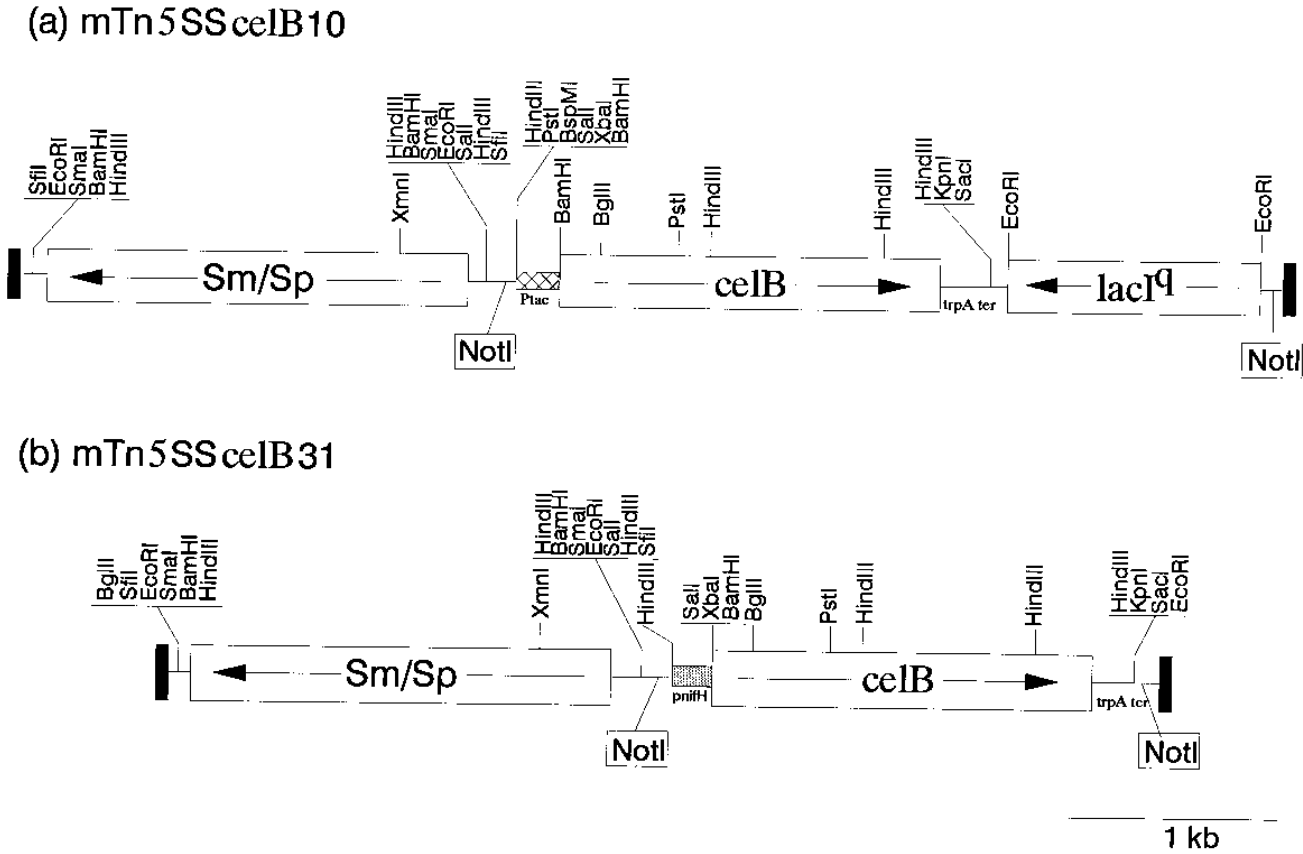

FIG. 1. Genetic and physical maps of *celB* transposons mTn*5*SS*celB10* and mTn*5*SS*celB31*. The spectinomycin-streptomycin cassette, flanked by transcriptional and translational terminators, is indicated, as is the *lacI*^{q} gene in mTn*5*SS*celB10*. Restriction sites of the delivery plasmid pUT are not shown.

was marked separately with the symbiotic *gusA* transposon mTn*5*SS*gusA30* (19). Transfer frequencies on the order of one per 10^7 recipients were obtained.

To determine the activity of the thermostable β-galactosidase in liquid culture, the transconjugants of CIAT899 were grown to mid-exponential phase in yeast-mannitol broth (15). All transconjugants showed growth similar to that of the recipient strain, CIAT899. Those marked with mTn*5*SS*celB10* were grown in duplicate cultures, one of which contained 2 mM IPTG (isopropyl-β-D-thiogalactopyranoside). Enzyme activity was assayed by measuring the amount of *o*-nitrophenol (ONP) produced from ONP-β-D-galactopyranoside. Cultures (1.5 ml) were centrifuged and the pellets were resuspended in 1 ml of enzyme assay buffer 1 (50 mM $NaPO_4$ [pH 7.0], 1 mM EDTA). An aliquot was taken for a viable-cell count by the Miles and Misra drop count method (2) prior to incubating the cells at 70°C for 30 min to destroy endogenous enzymes. Subsequently, the cells were permeabilized by vortexing for 10 s with a solution containing 20 μl of chloroform and 10 μl of 0.1% sodium dodecyl sulfate, and 50 μl of permeabilized cells was added to 450 μl of enzyme assay buffer 2 (50 mM $NaPO_4$ [pH 7.0], 1 mM EDTA, 10 mM β-mercaptoethanol, 1.1 mM ONP-β-D-galactopyranoside). The reaction mixtures were incubated at 37°C, reactions were stopped by the addition of 400 μl of 0.4 M Na_2CO_3, and then the A_{420} was determined. The transconjugants of CIAT899 harboring mTn*5*SS*celB10* or mTn*5*SS*celB31* were used to inoculate *Phaseolus vulgaris* L. cv. Riz 30 plants and had a nodulation efficiency similar to that of the unmarked strain. Enzyme activities in plants inoculated with either CIAT899::*celB10* or CIAT899::*celB31* were determined by crushing single nodules, harvested after 20 days, in 1 ml of enzyme assay buffer 1 and incubating them at 70°C for 30 min before carrying out the assay as described above. This heat treatment was sufficient to eliminate all endogenous β-glyco-

TABLE 1. β-Galactosidase activities in *R. tropici* CIAT899 and in derivatives containing IPTG-inducible or non-IPTG-inducible *celB* genes[a]

Strain or derivative	Enzyme activity in liquid culture (nmol of ONP min^{-1} 10^7 $cells^{-1}$)		Enzyme activity in nodules (nmol of ONP mg of $nodule^{-1}$)
	With IPTG induction	Without IPTG induction	
CIAT899	NA	<0.01	<0.01
CIAT899::*celB10* A	6.80 a	0.50 a	0.14 d
CIAT899::*celB10* B	6.55 a	0.53 a	0.14 d
CIAT899::*celB10* C	7.47 a	0.68 a	0.20 b,c
CIAT899::*celB10* D	7.76 a	0.63 a	0.20 b,c
CIAT899::*celB31* A	NA	<0.01	0.22 b
CIAT899::*celB31* B	NA	<0.01	1.22 a
CIAT899::*celB31* C	NA	<0.01	0.15 c,d
CIAT899::*celB31* D	NA	<0.01	0.23 b

[a] Enzyme activities in liquid culture are the means of three replicates; the enzyme activities in nodules are the means of six replicates. Means within each column which are not significantly different from each other (at $P = 0.05$), as determined with Duncan's multiple-range test, are followed by the same letters. NA, not assayed.

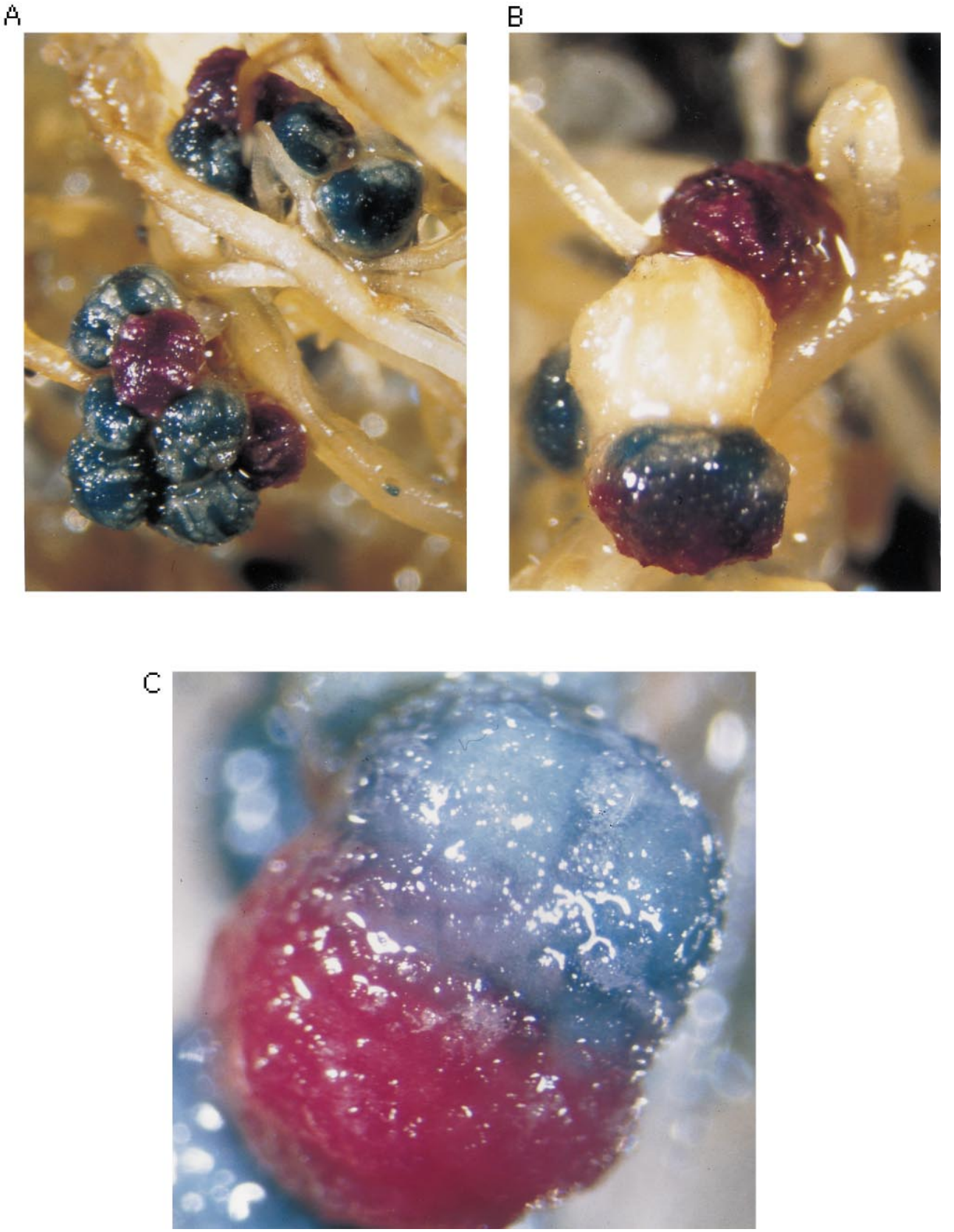

FIG. 2. Simultaneous detection of *celB*- and *gusA*-marked rhizobia in nodules. (A) Common bean root with nodules occupied by either CIAT899::*gusA10* (red) or CIAT899::*celB10* (blue); (B) detection of CIAT899, either unmarked (white) or marked with mTn*5*SS*gusA30* (red) or mTn*5*SS*celB31* (blue), in common bean root nodules; (C) common bean root nodule occupied by CIAT899::*gusA10* (red) and CIAT899::*celB10* (blue). Different magnifications of nodules that have a diameter of approximately 3 mm are shown.

sidase activity. Enzyme activities in cells or nodules were expressed as nanomoles of ONP produced per minute per 10^7 cells or as nanomoles of ONP per milligram of nodule (fresh weight), respectively (Table 1).

No significant differences in enzyme expression could be found among the *celB10*-marked derivatives in liquid culture, indicating that expression is largely independent of insertion position in the genome. In the presence of IPTG, which alleviated *lacI*^{q}-mediated repression, a 10-fold-higher level of expression compared with that of uninduced enzyme activity was found. The activity of the thermostable β-galactosidase in *E. coli* S17-1 λ-*pir* harboring pAS110 was 1.05 nmol of ONP min^{-1} 10^7 $cells^{-1}$ when the cells were grown in the presence of IPTG. No marker gene expression could be detected in S17-1 λ-*pir* containing pAS131 or in *celB31*-marked derivatives of strain CIAT899 in liquid culture. This was expected, as the *nifH* promoter is not normally active in free-living bacteria and is activated in response to microaerobic conditions encountered in a nitrogen-fixing nodule. By contrast, in nodules we observed a higher level of enzyme expression when *celB* was driven by the *nifH* promoter than when it was driven by the *tac* promoter. The reason why expression was about six times higher in nodules with one isolate, CIAT899::*celB31* B, than with the other *celB31*-marked derivatives of CIAT899 is unknown. Because of the position of the *celB* gene in Tn*5*SS*celB31* minitransposon, which is opposite that of the *gusA* gene in Tn*5*SS*gusA30* (19), polar effects of adjacent sequences would not be expected (Fig. 1).

For simultaneous detection of wild-type strain CIAT899 and its *gusA*- and *celB*-marked derivatives on roots, *P. vulgaris* L. cv. Riz 30 plants were grown in sterile modified Leonard jars (15). The seedlings were inoculated with a three-strain inoculum containing either a combination of CIAT899, CIAT899::*gusA10* A (11), and CIAT899::*celB10* or a combination of CIAT899, CIAT899::*gusA30*, and CIAT899::*celB31*. Plants were harvested after 25 days, and the roots were stained for GUS activity as described previously (11) except that the blue-dye-producing substrate X-GlucA (5-bromo-4-chloro-3-indolyl-β-D-glucuronic acid) was replaced with magenta-GlucA (5-bromo-6-chloro-3-indolyl-β-D-glucuronic acid; 200 μg/ml; Biosynth), which is converted by GUS to a magenta product. Following the GUS staining, the roots were kept for 1 h at 70°C in order to destroy endogenous β-galactosidases. Then, X-Gal (5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside) was added at a final concentration of 250 μg/ml, vacuum was applied again for 10 to 15 min, and roots were kept at 37°C overnight. The staining resulted in clearly distinguishable red and blue nodules formed by the marked strains (Fig. 2A). In control treatments in which plants were inoculated with parental strain CIAT899 only, no staining of nodules was observed, indicating that the heat treatment was sufficient to destroy endogenous enzyme activity. A clear advantage of using marker genes is the easy detection of dual-strain occupancy (9, 11). In this study all three possible combinations of double infection could be easily detected by the distinct zones formed by two different strains within a nodule (Fig. 2B and C).

GUS-marked rhizobial strains have shown to be very helpful in studies of rhizobial competition (12, 19) because no picking of nodules is required, enabling the whole root system to be analyzed. These advantages also apply to the *celB* marker gene, with the additional advantage that the histochemical substrates are substantially cheaper than the corresponding glucuronide substrates. The greatest advantage, however, is that *gusA*- and *celB*-marked strains can be localized simultaneously on a plant. Double staining by using the *E. coli lacZ* gene in combination with either the *gusA* gene (1, 10) or the *xylE* gene (3, 14) has been previously reported, but the thermostable and thermoactive marker gene allows faster detection and is better suited for rapid screening of rhizobial strains. This combined *gusA-celB* assay will now enable investigators to study multistrain rhizobial inocula in competition with indigenous populations of rhizobia, with nodules formed by the background population remaining noncolored.

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