MOLECULAR MARKERS TO STUDY COMPETITION AND DIVERSITY OF *RHIZOBIUM*

Angela Sessitsch

Promotor: dr. W.M. de Vos
hoogleraar in de microbiologie

Co-promotor: dr. A.D.L. Akkermans
universitair hoofddocent bij de vakgroep microbiologie

Angela Sessitsch

Molecular markers to study competition and diversity of *Rhizobium*

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Stellingen

1. The problem of inconsistent GUS expression in older nodules from constitutive gene fusions as observed by Streit et al., (1995) may be overcome by applying marker gene constructs containing *nif* promoters.

   Streit W., L. Botero, D. Werner, and D. Beck. 1995. Competition for nodule occupancy on *Phaseolus vulgaris* by *Rhizobium etli* and *Rhizobium tropici* can be efficiently monitored in an ultisol during the early stages of growth using a constitutive GUS gene fusion. Soil Biol. Biochem. 27, 1075-1081.

2. The assumption that marked microbial strains are simply equal to their parents is not valid as transposon-induced or spontaneous mutants may be impaired in one or more phenotypic traits.

   This thesis
   Brockman, F.J., L.B. Forse, D.F. Bezdicek, and J.K. Frederickson. 1991. Impairment of transposon-induced mutants of *Rhizobium leguminosarum*. Soil Biol Biochem. 23, 861-867.

3. The presence of large plasmids in *Rhizobium* complicates its systematics and different plasmid complements may explain the presence of similar 16S rRNA gene sequences but very low DNA-DNA homology among species.

   van Berkum, P., D Beyene, and B.D. Eardly. 1996. Phylogenetic relationships among *Rhizobium* species nodulating the common bean (*Phaseolus vulgaris* L.). Int. J. Syst. Bacteriol. 46, 240-244.
   Martinez-Romero, E. 1996. Comments on *Rhizobium* systematics. Lessons from *R. tropici* and *R. etli*. *In* Biology of Plant-Microbe Interactions. G. Stacey, B. Mullin, and P.M. Gresshoff (eds.). 503-508. Int. Soc. for Plant-Microbe Interactions, St. Paul, Minnesota.

4. The identification of diazotrophs and disease-suppressing microorganisms which colonize roots, stems and leaves endophytically may lead to the development of new inoculant formulations.

5. A recent poll has indicated that two thirds of the Austrian population are against biotechnology, while 87% feel that they are poorly informed on this issue. The question arises whether those who feel that they are well informed are the ones being in favor of biotechnology.

   Profil, March 1997

6. The discussion on the biosafety of genetically engineered organisms shows that more emphasis has to be given to pass on science to the general public.

7. The use of biotechnology in developing countries will not be sufficient to eliminate the world hunger but may play an important role in fighting plant diseases and environmental stresses on crop production.

8. Women should have more confidence in their own abilities.

9. Having a family is still a major problem in a woman's career, whereas this conflict does not seem to exist for men.

11. Scientists have to find their ecological niche, too.

12. Dutch cheese with Austrian wine helps to relax from unsuccessful experiments.

Stellingen behorende bij het proefschrift "Molecular markers to study competition and diversity of *Rhizobium*".

Angela Sessitsch Wageningen,3 juni 1997

Contents

General introduction

Symbiotic biological nitrogen fixation

Members of the genera *Rhizobium* and *Bradyrhizobium* are well known for their capacity to establish a symbiosis with legumes. During this symbiosis the bacteria inhabit root nodules where they reduce atmospheric nitrogen and make it available to the plant. The family of legumes (Fabaceae) is large and diverse, including herbs, trees, and many food crops. Many legumes are used in agriculture, the most commonly cultivated ones are grain legumes such as common bean, soybean, and pasture legumes, including clover and lucerne. Moreover, nitrogen-fixing trees play an important role in agroforestry. Although the most obvious benefit from this symbiosis is the nitrogen input obviating the need of expensive fertilizer application, this interaction has also other beneficial effects on the soil environment. Legumes stimulate the soil microflora and may favour the proliferation of plant pathogen antagonists while rhizobia may promote plant growth (Chabot et al., 1996). Nitrogen fixation is an important source of nitrogen and the various legume crops and pasture species often fix as much as 200-300 kg nitrogen per hectare (Peoples et al., 1995). Globally, symbiotic nitrogen fixation has been estimated to amount to at least 70 million metric tons of nitrogen per year (Brockwell et al., 1995). Furthermore, in many cases nitrogen fertilizers are not efficiently used by crops and the environmental costs are high due to nitrogen losses from fertilizers (Peoples et al., 1994). The contribution of biological nitrogen fixation (BNF) has been suggested to be more open to management than fertilizer nitrogen (Peoples et al., 1995). Natural plant communities, legume crops, pastures, tree plantations and various integrated cropping systems such as alley cropping, intercropping and crop rotations can gain from nitrogen inputs by BNF (Nambiar et al., 1983; Sanginga et al., 1995; Thomas ,1995; Wani et al., 1995).

Rhizobium taxonomy

Early *Rhizobium* taxonomy has been mainly based on the nodulation host range (Fred et al., 1932), although overlapping host ranges have already been reported more than fifty years ago (Wilson, 1944). Over time, rhizobia have been found to be diverse, both in their symbiotic properties and physiological properties. Fast-growing and slow-growing strains were described and Jordan (1982) proposed a new genus, *Bradyrhizobium*, consisting of slow-growing strains. The development of molecular

techniques accelerated the taxonomic evaluation and the current classification includes the use of the small subunit ribosomal RNA (rRNA) sequences. Based on the sequence of the 16S rRNA gene rhizobia could be grouped in the alpha subdivision of the *Proteobacteria* (Young and Haukka, 1996) and three genera, *Azorhizobium*, *Bradyrhizobium*, and *Rhizobium*, have now been well defined (Young et al., 1991; Willems and Collins, 1993; Yanagi and Yamasoto, 1993). In addition, *Sinorhizobium* (de Lajudie et al., 1994) has recently been accepted as another new genus, while others have been proposed (see below).

The genus *Azorhizobium* includes strains that are very distinct from other rhizobia in many characteristics and *A. caulinodans* is the only species characterized up to now nodulating the stems and roots of *Sesbania rostrata* (Dreyfus et al., 1988). Two species of *Bradyrhizobium* are well known to nodulate soybean, *B. japonicum* (Jordan, 1982) and *B. elkanii* (Kuykendall et al., 1992). Recently, another soybean-nodulating species, *B. liaoningensis*, consisting of extremely slow-growing strains has been described (Xu et al., 1995). In addition, yet unnamed species have been found that nodulate other legumes than soybean (Young and Haukka, 1996).

The first described *Rhizobium* species, *R. leguminosarum*, can be grouped in three biovars: *R. leguminosarum* bv. trifolii that nodulates clover, *R. leguminosarum* bv. viciae that nodulates peas and faba bean, and *R. leguminosarum* bv. phaseoli found on common bean (Jordan, 1984). Various common bean-nodulating species have been described, such as *R. etli* (Segovia et al., 1993), *R. tropici* (Martínez-Romero et al., 1991), and others have been proposed (see Chapter 6). Recently, *R. hedysari* obtained from *Hedysarium coronarium* nodules has been characterized based on various PCR fingerprinting techniques as well as phenotypic properties (Squartini et al., 1993; Selenska-Pobell et al., 1996). Several *Rhizobium* species (*R. loti*, *R. huakuii*, *R. ciceri*, *R. tianshanense* and *R. mediterraneum*) might be moved to a new genus for which the name *Mesorhizobium* has been coined (Lindström et al., 1995). The name refers to the intermediate growth rates of some members of this genus that are in between that of *Bradyrhizobium* and *Rhizobium* strains. Chen et al. (1995) demonstrated that *R. thianshanense* strains can be obtained from a variety of legumes such as soybean, *Glycyrrhiza* species, *Sophora alopecuroides* and *Caragana polourensis*. *R. loti* nodulates *Lotus* species (Jarvis et al., 1989), *R. huakuii* is found on *Astralagus sinicus* (Chen et al., 1991), while *R. ciceri* and *R. mediterraneum* strains were obtained from chickpea (Nour et al., 1994; Nour et al., 1995). *R. galegae* nodulating *Galega* species has been well defined (Lindström et al., 1983; Lindström et al., 1988; Lindström, 1989; Lipsanen and Lindström, 1989). However, the 16S rRNA gene sequence shows highest similarity to *Agrobacterium* and therefore it may be transferred to a different genus (Young and Haukka, 1996).

Sinorhizobium includes *S. fredii*, *S. meliloti*, *S. teranga* and *S. saheli* (de Lajudie et al., 1994; Young and Haukka, 1996). *S. fredii* comprises fast-growing strains nodulating soybean (Scholla and Elkan, 1984), although strains of this species are also able to nodulate and fix nitrogen on various legumes (Krishnan and Pueppke, 1994). *S. fredii* strains have 16S rRNA gene sequences identical to *Rhizobium* sp. NGR234 isolated from *Lablab purpureus* (L.) in Papua New Guinea (Trinick, 1980), which is well known because of its unusual broad host range (Jarvis et al., 1992). However, NGR234 has a distinctly wider host range than *S. fredii* (Krishnan and Pueppke, 1994). *S. meliloti* was isolated from *Medicago* (alfalfa), while *S. teranga* and *S. saheli* have been isolated from various tree legumes such as *Sesbania* and *Acacia* species (de Lajudie et al., 1994). Recently, a new species, *S. medicae*, was proposed and its members are able to nodulate various *Medicago* species but show a different host range than *S. meliloti* strains (Rome et al., 1996). Table 1 gives an overview of *Rhizobium*, *Bradyrhizobium*, *Azorhizobium*, *Sinorhizobium* and *Mesorhizobium* species whereas a phylogenetic tree of rhizobia and some related bacteria in the alpha subdivision of the *Proteobacteria* is shown in Figure 1.

Graham et al. (1991) proposed minimal standards for the description of new genera and species of root- and stem-nodulating bacteria. It was suggested that a proper description should be built on a set of independently isolated strains. As *Rhizobium* strains have been widely spread due to inoculation, isolates should be chosen from different geographic regions emphasizing the center of origin of the host legume. Graham et al. (1991) mentioned that the host range is still a valuable practical characteristic whereas phenotypic traits, 16S rRNA sequences and analysis of DNA:DNA relatedness were considered as the most important parameters. Colony characteristics, growth rate and carbon source utilization have been widely used in the taxonomy of rhizobia. *Rhizobium* strains can metabolize a wider range of carbohydrates than *Azorhizobium* strains (Dreyfus et al., 1988; Martínez-Romero et al., 1991), whereas bradyrhizobia are able to grow on various aromatic compounds (Parke and Ornston, 1984). The 16S rRNA gene has been considered to be a useful parameter for phylogenetic analysis (Woese, 1987) and based on the rising number of analyzed sequences a ribosomal database has been established (Maidak et al., 1994). In many bacteria a good correlation between the 16S rRNA gene sequence and the relatedness of the whole genome has been found (Ward et al., 1992), but in some cases there is little congruence. This was reported for *Rhizobium* and *Bradyrhizobium* (Oyaizu et al., 1992) but also for other bacterial species such as *Aeromonas* and *Plesiomonas* (Martínez-Murcia et al., 1992). Stackebrandt and Goebel (1994) demonstrated that the correlation between the percentage of sequence homology of the 16S rRNA genes and the percentage of DNA relatedness is not necessarily linear.

This suggests that conclusions on the phylogeny of bacteria cannot be drawn from the 16S rRNA gene sequence only and that assessment of DNA relatedness is an important criterion. Recently, it has been shown that the 16S rRNA gene sequence similarity among several common bean rhizobia is high although the DNA relatedness data indicate different species (van Berkum et al., 1996).

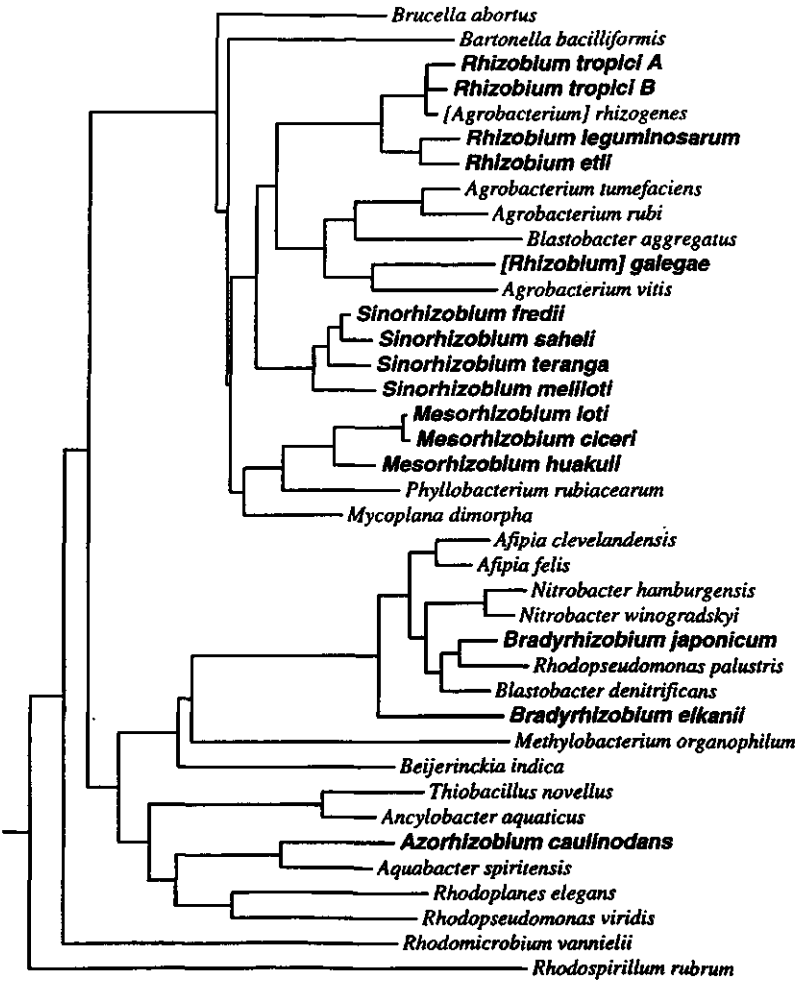

Figure 1. Phylogenetic tree of rhizobia and related bacteria in the alpha subdivision of the *Proteobacteria* based on 16S rDNA sequences. Taken from Young and Haukka (1996).

Table 1. Recognized and proposed species of *Rhizobium*, *Bradyrhizobium*, *Azorhizobium*, *Sinorhizobium*, and *Mesorhizobium*.

Genus	Species	Host plant	Reference
Rhizobium	*R. leguminosarum* bv. trifolii	clover	Jordan, 1984
	R. leguminosarum bv. viciae	pea, faba bean	Jordan, 1984
	R. leguminosarum bv. phaseoli	common bean	Jordan, 1984
	R. tropici	common bean, *Leucaena*, *Gliricidia maculata* etc.	Martínez-Romero et al., 1991
	R. etli	common bean, *Sesbania exaltata*, *Vigna umbellata* etc.	Segovia et al., 1993
	R. galegae	*Galega* spp.	Lindström, 1989
	R. hedysari	*Hedysarium coronarium*	Squartini et al., 1993; Selenska-Pobell et al., 1996
Bradyrhizobium	*B. japonicum*	*Glycine* spp.	Jordan, 1982
	B. elkanii	*Glycine* spp.	Kuykendall et al., 1992
	B. liaoningensis	*Glycine* spp.	Xu et al., 1992
Azorhizobium	*A. caulinodans*	*Sesbania rostrata*	Dreyfus et al., 1988
Sinorhizobium	*S. meliloti*	*Medicago* spp.	Fred et al., 1932; de Lajudie et al., 1994
	S. fredii	*Glycine* spp., *Albizia lebbeck* (L.), *Indigofera tinctoria* (L.) etc.	Scholla and Elkan, 1984; de Lajudie et al., 1994
	S. teranga	*Sesbania* spp., *Acacia* spp., etc.	de Lajudie et al., 1994
	S. sahelii	*Sesbania* spp.	de Lajudie et al., 1994
	S. medicae	*Medicago* spp.	Rome et al., 1996
Mesorhizobium	*M. loti*	*Lotus* spp.	Jarvis et al., 1982
	M. huakuii	*Astralagus sinicus*	Chen et al., 1991
	M. ciceri	chickpea	Nour et al., 1994
	M. thianshanense	soybean, *Glycyrrhiza* spp., *Sophora alopecuroides*, etc.	Chen et al., 1995
	M. mediterraneum	chickpea	Nour et al., 1995

Unnamed species have not been included in this table

Problems related to *Rhizobium* inoculation from a microbial ecology point of view

Although rhizobia and bradyrhizobia are widely distributed in soils, inoculation is often required as in many soils suitable strains are absent or the population density is too low to sustain legume growth. Proposals for determining the need for inoculation have been made (Thies et al., 1994) and models have been established to predict the success of inoculation (Brockwell, 1963; Thies et al., 1991a).

Various ways of preparation and use of inoculants have been developed (Brockwell, 1977; Thompson, 1980; Somasegaran, 1991; Keyser et al., 1992). Most of these are based on non-sterile or sterilized peat carriers for preparation of inoculants for posterior seed coating or inoculation into the seed bed. Alternative methods include the use of vermicilite carriers (Graham-Weiss et al., 1987) or cellulose gels (Jawson et al., 1989). Liquid inoculants have been applied in Australia (Brockwell et al., 1995) and in the USA (Gault, 1978) and rhizobia could be successfully introduced into rhizobia-free soils under irrigation water-run inoculation (Ciafardini and Barbieri, 1987).

Based on studies by Brockwell et al. (1982), Burton (1979), Date (1982), Howieson and Ewing (1986) and Keyser et al. (1992), Brockwell et al. (1995) summarized the advantagous properties of inoculant strains which are listed in Table 2.

Table 2. Desirable traits of *Rhizobium* inoculant strains

Rhizobium-host plant interaction dependent characters
High nitrogen-fixation with the target legume
Broad host range
Rhizobium-Rhizobium interaction dependent characters
High competitive ability for nodulation
Persistence in soil and saprophytic competence
Genomic stability
Rhizobium-environment interaction dependent characters
Tolerance to environmental stresses
Ability to fix nitrogen at various environmental conditions
Nodulation and fixing ability in the presence of soil nitrate

The Rhizobium competition problem

Numerous rhizobial strains have been identified that show high nitrogen-fixing ability. Moreover, genetic engineering may lead to the development of even more effective strains (Chen et al., 1991; Scupham et al., 1996; Streit and Phillips, 1996). Nevertheless, attempts to increase legume yields in agricultural fields by inoculation with such superior strains often failed. This is due to the inability of many inoculant strains to compete with indigenous rhizobial strains for nodule formation on the plant host. Furthermore, the ability of an inoculant strain to multiply in the absence of the host plant and to establish itself in the soil is referred to as saprophytic competence and will influence later inoculations. Many efforts have been undertaken to understand rhizobial competition and the various factors contributing to the success of inoculation have been reviewed by Dowling and Broughton (1986), Bottomley (1992) and Streeter (1994).

During the legume-*Rhizobium* symbiosis the macro- and microsymbiont, i.e. the plant and the microbe, interact but also environmental conditions influence the outcome of inoculation. Important parameters are the inoculum size, i.e. the amount of *Rhizobium* cells added to the seed or to the soil, and the size of indigenous soil populations being able to nodulate the host (Thies et al., 1991b). Weaver and Frederick (1974a and 1974b) found that the number of nodules formed on soybean increased with increasing amounts of *Bradyrhizobium japonicum* inoculant, but this increase was not observed in soils containing more than 1000 rhizobia g^{-1} soil. Carter et al. (1995) also indicated that the inoculation of soils containing high numbers of native strains with non-adapted strains is problematic. In addition, inoculant bacteria often fail to persist and are replaced by indigenous or naturalized strains (McLoughlin et al., 1990a and 1990b). Besides the indigenous population size, the population structure plays an important role and various environmental factors as well as agricultural practices may contribute to field dominance. Furthermore, genetic exchange among rhizobia in soil (Johnston et al., 1978; Schofield et al., 1987) and genomic instability of *Rhizobium* (Flores et al., 1988; Brom et al., 1991) may lead to altered competitivity. Moreover, loss of symbiotic functions has also been found as a result of genomic variability (Weaver and Wright, 1987). Soil textural and structural properties affect rhizobial competition (May and Bohlool, 1983; Moawad and Bohlool, 1984) and persistence in soil as well as nodulation may be influenced by the water potential (Chatel et al., 1973) or soil temperature (Hardarson and Jones, 1979; Montañez et al., 1995). Rhizobial strains differ in their motility in soil and it has been suggested that motile strains are able to occupy lateral roots resulting in higher nitrogen fixation (Wadisirisuk et al., 1989). Rhizobia show a varying sensitivity to

acidity (Coventry and Evans, 1989) and differ in their ability to sequester low concentrations of inorganic phosphorus (Alemendras and Bottomley, 1987; Leung and Bottomley, 1987). All these characteristics contribute to the competitivity of a microbial strain. In addition, the plant genotype plays an essential role in selecting the microsymbiont (Hardarson et al., 1981; Cregan and Keyser, 1988; Cregan and Keyser, 1989) and different genotypes may prefer more or less effective rhizobial partners (Hardarson at al., 1982). The method of rhizobial inoculation has been reported to affect the nodulation pattern (Hardarson et al., 1989; Danso and Bowen, 1989) demonstrating that soil inoculation gives increased nitrogen fixation over seed inoculation.

Several suggestions have been made how to overcome the rhizobial competition problem. The application of extremely high inoculation rates has the potential to at least partially or temporarily increase the nodule numbers formed by the inoculant strain (McLoughlin et al., 1990a and b). Numerous efforts have been made to increase nodulation success by improvement of the inoculant formulation (Zdor and Pueppke, 1990; Zablotowicz et al., 1991) or the inoculum placement (McDermott and Graham, 1989). In order to avoid competition for nodulation plant breeding programmes have been carried out using two approaches. The first approach has been directed towards the selection of highly effective combinations of host plant and bacterial cultivar (Alwi et al., 1989) or the development of lines with a restricted nodulation that are able to bypass the native soil rhizobia (Cregan and Keyser, 1986; Montealegre and Kipe-Nolt, 1994). The second approach is to screen for plants that are nodulated by the most effective strains present in a natural soil population (Kueneman et al., 1984; Herridge and Rose, 1994). This is a promising solution for field situations with high numbers of rhizobial strains and high diversity (Herridge and Danso, 1995). Regarding the bacterial symbiont, rhizobial competition can be overcome in two ways. The first possibility is to make use of genetic engineering and on the long term this approach may lead to the development of more competitive inoculant strains. Strains have been generated that produce trifolitoxin, an antirhizobial peptide (Triplett, 1990), or that harbour the root-inducing plasmid of a *Agrobacterium rhizogenes* strain (Novikova and Pavlova, 1993). In addition, Martínez-Romero and Rosenblueth (1990) constructed *R. etli* strains carrying a non-symbiotic plasmid from *R. tropici* that conferred enhanced competitive ability. The second possibility is to use as inoculants dominant field strains with sufficient nitrogen fixation ability. Promising results with this approach were reported by McLoughlin et al. (1984). Recently developed molecular techniques to characterize microbial populations could facilitate the latter approach. Reliable and fast methods are required to assess the competitivity of a bacterial strain in a particular

environment. Conventional techniques include the use of intrinsic (Broughton et al., 1987) or induced (Turco et al., 1986) antibiotic resistances as identification markers while also immunological approaches have been applied (Berger et al., 1979; Schmidt et al., 1968). Furthermore, plasmid profiles have been used for the determination of nodule occupancy (Broughton et al., 1987; Pepper et al., 1989). The use of marker genes for identification, such as *gusA* and *celB*, has several advantages over these techniques including a high degree of specificity and the fact that the assay can be carried out on intact nodulated root systems. The use of marker genes in competition studies of *Rhizobium* has been reviewed in Chapter 1.

Quality of inoculants

Crop failures can be due to the poor quality of the inoculant used because of the presence of a large number of contaminant cells or insufficient viable cells of the desired *Rhizobium* strain. In some countries, such as Canada and Uruguay, quality control is recognized and supported by appropiate legislation (Olsen et al., 1994a) while in other parts of the world (Australia, New Zealand, India and South Africa) inoculant producers perform a voluntary quality control. However, in many countries appropiate quality control is lacking. Inoculants produced in the USA have been tested by Olsen et al. (1994b) and they, as well as other researchers, reported alarming results (Skipper et al., 1980; Vincent and Smith, 1982). This is due to the fact that most of the world's inoculant production uses non-sterile peat as carrier although many alternatives have been developed (see above). Quality control cannot be based on non-selective plate-count methods and is currently based on most probable number (MPN) counts carried out on plants. This latter approach requires often 4 weeks for completion. However, the application of molecular techniques could overcome this limitation.

Genomic instability

Genomic variability and losses of symbiotic functions have been reported for *Rhizobium* and *Bradyrhizobium* (Gibson et al., 1975; Herridge and Roughley, 1975; Soberon-Chavez et al., 1986; Weaver and Right, 1987). Potential changes in symbiotic effectiveness have to be carefully considered by inoculant producers, particularly if a mutant becomes dominant. The *Rhizobium* genome carries a high number of repeated sequences (Flores et al., 1987; Martínez et al, 1990) and it has

been suggested that they cause recombination leading to rearrangements and deletions (Hahn and Hennecke, 1987). High-frequency rearrangements have been found in the symbiotic plasmid in *R. etli* due to the presence of several copies of the *nifH* gene (Brom et al., 1991). There are several studies on the molecular mechanisms of instability in *R. etli* symbiotic plasmids where amplification and deletion events are concentrated in a zone covering most of the symbiotic genes (Romero et al., 1991; Flores et al., 1993; Romero et al., 1995). Nevertheless, genomic instability in *Rhizobium* is poorly understood and more studies are needed to understand the biological function of this plasticity.

Molecular techniques relevant for rhizobial ecology

Multilocus enzyme electrophoresis (MLEE) has been a standard method in eukaryotic population genetics before it was used for the analysis of microbial populations (Selander et al., 1986). This methodology is based on the relative electrophoretic mobilities of a large number of water-soluble cellular enzymes and mobility variants of an enzyme can be directly equated with alleles at the corresponding structural gene locus. In *Rhizobium,* where symbiotic functions as well as the genes determining host-range are plasmid-encoded, MLEE has been particularly useful to characterize the chromosomal genotype of bacteria (Young, 1985; Eardly et al., 1990; Leung et al., 1994; Nour et al., 1994a). Total protein profiles and patterns of membrane proteins analyzed with SDS polyacrylamide gels have also been employed for the characterization of *Rhizobium* (de Maagd et al., 1988; Lipsanen and Lindström, 1989). Roberts et al. (1980) classified *Rhizobium* strains relying on two-dimensional polyacrylamide gel electrophoresis but nowadays protein-based methods have for a good deal been replaced by techniques that analyze the DNA of an organism.

In *Rhizobium,* plasmids carry up to 25% of the genetic information (Prakash and Atherly, 1986) and the size as well as the number of plasmids can vary among strains. Usually the number of different plasmids varies from one to six within one strain and their molecular weights range from 30 to up to 1000 MDa. Specific dominant plasmid types of *R. leguminosarum* bv. viciae populations were found in different topographic positions (Brockman and Bezdicek, 1989) and Shishido and Pepper (1990) determined *R. meliloti* field strains that are dominant in a desert soil by plasmid profiles. Other studies correlated variation in Sym plasmids with chromosomal variation of *R. leguminosarum* bv. viciae field populations (Laguerre et

al., 1992) and investigated the evolution of symbiotic and chromosomal loci in the *Rhizobium* genome (Broughton et al., 1987). Both studies indicated lateral transfer of plasmids between strains. Hybridization of genomic DNA with plasmid-encoded genes such as *nod* or *nif* operons has been employed to analyse variation in symbiotic genes. Genetic diversity of Italian *R. meliloti* populations was suggested to be distributed on both the chromosome and the symbiotic plasmid (Pafetti et al., 1996). This study was based on hybridization of total restricted DNA with a *nod* gene probe together with fingerprinting techniques targeting the whole genome or the 16S-23S ribosomal intergenic spacer. Similarily, Madrzak et al. (1995) found a considerable degree of variation in symbiotic loci among Polish *B. japonicum* field populations by using *nod* and *nif* gene probes. Sadowsky et al. (1990) suggested that *nod* gene probes may be useful in selecting compatible host plant-*Rhizobium* combinations. Furthermore, hybridization of restricted genomic DNA with chromosomal genes has been applied frequently.

Various insertion sequences are distributed among the genomes of some *Rhizobium* species and they have been used as markers in several studies analyzing the genetic structure of *Rhizobium* field populations (Hartmann and Amarger, 1991; Bromfield et al., 1995; Mazurier et al., 1996). Hartmann et al. (1992) used RSα, a repeated sequence common in *B. japonicum* and *S. fredii*, as a probe for strain identification. A probe for hydrogenase uptake genes was applied to demonstrate variability among members of the *B. japonicum* serogroup 110 (van Berkum et al., 1993). Similarily, Laguerre et al. (1993a) used LPS gene regions and *lac* genes as chromosomal probes in combination with symbiotic plasmid probes to demonstrate genetic transfer and recombination among members of a rhizobial soil population.

The sequence of the small subunit of ribosomal RNA, the 16S rRNA gene, plays an important role in the current classification of bacteria as described above. The ribosomal DNA is highly suitable for phylogenetic studies as it is constant in its function, present in all bacteria and contains highly conserved as well as more variable regions (Woese, 1987; Schleifer and Ludwig, 1989). Therefore, the 16S rRNA gene sequences have become an indispensable parameter in *Rhizobium* taxonomy (Young and Haukka, 1996) but also restriction fragment length polymorphism (RFLP) analysis of PCR-amplified 16S rRNA genes has been proven to be a valuable tool for *Rhizobium* species identification (Laguerre et al., 1994). Nevertheless, the conservative nature of 16S rRNA genes limits its use for discrimination at the strain level. The intergenic spacer (IGS) between the 16S rRNA and the 23S rRNA genes was described to be more variable (Massol-Deya et al., 1995) and RFLP of the PCR-amplified IGS was used for the characterization of *Rhizobium* strains (Nour et al., 1994a and b; Selenska-Pobell et al., 1996). The 23S

rRNA gene might be a promising tool to study microbial ecology as it has been found that in *Rhizobium* as well in other genera (e.g. *Rhodobacter*, *Salmonella*, *Yersinia*) the 23S rRNA genes contain in their 5' end highly variable extra stem-loop structures, where cleavage and fragmentation occurs (Selenska-Pobell and Evguenieva-Hackenberg, 1995).

Pulsed-field gel electrophoresis (PFGE) and field inversion gel electrophoresis (FIGE) allow the resolution of large DNA fragments obtained with rare-cutting restriction endonucleases. The resulting total DNA profiles can be used for genome size estimation and for the genotypic characterization of strains (Sobral et al., 1990; Sobral et al., 1991; Haukka and Lindström, 1994; Huber and Selenska-Pobell, 1994). The development of the polymerase chain reaction (PCR) led to new fingerprinting methods. Arbitrary oligonucleotide PCR primers of random sequence (RAPD) have been proven to be valuable means for the differentiation of complex genomes (Williams et al., 1990; Welsh and McClelland, 1990; Caetano-Anolles et al., 1991) and random primers have been used to generate strain-specific fingerprints of *Rhizobium* (Dooley et al, 1993; Kay et al., 1994; Selenska-Pobell et al., 1995). The RP01-directed primer developed by Richardson et al. (1995) was based on a reiterated *Rhizobium nif* promoter consensus element and has been demonstrated to be suitable to fingerprint rhizobial genomes. Short intergenic repeated sequences such as repetitive extragenic palindromic (REP) and enterobacterial repetitive intergeneric consensus (ERIC) sequences have been found in enteric bacteria. Based on these elements Versalovic et al. (1991) designed REP- and ERIC-specific PCR primers in order to probe bacterial genomes for the presence of these repetitive sequences. The use of REP and ERIC primers and PCR to fingerprint *Rhizobium* genomes was first demonstrated by de Bruijn (1992) and this PCR-based approach became a frequently employed technique for analyzing bacterial communities (Judd et al., 1993; Leung et al., 1994; Laguerre et al., 1996). Table 3 gives an overview of the most frequently used PCR-based fingerprinting techniques in rhizobial ecology.

Rhizobium - Phaseolus vulgaris interactions

Common bean (*Phaseolus vulgaris* L.) is an important food crop in Central and South America, Asia and Africa and has been considered as a poor nitrogen-fixing grain legume. However, great variability in nitrogen fixation has been observed between bean genotypes and it has been shown that bean lines supporting high nitrogen fixation exist (Hardarson et al., 1993). Inoculation with effective *Rhizobium*

strains failed in many cases often due to the high numbers and the extreme diversity of bean-nodulating strains in soils of Latin America and Africa (Graham, 1981; Piñero et al., 1988; Anyango et al., 1995). These native strains represent well-adapted populations that show superior competitive ability but many of them are poor nitrogen fixers. In order to overcome this problem, efforts have been directed to the identification of bean lines

Table 3. PCR-based fingerprinting methods used in *Rhizobium*

Method	Target sequence	Primers	Reference
REP-PCR	repetitive extragenic palindromic sequences	REPIR-I + REP2-I	de Bruijn, 1992
ERIC-PCR	enterobacterial repetitive intergeneric consensus sequences	ERICIR + ERIC2	de Bruijn, 1992
RP01-PCR	*nif* promoter region	RP01	Richardson et al., 1995
RAPD-PCR	random sequences	RP04, RP05, SPH1	Richardson et al., 1995 Dooley et al.,1993
RFLP of the 16S rDNA	16S rRNA gene	rD1 + fD1	Laguerre et al., 1994
RFLP of the 16S-23S rDNA	16S-23S rRNA IGS	pHr + p23SROI	Massol-Deya et al., 1995
RFLP of *nod* genes	*nodD2*, IGS between *nodD2* and *nodD3*, part of *nodD3*	NODD2PH678 + NODD3PH2152'	Laguerre et al., 1996
RFLP of *nif* genes	intergenic spacer between *nifD* and *nifH*	FGPD807 + FGPK492'	Laguerre et al., 1996

with restricted nodulation (Kipe-Nolt et al., 1992; Montealegre and Kipe-Nolt, 1994), the identification of highly competitive inoculant strains, and the generation of recombinant strains that have increased competitive capacity (Martínez-Romero and Rosenblueth, 1990). Souza et al. (1994) suggested that the enormous genetic pool may provide strains that are able to fix nitrogen and nodulate legumes in many different habitats and with high efficiency. *Phaseolus vulgaris* (L.) has two centers of origin, in Mesoamerica and in the Southern Andes (Gepts, 1990) and was introduced into other parts of the world after the discovery of America in 1492. Traditionally, common bean nodulating rhizobia have bean classified as *R. leguminosarum* bv.

phaseoli according to their host plant (Jordan, 1984) but later on two new species have been recognized, i.e. *R. tropici* (Martínez-Romero et al., 1991) and *R. etli* (Segovia et al., 1993). The latter dominates soils in Central America whereas *R. tropici* can be found in tropical soils. *R. leguminosarum* bv. phaseoli strains have been found in European soils although it is most probable that the *Phaseolus* microsymbionts originiated from the same region as their host plant. Martínez-Romero (1994) suggested that *R. leguminosarum* bv. phaseoli strains are a result of plasmid transfer in historic times. *R. leguminosarum* bv. phaseoli was believed to be the only microsymbiont of common bean in Europe but recently *R. tropici*, *R. etli* and two new species have been detected in French and Austrian soils (see Chapters 5 and 6; Laguerre et al., 1993b; Amarger et al.,1994). The recognized common bean nodulating species have different 16S rDNA sequences and share low DNA homology (van Berkum et al., 1996) but they differ also in other characteristics. *R. leguminosarum* bv. phaseoli and *R. etli* maintain three copies of the nitrogenase reductase (*nifH*) gene on the symbiotic plasmid (Martínez et al., 1985; Segovia et al., 1993), whereas *R. tropici* contains only one copy (Martínez-Romero et al., 1991). Romero et al. (1988) demonstrated that at least two *nif* regions are required in *R. etli* for full symbiotic effectivity. Due to the presence of reiterated *nifH* genes, high-frequency rearrangements including amplifications and deletions in the symbiotic regions have been reported in *R. etli* (Romero et al., 1991; Romero et al., 1995). Furthermore, *R. etli* strains lacking the symbiotic plasmid have been isolated from the bean rhizosphere (Segovia et al., 1991) and a large diversity among *R. etli* strains has been reported (Piñero et al., 1988). Recently, an isoform of glutamine synthetase II has been found to be common in *R. etli* but distinct from other rhizobia (Taboada et al., 1996). *R. etli* as well as *R. tropici* are able to nodulate other legumes than bean but their host ranges are different (Hernández-Lucas et al., 1995). In addition, *R. tropici* tolerates high temperatures and high levels of acidity and is symbiotically more stable probably due to the presence of single *nif* gene copies (Martínez-Romero et al., 1991). Among *R. tropici* two groups, designated type A and type B, can be distinguished based on their group-specific megaplasmids (Geniaux et al., 1995) but they show the same host range (Hernández-Lucas et al., 1995). Rhizobia that can form nitrogen-fixing nodules were found to be genetically distant and phylogenetically diverse (Piñero et al., 1988; Eardly et al., 1995). Eardly et al. (1995) studied the phylogenetic relationships of bean rhizobia and suggested that recombination of horizontally transferred chromosomal genes or gene segments has been involved in their evolution. In addition to the three recognized bean-nodulating species, several new species have been proposed such as *Rhizobium* sp. (*Phaseolus*) strain RCR 3618D with unknown geographical origin (van Berkum et al., 1996). Two possible new species have been

isolated in France, *Rhizobium* sp. (*Phaseolus*) strain R602sp and *Rhizobium* sp. (*Phaseolus*) strain H152 (Laguerre et al., 1993b). However, the partial 16S rRNA gene sequence of R602sp was found to be identical to strain FL27 (Laguerre et al., 1993b), a Mexican isolate from common bean (Piñero et al., 1988). Bean-nodulating strains isolated in Austria were demonstrated to be highly related to the French strain R602sp based on several approaches targeting symbiotic and chromosomal regions of the genome as well as the nodulation phenotype (see Chapter 5).

Outline of the thesis

A major aim of this work was to develop a technique that can be used to determine the competitive ability of a rhizobial strain or to assess the effect of environmental factors on competition. Another objective was to investigate the genetic structure of common bean-nodulating rhizobia and to determine the taxonomy of new isolates. As discussed above, in many cases the poor competitivity of inoculant strains contributes to failures in the practical application of nitrogen fixation. As conventional techniques to measure nodule occupancy have several drawbacks, various systems based on reporter genes have been developed. In Chapter 1, the use of such marker genes in competition studies is reviewed. Chapter 2 describes the construction of various ß-glucuronidase (GUS) transposons to be used for genetic and ecological studies of rhizobia. Constitutively expressed, regulated or symbiotically active *gusA* reporter gene cassettes were developed and the assay conditions for studying rhizobial infections were optimized. Although potential ecological impacts were anticipated during the design of these transposons, the effect of the marker gene system on the fitness of the host organism has to be evaluated rigorously. This point has been addressed in Chapter 3, that describes the effect of introduction of the *gusA* gene on a representative and easily studied phenotype such as nodulation competitiveness. Five isolates of the common bean-nodulating *R. tropici* strain CIAT899, marked with the mini-transposon mTn*5*SS*gusA*10, were characterized for their nodulation characteristics and their competitive ability was compared to the wild-type strain. The development of an additional marker gene, the *Pyrococcus furiosus celB* gene encoding a thermostable ß-glucosidase, is presented in Chapter 4. This chapter is focussed on the construction of *celB* mini-transposons and their use in detecting *Rhizobium* strains on plant. A combined *gusA/celB* assay is described that allows simple, sensitive, and simultaneous detection of differently marked bacteria within nodules and the detection of dual nodule occupancy. Chapter

5 analyses common bean nodulating *Rhizobium* populations of an Austrian soil located close to the Seibersdorf laboratory. Common bean nodulates well in this soil although this crop has not been cultivated during the last decades. The application of approaches targeting chromosomal and symbiotic regions of the genome as well as the nodulation phenotype revealed the presence of two species, *R. etli* and a not yet recognized *Rhizobium* sp (*Phaseolus*). As the latter showed high relatedness to a genomic species found in France and a bean isolate from Mexico, the phylogeny and taxonomy of these strains were studied (Chapter 6) and found to belong to a novel species, for which the name *Rhizobium pueblae*, is proposed. Finally, in Chapter 7 the results of the experimental chapters are summarized and discussed in a wide perspective.

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CHAPTER 1

Use of marker genes in competition studies of *Rhizobium*

A. Sessitsch, G. Hardarson, W. M. de Vos and K. J. Wilson

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Use of marker genes in competition studies of *Rhizobium*

Angela Sessitsch[1], Gudni Hardarson[1], Willem M. de Vos[2] and Kate J. Wilson[3]

1 Soil Science Unit, FAO/IAEA Agriculture and Biotechnology Laboratory, Agency's Laboratories, A-2444 Seibersdorf, Austria

2 Department of Microbiology, Wageningen Agricultural University, Hesselink van Suchtelenweg 4, 6703 CT Wageningen, The Netherlands

3 Center for the Application of Molecular Biology to International Agriculture (CAMBIA), GPO Box 3200, Canberra, ACT 2601, Australia

Summary

Use of marker genes has several advantages in studying rhizobial competition compared to traditional approaches. Reporter genes such as the ß-glucuronidase gene (*gusA*) or a thermostable ß-glucosidase gene (*celB*) allow detection of rhizobial strains in nodules when they are still attached to the root system. Analysis is extremely simple, fast and permits a high data throughput. This detection technique is therefore highly suitable for the study of rhizobial competition and studies using *gusA*-marked strains of *Rhizobium* are presented. By making use of *gusA* and *celB*, differentially marked strains can be produced and distinguished easily on roots. The availability of two marker genes permits competition studies of two or more than two strains and analysis of dual nodule occupancy. As this methodology does not require sophisticated equipment, a GUS Gene Marking Kit was developed.

Introduction

The competitive ability of an inoculant strain is a major factor determining the success of rhizobial inoculation. Many soils contain high numbers of indigenous rhizobia which are often poor in nitrogen fixation ability but highly competitive as they are well adapted to local conditions. Therefore, effective inoculant strains have to be selected which are able to compete with the native rhizobia and thus form a high percentage of nodules. An additional desirable property is high saprophytic competence in order to enable persistence of the inoculant strain in the soil.

Evaluation of the competitive ability of rhizobial strains has been done by employing intrinsic (Josey et al., 1979; Broughton et al., 1987) or induced (Bushby, 1981; Turco et al., 1986) antibiotic resistances as identifying markers. Other markers used in strain detection are antigenic molecules located on the cell surface which react with specific antibodies. This immmunological response can be detected by ELISA (Berger et al., 1979), fluorescently-labelled antibodies (Schmidt et al., 1968) or immunodiffusion (Dudman, 1971). Analysis of plasmid profiles has also been used in rhizobial competition studies (Broughton et al., 1987; Pepper et al., 1989; Shishido and Pepper, 1990). Recently, several nucleic acid detection methods have been developed for use in rhizobial ecology. They are mainly based on detection of specific sequences by either hybridization (Frederickson et al., 1988; Springer et al., 1993) or amplification (Steffan and Atlas, 1991). Amplification profiles of rhizobial strains using random (Harrison et al., 1992; Richardson et al., 1995) or directed (de Bruijn, 1992; Judd et al., 1993) primers have proven to be useful in ecological studies.

The addition of specific genes such as *gusA*, encoding the enzyme ß-glucuronidase (GUS), to a strain of interest has proved to be particularly suitable for ecological studies of *Rhizobium* (Wilson et al., 1994; Akkermans et al., 1994; Wilson et al., 1995). This methodology has a number of advantages in competition studies of *Rhizobium* over the above mentioned techniques. This technique for detecting rhizobial strains in nodules is based on introduced marker genes and its use in competition studies will be presented here.

Marker genes used in competition studies

The main advantage of using introduced marker genes is that the assay for the presence of the marker is simpler than that of other methods. Most reporter genes used in ecological studies allow detection of the marked organism by eye, because the marker gene encodes an enzyme which gives rise to a coloured product following

incubation with a histochemical substrate. However, there are several other criteria that a suitable marker gene has to fulfill. These include lack of background activity in the environment to be studied, versatility of substrates, affordability, possibility of quantitative assays and lack of interference with physiology of the host (Wilson, 1995).

Various marker gene systems are available to detect microbes but not all of them are suitable for use in rhizobial competition studies (see Table 1). The *lacZ* gene, encoding ß-galactosidase, has been used to assess rhizobial competition for the nodulation of soybean (Krishnan and Pueppke, 1992) and to study root colonization by *Azospirillum* (Katupitiya et al., 1995). The *phoA* gene, encoding alkaline phosphatase, can serve as a reporter gene in *Rhizobium* (Reuber et al., 1991). Although several substrates are commercially available for the simple detection of the *lacZ* and *phoA* products, high background activity in rhizobia and plants prohibits easy use of these marker genes. However, catechol 2,3-dioxygenase encoded by *xylE* has also been used for detection of microbes (Winstanley et al., 1991) but this gene is not suitable for rhizobial strain detection in nodules on intact roots as the product of the assay is soluble. Luciferase genes, either the bacterial *luxAB* genes or the firefly *luc* gene, have been also used to study nodule occupancy and root colonization by *Rhizobium* (O'Kane et al., 1988; Cebolla et al, 1991; Cebolla et al., 1993). This marker gene system suffers from the disadvantage that sophisticated amplification devices or long photographic exposure times are required for detection.

The *E. coli gusA* gene, encoding ß-glucuronidase (GUS), is a widely used reporter gene in plant molecular biology (Jefferson et al., 1987). It has also proved to be a highly suitable marker for studying plant-microbe interactions as GUS activity is not detected in plants or in many bacteria of agricultural importance such as *Rhizobium, Bradyrhizobium, Agrobacterium, Azospirillum* and *Pseudomonas* species (Wilson et al., 1992). GUS cleaves glucuronide substrates such as X-glcA (5-bromo-4-chloro-3-indolyl-ß-D-glucuronide) or Magenta-glcA (5-bromo-6-chloro-3-indolyl-ß-D-glucuronide) releasing an indigo or magenta coloured precipitate by which the marked strain can be visualized. In addition, several other substrates are available and hence a large number of possible assays exist (Jefferson and Wilson, 1991). Wilson et al. (1994) described quantitative assays for counts of soil bacteria based on the detection of *gusA*-marked cells on plates. Quantitative assays of GUS activity can also be done in pure cultures by measuring GUS activity using substrates that form coloured (e.g. p-nitrophenol glucuronide, pNPG) or fluorescent products (4-methyl umbelliferyl glucuronide, MUG) (Jefferson and Wilson, 1991; Wilson et al., 1995). In certain circumstances the rate of GUS activity is directly proportional to cell number.

The *gusA* marker is particularly appropriate for rhizobial competition studies since the assay to detect the marked strain within nodules or on the root system is extremely easy to perform. *gusA*-marked cells turn blue when the washed root is incubated in a phosphate buffer containing a GUS substrate such as X-glcA (Wilson et al., 1995). This procedure eliminates the time-consuming step of picking nodules and of preparing bacterial isolates that is required for other detection techniques. Using conventional methods only a percentage of the nodules is analyzed, data produced by this method are obtained from the total nodule number. This is an advantage as it is obvious that a larger sample size will substantially reduce error in statistical analysis (Beattie and Handelsman, 1989; Wilson, 1995).

The *celB* gene from the hyperthermophilic archaeon *Pyrococcus furiosus* encodes a thermostable ß-glucosidase with a high ß-galactosidase activity, which can be determined at temperatures up to 100°C (Voorhoorst et al., 1995). The latter enzyme activity can be used for detection of microbes. As discussed above, high background activity of ß-galactosidase is found in strains of *Rhizobium* and in the host plants. Since the endogenous enzymes in both plant and bacterium can be destroyed easily at high temperature, including those in nodules, the thermostable ß-galactosidase has proved to be a suitable marker for rhizobial competition studies. Assays for detection of *celB* activity in the plant are simple. The washed roots are incubated in phosphate buffer at 70°C in order to destroy endogenous enzymes. Subsequently the roots are incubated in the presence of a chromogenic substrate for the *celB* product such as X-gal (5-bromo-4-chloro-3-indolyl-ß-D-galactoside) (Sessitsch, Wilson, Akkermans and de Vos; unpublished results). Nodules containing *celB* and *gusA* marked strains of *Rhizobium* are shown in Fig. 1 and 2.

Differentially marked strains and dual occupancy of nodules

The availability of different reporter genes allows simultaneous detection of several strains on a single plant. The *gusA* and the *celB* markers are easy to use together as their enzyme activities can readily be distinguished. By using the substrate Magenta-glcA, it is possible to obtain magenta coloured nodules containing the *gusA*-marked strain and by subsequently using the substrate X-gal, following heat-inactivation of endogenous enzymes, blue nodules are formed by the *celB*-marked strain. Hence, simultaneous localization of two specific strains plus the unmarked background population on the plant is possible. Rhizobial competition and other aspects of microbial ecology of several, even very similar, strains of *Rhizobium* can

thus be studied with marker genes under natural conditions, and in the presence of indigenous populations.

Table 1. Marker genes used in studies on microbial ecology

Gene(s)	Gene product	Comments for use in rhizobial competition studies	Use of marker	References
gusA	ß-glucuronidase	No background in rhizobia or plants. The assay is easy and fast to perform.	Rhizobial competition studies, symbiotic gene expression, risk assessment studies on release of recombinant microbes	Sharma and Signer (1990), Streit et al. (1992), Selbitschka et al. (1992), Streit et al. (1995)
lacZ	ß-galactosidase	High background in both bacteria and plants does not allow easy use	Rhizobial competition studies, tracking of recombinant microbes in the environment, root and rhizosphere colonization studies	Drahos et al. (1986), Lam et al. (1990), Krishnan and Pueppke (1992), Katupitiya et al. (1995)
phoA	Alkaline phosphatase	High background in both bacteria and plants does not allow easy use	Regulation of gene expression	Reuber et al. (1991)
xylE	Catechol 2,3-dioxygenase	Product of assay is soluble and therefore not suitable for easy strain detection in nodules still attached to roots	Monitoring survival of recombinant microbes	Winstantley et al. (1991)
luxA, luc	Luciferase	Sophisticated amplification devices or long photographic exposure times are required for detection	Marker of gene expression, environmental monitoring, rhizosphere colonization, detection of GEM's in soil	O'Kane et al. (1988), Cebolla et al. (1991), de Weger et al. (1991), Silcock et al. (1992), Cebolla et al. (1993)
celB	ß-glucosidase	Simple detection possible after denaturation of endogenous enzymes		Voorhorst et al. (1995)

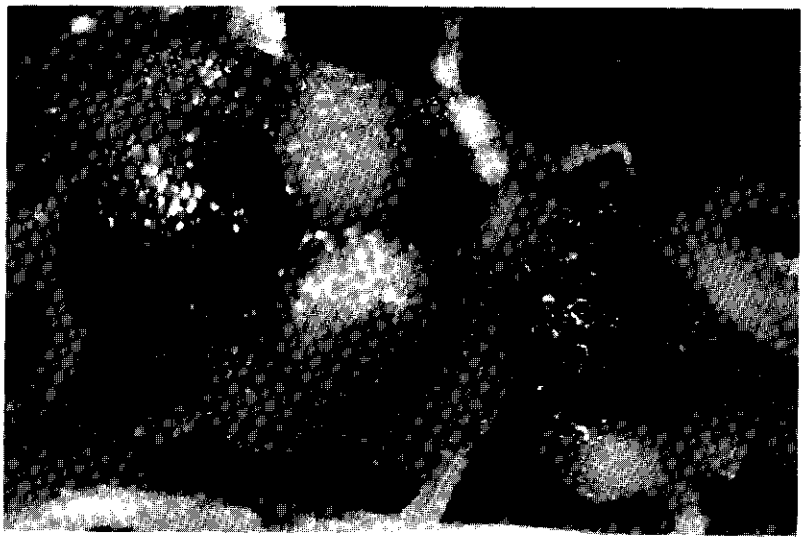


Fig. 1. *Phaseolus vulgaris* root nodules occupied either by *R. tropici* strain CIAT899 (unstained nodules) or by the *gusA*-marked derivative CIAT899::*gusA*10 (blue nodule; here black), and one nodule by both strains, after staining with X-gluc.

Fig. 2. *Phaseolus vulgaris* root nodules occupied either by *R. tropici* strain CIAT899::*gusA*10 (magenta coloured; here black) or by CIAT899::*celB*10 (blue coloured; here black) after staining with Magenta-gluc and X-gal.

When using *gusA* as a marker gene, double strain nodule occupancy by marked and unmarked rhizobia can be detected by partial staining of nodules (Sessitsch et al., submitted). No partially stained nodules were found on plants inoculated with single strains. Double strain occupancy was confirmed by isolating bacteria from nodules. Partially stained nodules were also observed by Krishnan and Pueppke (1992) when examining plants that were inoculated with an unmarked and a *lacZ*-marked *R. fredii* strain. The ability to mark several strains of *Rhizobium* facilitates the visualization of nodules containing more than one strain. When plants were inoculated with differently marked strains and an unmarked strain, all three possible combinations of double infection could be easily detected by eye (Sessitsch, Wilson, Akkermans and de Vos, unpubl. data).

Marking *Rhizobium* and other Gram-negative bacteria with *gusA* and *celB*

For ecological experiments, it is advantageous to insert foreign genes into the chromosome of a bacterial strain. When located on the chromosome, they are not over-expressed as a result of high plasmid copy number and are as stable as chromosomal genes. This is important as the marker gene itself or the gene product should have minimal interference with the physiological properties of the strain. Based on the Tn*5* transposable element, a simple procedure for insertion of foreign genes into the chromosome of Gram-negative bacteria has been developed. Herrero et al. (1990) and de Lorenzo et al. (1990) constructed mini-transposons, located on a suicide delivery plasmids. The mini-transposons contain unique cloning sites in which the reporter gene of choice can be inserted. The delivery plasmid carrying the marker can be transferred from *E. coli* to *Rhizobium* through bacterial conjugation, a procedure that requires only basic microbiological techniques. As specific proteins from *E. coli* are required for plasmid replication, the plasmid itself cannot be maintained in the recipient cells. However, the mini-transposon containing the marker is moved by transposition to a new location in the genome of the host. A special feature of the mini-transposons used is that the transposase gene required for moving the transposonable element is located external to the transposon and is therefore not inserted into the genome. This reduces the probability of further transposition of the introduced marker gene and hence increases its stability and acceptability under current biosafety regulations (de Lorenzo et al., 1990).

gusA and *celB* marker gene cassettes

Depending on the experimental situation and on the question asked in a particular study, different marker gene cassettes might be preferred. A gene cassette consists of the marker gene itself and of sequences that regulate gene expression. Such sequences are primarily known as promoters and terminators that are essential for switching on and off gene expression. Promoters may be regulated, generally either by gene products of other regulating sequences or environmental signals.

Wilson et al. (1995) designed *gusA* transposons using different regulation systems for measuring microbial population changes in soil, in the rhizosphere and particularly, for studying rhizobial competition (Table 2). For creating the *gusA* transposon mTn*5*SS*gusA*10, the *tac* promoter was used to regulate expression of the structural gene in combination with the *lacI*^{q} repressor gene. As long as the *lacI*^{q} product blocks expression, enzyme production remains at a low level, but can be increased in active cells by addition of the inducer IPTG (isopropyl-ß-D-thiogalactoside). The advantage of this construct is that the marker gene is not induced until the moment of assay. Therefore, possible effects on the ecological fitness of the host should be reduced. mTn*5*SS*gusA*10 can be used to detect marked cells both in the free-living state and to study nodule occupancy. Two mini-transposons, mTn*5*SS*gusA*11 and mTn*5*SS*gusA*20, were made in which the marker gene is constitutively expressed. mTn*5*SS*gusA*11 contains the *tac* promoter without the repressor gene, while for the construction of mTn5SS*gusA*20, the *aph* promoter which drives the kanamycin resistance gene in Tn*5* was chosen for constitutive *gusA* expression as this promoter is known to function in a wide range of Gram-negative bacteria. The same GUS cassette is also located on the transposon Tn*5gusA*KW107 on the plasmid pKW107 and was used to study population dynamics of *Pseudomonas putida* in soil (Wilson et al., 1994). mTn*5*SS*gusA*11 and mTn*5*SS*gusA*20 are suitable for experiments on rhizosphere colonization whereas they are not optimal for detection of marked strains in nodules due to a decline in gene expression in older nodules (Streit et al., 1995).

For symbiotic expression of the *gusA* gene, gene fusions were made by Wilson et al. (1995) using promoters of the *nifH* gene. *nifH* codes for the Fe-component of the enzyme nitrogenase and gene expression occurs only in symbiotic or other micro-aerobic conditions (Fischer, 1994). mTn*5*SS*gusA*30 contains the *nifH* promoter of *R. etli* strain CFN42 including an upstream activating sequence (UAS) which can confer enhanced activity in nodules. For making mTn5SS*gusA*31, the *nifH* promoter of *Bradyrhizobium* sp. (*Parasponia*) strain Rp501 was used without the UAS. In fact, GUS expression in symbiotic conditions was found to be very similar with both

transposons and also independent of the origin of the *nifH* promoter and of the presence or absence of the UAS (Wilson et al., 1995; unpublished data). The symbiotic gene fusions are recommended for the study of nodule occupancy, especially in longer-term experiments.

Finally, mTn*5*SS*gusA*40 was designed for molecular genetic studies and to screen bacteria which respond to specific environmental signals (Wilson et al., 1995). This construct lacks a promoter and GUS expression is dependent on promoters of the host genome that are located adjacent to the inserted marker gene.

Two transposons containing the *celB* marker gene were constructed for parallel detection of differently marked strains. In mTn*5*SS*celB*10, the *tac* promoter regulated by the *lacI*^{q} gene product promotes gene expression. In mTn*5*SS*celB*31, the marker gene is expressed symbiotically as gene expression is driven by the *nifH* promoter of *Bradyrhizobium* sp. (*Parasponia*) strain Rp501. These transposons are designed for use in combination with their corresponding GUS transposons. Molecular aspects of these *celB* transposons and detailed staining procedures will be published elsewhere.

Competition studies using marker genes

Competition studies known to the authors that employed introduced marker genes for rhizobial strain detection are summarized in Table 3. Wilson et al. (1991) used the *gusA* gene as a marker for detection of nodule occupancy by *R. meliloti* on *Medicago sativa* and *Bradyrhizobium* sp. on *Macroptilium atropurpureum* and suggested its general use in rhizobial competition studies. Krishnan and Pueppke (1992) used *R. fredii* strain USDA257 marked with a constitutively expressing *nolC-lacZ* gene fusion in order to directly measure rhizobial competition for nodule occupancy. The competitive abilities of the mutant strain and *R. fredii* strain USDA208 were compared in sterile conditions by inoculating soybean seedlings with mixtures containing various ratios of both strains. Randomly selected nodules were picked and tested for ß-galactosidase activity by performing an assay consisting of a staining and a fixing procedure.

The *gusA* marker gene technique has been used in various competition experiments because the assay is extremely simple, not requiring any pre-treatment or analysis of individual nodules and because large numbers of nodules can be analyzed. Streit et al. (1992) compared the capacities of 17 *R. leguminosarum* bv. *phaseoli* and three *R. tropici* strains to compete for nodulation by co-inoculating them with a *gusA*-marked derivative of the *R. leguminosarum* bv. *phaseoli* strain KIM5s. The

Table 2. Mini-transposons for studies on rhizobial ecology containing *gusA* or *celB* as marker gene

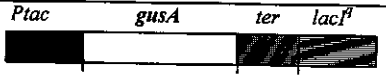
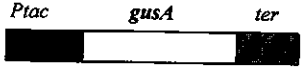
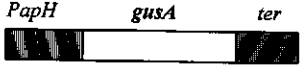
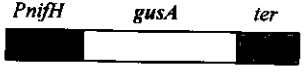
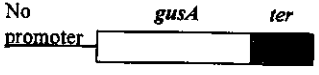
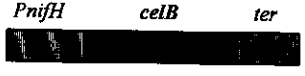
Name of minitransposon	Marker gene cassette	Promoter type	Use
mTn*5*SS*gusA*10		Repressible (by *lacI*^{q} gene product)	To detect rhizobial strains in soil and rhizosphere; To study nodule occupancy
mTn*5*SS*gusA*11		Constitutive	To detect rhizobial strains in soil and rhizosphere
mTn*5*SS*gusA*20		Constitutive	To detect rhizobial strains in soil and rhizosphere
mTn*5*SS*gusA*30 mTn*5*SS*gusA*31		Symbiotic	To study nodule occupancy
mTn*5*SS*gusA*40		No promoter	To select strains that produce GUS only in response to environmental signals
mTn*5*SS*celB*31		Symbiotic	To study nodule occupancy
mTn*5*SS*celB*10		Repressible (by *lacI*^{q} gene product)	To detect rhizobial strains in soil and rhizosphere; To study nodule occupancy

Table 3. The use of introduced marker genes in rhizobial competition studies

Marker gene cassette*	Organism	Objectice of study	Reference
Tn*5**gusA*KW107, pKW210 (***gusABC***)	*Bradyrhizobium* sp. (*Arachis*) *R. meliloti*	To demonstrate the potential as a marker for detecting strains of *Rhizobium* and *Bradyrhizobium* in symbiosis with their host plants	Wilson et al. (1991)
nolC-lacZ	*R. fredii*	To compare competitive abilities of two strains of *R. fredii*; to demonstrate that gene fusions simplify the assessment of nodulation competitiveness	Krishnan and Pueppke (1992)
Tn*5**gusA*KW107	*R. leguminosarum* bv. phaseoli *R. tropici*	To correlate physiological and genetic characteristics of different strains of *R. leguminosarum* bv. phaseoli and *R. tropici* with competitive abilities	Streit et al. (1992)
Tn*5**gusA*KW107	*R. leguminosarum* bv. phaseoli *R. tropici*	To compare competitive abilities in a non-sterile tropical soil	Streit et al. (1995)
Tn*5**gusA*KW107	*Bradyrhizobium japonicum*	To compare symbiotic characteristics, motility on roots and competitive ability of a *gusA*-marked derivative with the parent strain	Herndl-Silmbrod et al. (unpublished results)
mTn*5*SS***gusA***10	*R. tropici*	To study the impact on symbiotic properties and competitive ability due to insertion of the GUS transposon	Sessitsch et al. (submitted)
mTn*5*SS***gusA***10	*R. tropici*	To compare competitive abilities of independent *gusA* derivatives in relation to native rhizobial strains	This study

* The marker gene itself is typed in bold letters.

competition experiments were carried out at two different pH values, pH 5.2 and pH 6.4. A range of competitive abilities was obtained lying between 4% for the least competitive to 96% for the most competitive strain. Strains of *R. tropici* showed little nodule occupancy at pH 6.4, but their competitive abilities relative to strain KIM5s increased significantly at lower pH. Although the main objective of this study was to correlate physiological and genetic characteristics with nodulation competitiveness, the usefulness of this methodology for rapid screening of rhizobial strains was clearly demonstrated. The transposon used for introducing the *gusA* gene was Tn*5gusA*KW107 in which the marker gene is expressed constitutively. This gene fusion was also employed in a study by Streit et al. (1995) for monitoring nodulation competitiveness of strains of *R. leguminosarum* bv. *phaseoli* and *R. tropici* in an non-sterile ultisol. Clear and reliable differentiation between nodules containing a marked strain and nodules produced by indigenous rhizobia was achieved when performing the GUS assay at 14 and 21 days after planting (DAP). However, at 30 DAP incubation of nodules in the staining buffer resulted only in weak colouration of nodules. This is most probably due to the spatial restriction of GUS expression in the *gusA* transposon used.

B. japonicum strain 61A124a was marked with Tn*5gusA*KW107 and competitive ability, shoot dry weight and motility on roots of the wild-type strain were compared against the *gusA*-marked derivative (Herndl-Silmbrod and Hardarson, unpublished results). *Glycine max.* seedlings were inoculated with various ratios of both strains and plants were harvested at 47 DAP and 67 DAP. The symbiotic characteristics, i.e. shoot dry weight and number of nodules per plant as well as the motility along the root were very similar for both the wild-type and the mutant strains. However, the competitive ability of the *gusA*-marked strain was negatively affected, even in treatments where the marked strain outnumbered the parent strain. This decrease in competitive ability could be explained by the constitutive expression of *gusA* in the transposon used or by the fact that just a single *gusA* derivative was used in this study. It became obvious in this early study using this methodology that the possible impact on symbiotic and competitive behaviour has to be carefully investigated before using marked derivatives in competition experiments. In other investigations (Streit et al., 1992; Streit et al., 1995), the competitive abilities of the mutant and the parent strain were compared by performing 1:1 co-inoculation treatments. Subsequently, only those *gusA* derivatives were used that were found to be equal in competitiveness to the wild type parents.

As it is a key requirement for the marker gene that it should have no intrinsic effect on the ecological property studied, the impact of introduction of the *gusA* gene on rhizobial nodulation and competition was examined thoroughly (Sessitsch et al.,

submitted). The nodulation characteristics and competitive abilities of five independent isolates of *R. tropici* strain CIAT899 marked with the mini-transposon mTn*5*SS*gusA*10 were compared with the wild-type strain. Competitiveness indices according to Beattie et al. (1989) were calculated from different inoculation treatments where different ratios of parent strain and *gusA* derivative were applied. The indices obtained varied both between isolates and between independent experiments (Table 4). One isolate showed consistently lower competitive ability compared to the parent strain in all three experiments. The other four isolates showed competitiveness indices which varied between experiments but they appeared either equally competitive or more competitive than the wild-type strain. By contrast, no significant differences in nodulation or nitrogen fixation ability were found due to insertion of the mTn*5*SS*gusA*10 minitransposon. Although the results indicate that there is no impact due to insertion of the GUS transposon *per se* as four out of five isolates were similar to the parent, initial screening is necessary before using marked strains in rhizobial competition experiments. It is sufficient to ensure that the proportion of blue nodules does not significantly differ from 50% after co-inoculating the plant with marked and unmarked strain in a 1:1 ratio.

An experiment was carried out at the FAO/IAEA Agriculture and Biotechnology Laboratory, Seibersdorf, testing the competitive abilities of five mTn*5*SS*gusA*10-marked isolates of strain CIAT899 described above relative to indigenous soil rhizobia. *Phaseolus vulgaris* cv. Extender was grown in Leonard jars filled with a 1:1 mixture of sand and soil from the fields around the Seibersdorf laboratory. Each marked isolate was separately inoculated at two inoculum densities, 10^5 and 10^8 cells per seed. There were four replicates used. The indigenous bacteria outcompeted the inoculant strain at both inoculation levels (Table 5). No significant difference could be found between strains in percentage nodule occupancy when inoculating the marked strain at the 10^8 cells per seed. However, one isolate, isolate E, formed a significantly higher proportion of nodules than the other *gusA*-marked derivatives when applied at the 10^5 level. This isolate also showed an increased competitive ability compared to the parent strain in two competition experiments out of three (Table 4; Sessitsch et al., submitted). Unexpectedly, isolate B, which had significantly reduced competitive ability relative to the parent in sterile conditions, appeared to be as competitive as the other isolates against indigenous rhizobia in soil.

Table 4. Competitiveness indices (Cx,y) of mTn*5*SS*gusA*10-marked derivatives of *R. tropici* strain CIAT899 relative to the parental strain in three independent experiments

Strain	Experiment	Cx,y[a]	Probability that Cx,y = 0[b]	Competitive ability relative to the wild-type strain[c]
CIAT899::*gusA*10 A	1	-0.12	0.24	S
	2	-0.33	0.13	S
	3	+0.21	<0.01	+
CIAT899::*gusA*10 B	1	-0.38	0.04	-
	2	-0.43	<0.01	-
	3	-0.60	<0.01	-
CIAT899::*gusA*10 C	1	+0.42	0.01	+
	2	+0.38	0.02	+
	3	+0.05	0.29	S
CIAT899::*gusA*10 D	1	+0.41	0.11	S
	2	+0.42	<0.01	+
	3	+0.07	0.07	S
CIAT899::*gusA*10 E	1	+0.05	0.67	S
	2	+0.55	<0.01	+
	3	+0.32	<0.01	+

[a] A significantly positive value indicates that the GUS-marked strain is more competitive than the parent strain, a significantly negative value that it is less competitive

[b] This column gives the probability that the calculated competitiveness index is not significantly different from zero, i.e. that the two strains are equal in competitiveness.

[c] Values in this column show the significant position of the marked strain relative to the parent strain: + = more competitive, S = same competitive index, - = less competitive

Table 5. The ability of five GUS-marked derivatives of *R. tropici* strain CIAT899 to compete with indigenous soil rhizobia for nodule occupancy on *P. vulgaris* cv. Extender

Isolate	Percentage nodule occupancy when applied at 10^5 cells per seed[a]	Percentage nodule occupancy when applied at 10^8 cells per seed[a]
CIAT899::*gusA*10 A	0	35 ± 14
CIAT899::*gusA*10 B	2 ± 1	56 ± 7
CIAT899::*gusA*10 C	1 ± 1	35 ± 18
CIAT899::*gusA*10 D	0	25 ± 8
CIAT899::*gusA*10 E	11 ± 3	39 ± 8

[a] Values given represent the mean ± standard error for four plants. The percentage values were subjected to a square-root transformation before the analysis of variance was performed.

Advantages of using GUS transposons in rhizobial competition studies

Determining nodule occupancy by using GUS transposons allows rapid screening of competitive ability of inoculant strains. This is because the marked strain can be detected directly on the plant and therefore picking of nodules and preparation of bacterial isolates are not required. These steps are time-consuming and are required in most conventional techniques. The assay itself is also simple to perform. Additionally, the technique is highly suitable to study saprophytic competence as the marked strain can be detected easily in soils (Wilson et al., 1994).

A very important advantage of this methodology is the greatly increased sample size. Using conventional techniques, a sampling strategy has to be employed and, in general, 20 nodules are analyzed per plant (Somasegaran and Hoben, 1985). Several studies suggest that large nodule numbers should be analyzed in rhizobial competition studies (Gault et al., 1973; Beattie and Handelsman, 1989a) and Wilson (1995) illustrated how 95% confidence intervals decrease tremendously when increasing sample sizes for a binomial proportion, such as percentage nodule occupancy.

As nodules are analyzed when still attached to the root, information on the position of the marked strain is conserved. It was suggested that more competitive

strains occupy more beneficial sites of the root system (McDermott and Graham, 1989) and therefore, placement of an inoculant strain also plays a role in rhizobial competition. Results can therefore easily be biased when sampling strategies do not consider differences in the pattern of nodulation.

No inherent impact of *gusA* insertion on competitive ability was found (Sessitsch et al., submitted) and therefore transposons could be used to study the genetic basis of rhizobial competition. Large-scale screening for competition mutants is possible and mutants could rapidly be tested in a variety of conditions to see whether the effect is general or related to other factors. Using GUS transposons would facilitate isolation and genomic mapping of genes involved in competition. All of these advantages are greatly amplified by the availability of additional markers, such as *celB*, which can be used in combination with GUS.

The GUS Gene Marking Kit

A kit has been developed at the FAO/IAEA Agriculture and Biotechnology Laboratory, Seibersdorf, particularly for microbiologists and agronomists who wish to use the system but are not familiar with the methodology and do not have the resources to establish it in their laboratories. The GUS gene marking technique is highly suitable for transfer in a kit-type approach as only basic microbiological skills are required for marking and detection. Additionally, no sophisticated instruments are needed. Limitations common in developing countries such as water quality and electricity problems do not severely affect the assay. At present, scientists in developing countries have access to the kit if they are participating in one of they ongoing Coordinated Research Programmes or Technical Cooperation Projects of the Soil Fertility, Irrigation and Crop Production Section of the Joint FAO/IAEA Division. The kit is supposed to be used to introduce the methodology so that later on, researchers should become independent of the kit. Therefore, the GUS Gene Marking Kit consists of two parts, the Marking Kit, that is provided only once, and the Detection Kit that will be provided until the methodology has been established in the laboratory.

Conclusions

The use of marker genes has been demonstrated to work with a number of strains on a variety of legumes and the introduction of the marker gene *per se* does not

show any impact on important symbiotic properties. Hence the next step is to try to use it in more practical situations, e.g. for the selection of inoculant strains that are highly competitive or resistant to environmental stresses. A kit has been made available for researchers who are not familiar with this methodology but want to use it. As nodule occupancy can be studied in any given crop and soil environment, marker genes could be potentially used in the field as a tool for screening. However, many countries have strict regulations concerning the release of genetically engineered microorganisms. Therefore it is recommended to use this methodology in carefully planned greenhouse experiments that replicate the field situation.

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CHAPTER 2

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

K. J. Wilson, A. Sessitsch, J. C. Corbo, K. E. Giller, A. D. L. Akkermans and R. A. Jefferson

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

Kate J. Wilson,[1] Angela Sessitsch,[2] Joseph C. Corbo,[2]† Ken E. Giller,[3] Antoon D. L. Akkermans[4] and Richard A. Jefferson[1]

Author for correspondence: Kate J. Wilson. Tel: +61 6 246 5302. Fax: +61 6 246 5303. e-mail: wilson@cambia.org.au

[1] Center for the Application of Molecular Biology to International Agriculture (CAMBIA), GPO Box 3200, Canberra ACT 2601, Australia

[2] Soil Science Unit, FAO/IAEA Agriculture and Biotechnology Laboratories, A-2444 Seibersdorf, Austria

[3] Department of Biological Sciences, Wye College, University of London, Wye, Ashford, Kent TN25 5AH, UK

[4] Department of Microbiology, Wageningen Agricultural University, Hesselink van Suchtelenweg 4, 6703 CT, Wageningen, The Netherlands

A series of transposons are described which contain the *gusA* gene, encoding β-glucuronidase (GUS), expressed from a variety of promoters, both regulated and constitutive. The regulated promoters include the *tac* promoter which can be induced by IPTG, and *nifH* promoters which are symbiotically activated in legume nodules. One transposon contains *gusA* with a strong Shine–Dalgarno translation initiation context, but no promoter, and thus acts as a promoter-probe transposon. In addition, a *gus* operon deletion strain of *Escherichia coli*, and a transposon designed for use in chromosomal mapping using PFGE, are described. The GUS transposons are constructed in a mini-Tn*5* system which can be transferred to Gram-negative bacteria by conjugation, and will form stable genomic insertions. Due to the absence of GUS activity in plants and many bacteria of economic importance, these transposons constitute powerful new tools for studying the ecology and population biology of bacteria in the environment and in association with plants, as well as for studies of the fundamental molecular basis of such interactions. The variety of assays available for GUS enable both quantitative assays and spatial localization of marked bacteria to be carried out.

Keywords: GUS transposons, microbial ecology, rhizobial competition, rhizosphere colonization

INTRODUCTION

Reporter genes are powerful molecular biological tools with a diversity of applications. They may be used to substitute for a structural gene-of-interest and hence to report on regulation of gene expression through creation of a gene fusion. They are used in microbial ecology to facilitate the detection of individual marked strains of bacteria (Drahos, 1991; Wilson, 1995). Additionally they can be used to report on properties of the surrounding environment, e.g. bioavailability of phosphate (de Weger *et al.*, 1994) or naphthalene (Heitzer *et al.*, 1992).

The key advantage of reporter genes as tools in microbial ecology is that they enable closely related strains of bacteria to be readily distinguished, and provide a rapid means of identifying the strain of interest (Wilson, 1995). The extent to which these advantages are realized depends largely on the properties of the reporter gene used. To date, reporter genes used as markers for Gram-negative bacteria in microbial ecology have included *lacZ*, encoding β-galactosidase, the *xylE* gene, encoding catechol 2,3-dioxygenase, and the different sets of luciferase genes – the bacterial *luxAB* genes or the *luc* gene from fireflies. Each has different advantages and limitations (reviewed in Drahos, 1991; Wilson, 1995).

The *gusA* gene, encoding β-glucuronidase (GUS), is the most widely used reporter gene in plant molecular biology (Jefferson *et al.*, 1987). It has the major advantages that there is no background activity in plants, and the wide variety of GUS substrates available enable both quantitative assays and spatial localization of reporter gene activity (Jefferson, 1987). Although the *gusA* gene was

†**Present address:** University of California, San Diego, Department of Biology, 2425 Bonner Hall, 9500 Gilman Drive, La Jolla, CA, USA.

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

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

isolated from *Escherichia coli* (Jefferson *et al.*, 1986), GUS activity is not found in many bacteria of economic and agricultural importance, including *Rhizobium*, *Bradyrhizobium*, *Agrobacterium*, *Pseudomonas* and *Azospirillum* species (Wilson *et al.*, 1992) nor in many fungi, including *Saccharomyces*, *Schizosaccharomyces*, *Aspergillus*, *Neurospora* or *Ustilago*. In addition to its widespead use in plant molecular biology, *gusA* is therefore also of great utility as a reporter gene in microbes.

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

METHODS

Bacterial strains, plasmids and media. Bacterial strains are given in Table 1 and plasmids are given in Table 2. Media used for growth of *E. coli* were: LB (Miller, 1972) supplemented as appropriate with ampicillin (50 μg ml^{-1}), tetracycline (10 μg ml^{-1}), kanamycin (50 μg ml^{-1}), spectinomycin (50 μg ml^{-1}), X-GlcA (5-bromo-4-chloro-3-indolyl β-D-glucuronide; 50 μg ml^{-1}), X-Gal (20 μg ml^{-1}) or IPTG (100 μM). Minimal medium for growth of *E. coli* was M9 salts (l^{-1}: 3 g Na_2HPO_4, 1·5 g KH_2PO_4, 0·5 g NH_4Cl, 0·25 g NaCl) with 0·2% glucose, 0·2% casamino acids, 1 mM $MgSO_4.7H_2O$, 0·5 μg thiamine hydrochloride ml^{-1}. For growth of strain KW1, minimal medium was supplemented with 15 μg hypoxanthine ml^{-1} and 15 μg adenine ml^{-1}. Agar was added to 1·5% (w/v) for solid media.

Rhizobia were grown in yeast-mannitol (YM) medium (Vincent, 1970) or in modified minimal BD medium (Brown & Dilworth, 1975) which contains (l^{-1}): 0·7 g KNO_3, 0·25 g $MgSO_4.7H_2O$, 0·02 g $CaCl_2$, 0·2 g NaCl, 0·36 g KH_2PO_4, 1·4 g K_2HPO_4, 6·6 mg $FeCl_3$, 0·15 mg EDTA, thiamine HCl (1 μg ml^{-1}), biotin (1 ng ml^{-1}), calcium pantothenate (2 μg ml^{-1}), and glucose or glycerol 0·2% (w/v) as carbon source.

Construction of *E. coli* strain KW1. Two successive phage P1 transductions (Miller, 1972) were used to convert *E. coli* strain SØ200 to an *hsdR* genotype. First, strain SØ200 was infected with a P1 lysate of *E. coli* strain TPC48 and colonies that grew on LB/tet plates at 32 °C were checked for temperature-sensitivity due to co-transduction of the temperature-sensitive *dnaC325* allele with the tetracycline resistance marker from transposon Tn*10*. One such derivative was infected with a second P1 lysate made on strain K802 and transductants that regained the ability to grow at 37 °C were shown to be tetracycline sensitive, indicating replacement of the region containing the Tn*10* with the corresponding region from strain K802.

Isolates were checked for acquisition of the linked *hsdR* genotype from strain K802 by examining the efficiency of transformation with pUC18 DNA prepared from strain DH5α (r_K^- m_K^+) and from strain NM522 (r_K^- m_K^-). The efficiency of transformation of strain SØ200 was three orders of magnitud higher with DNA prepared from strain DH5α than with DNA

Table 1. Bacterial strains

Strain	Relevant characteristics	Source/reference
E. coli		
DH5α	*endA1 hsdR17* (r_K^- m_K^+) *supE44 thi-1 recA1 gyrA96* (Nal^r) *relA1* Δ(*lacZYA–argF*) *U169* Φ*80dlac*(*lacZ*)M15	Woodcock *et al.* (1989)
SØ200	*metB strA purB* Δ(*add–gus–man*)	Jochimsen *et al.* (1975)
K802	*hsdR*$^-$ *hsdM*$^+$ *gal*$^-$*met*$^-$ *supE mcrA*$^-$ *mcrB*$^-$	Noreen Murray, University of Edinburgh, Edinburgh, UK
TPC48	*dnaC 325* Zjj::Tn*10*	Millie Masters/Noreen Murray, University of Edinburgh, Edinburgh, UK
NM522	F′ *lacI*^{q}Δ(*lacZ*)M15 *proA*$^+$*B*$^+$/*supE thi* Δ(*lac–proAB*) Δ(*hsdMS–mcrB*)*5* ($r_K^-m_K^-$McrBC$^-$)	Woodcock *et al.* (1989)
KW1	*metB strA purB* Δ(*add–gus–man*) *hsdR*$^-$ *hsdM*$^+$	This work
S17–1	*thi pro hsdR*$^-$ *hsdM*$^+$ *recA* RP4 2-Tc::Mu–Km::Tn*7*(Tp^r/Sm^r)	Simon *et al.* (1983)
S17–1 λ-*pir*	λ-*pir* lysogen of S17–1	Victor de Lorenzo, Centro de Investigaciones Biologicas, Madrid, Spain
Rhizobium		
NGR234	*Rhizobium* sp.; nodulates broad range of tropical legumes	Trinick (1980)
CIAT 899	R. *tropici*; nodulates *P. vulgaris* and *Leucaena leucocephala*	Martinez-Romero *et al.* (1991)

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

Name	Relevant characteristics	Reference/source
pDR540	Ap; plasmid containing hybrid *trp–lac* (*tac*) promoter	Russell & Bennett (1982)
pUT/mini-Tn*5* Sm/Sp	Ap, Sm, Sp; mini-Tn*5* encoding Sm^r/Sp^r with a unique *Not*I site for insertion of cloned fragments on broad-host-range suicide plasmid.	de Lorenzo *et al.* (1990)
pUC18Not	Ap; pUC18 derivative with *Not*I sites flanking the polylinker	Herrero *et al.* (1990)
pWM74	Ap; 1·2 kb $lacI^q$ gene as an *Eco*RI fragment	W. Margolin, Stanford University, CA, USA
pCQ15	*nifH* from R. *etli* strain CFN42	Quinto *et al.* (1985)
pBN370	Ap; 2·8 kb *Hin*dIII fragment in pBR322 containing *nifH* from *Bradyrhizobium* sp. (*Parasponia*) strain Rp501	B. Tracy Nixon, Pennsylvania State University, PA, USA
pBK*uidA*	Ap, Tc; 6·5 kb *Eco*RI–*Hin*dIII insert in pBR325 containing *gusABC* and downstream convergently transcribed ORF	Jefferson *et al.* (1986)
pTTQ18	Ap; high copy vector with the *tac* promoter and $lacI^q$ enabling regulated expression of cloned genes	Stark (1987)
pBI101.1	Km; *gusA* plus *nos* polyA site in pBIN19; reading frame 1	Jefferson *et al.* (1987)
pBI101.2	Km; *gusA* plus *nos* polyA site in pBIN19; reading frame 2	Jefferson (1987)
pBI101.3	Km; *gusA* plus *nos* polyA site in pBIN19; reading frame 3	Jefferson (1987)
pRAJ289	Ap: 6·2 kb insert in pTTQ18 containing promoterless *gusABC* and downstream convergently transcribed ORF	This work
pRAJ294	Ap; promoterless *gusA* gene with bacterial Shine–Dalgarno sequence in pTTQ18	This work
pTacter	Ap; *tac* promoter and *trpA* terminator flanking unique cloning sites in pUC8	This work
pKW28	Ap, Km; Tn*5*-containing *Eco*RI fragment from a *Bradyrhizobium* mutant in pUC13	Wilson (1987)
pKW106	Ap; 2·3 kb P*aph–gusA*–ter *Hin*dIII cassette in pUC13	This work
pKW117	Ap; *gusA* plus *trpA* ter for translational fusions: reading frame 1 in pTacter	
pKW118	Ap; *gusA* plus *trpA* ter for translational fusions: reading frame 2 in pTacter	
pKW119	Ap; *gusA* plus *trpA* ter for translational fusions: reading frame 3 in pTacter	
pKW120	Ap; promoterless *gusA* fragment from pRAJ294 in pUC18Not	
pKW121	Ap; 2·4 kb *Xba*I–*Sst*I fragment from pKW111 with P*aph–gusA*–ter cassette and adjacent *Spe*I site in pUC18Not	
pJC63	Ap; 1·2 kb *Eco*RI $lacI^q$ fragment in pUC18Not	
pJC64	Ap; 2 kb P*tac–gusA*–ter fragment plus 1·2 kb *Eco*RI *lacI* fragment in pUC18Not	
pJC66	Ap; 2 kb P*tac–gusA*–ter fragment in pUC18Not	
pJC67	Ap; 2·3 kb P*aph–gusA*–ter cassette from pKW106 in pUC18Not	
pAS12	Ap; R. *etli nifH–gusA* translational fusion in pUC18Not with ~ 1 kb of upstream sequence including the UAS	
pAS22	Ap; *Bradyrhizobium nifH–gusA* translational fusion in pUC18Not with the *nifH* promoter but no UAS	
pCAM110	Sm/Sp, Ap; mTn*5*SS*gusA10* (P*tac–gusA–trpA* ter transcriptional fusion and $lacI^q$ gene) in pUT/mini-Tn*5* Sm/Sp	
pCAM111	Sm/Sp, Ap; mTn*5*SS*gusA11* (P*tac–gusA–trpA* ter transcriptional fusion) in pUT/mini-Tn*5* Sm/Sp	
pCAM120	Sm/Sp, Ap; mTn*5*SS*gusA20* (P*aph–gusA–trpA* ter translational fusion) in pUT/mini-Tn*5* Sm/Sp	
pCAM121	Sm/Sp, Ap; mTn*5*SS*gusA21* (P*aph–gusA–trpA* ter translational fusion with adjacent unique *Spe*I site) in pUT/mini-Tn*5* Sm/Sp	
pCAM130	Sm/Sp, Ap; mTn*5*SS*gusA30* (R. *etli nifH–gusA–trpA* ter translational fusion) in pUT/mini-Tn*5* Sm/Sp	
pCAM131	Sm/Sp, Ap; mTn*5*SS*gusA31* (*Bradyrhizobium nifH–gusA–trpA* ter translational fusion) in pUT/mini-Tn*5* Sm/Sp	
pCAM140	Sm/Sp, Ap; mTn*5*SS*gusA40* (promoterless *gusA* for transcriptional fusions) in pUT/mini-Tn*5* Sm/Sp	

prepared from strain NM522, due to restriction of the unmodified DNA prepared from strain NM522. By contrast, equal transformation efficiencies of both modified and unmodified DNA were obtained in the isolates derived from the two successive P1 transductions, indicating co-transduction of the *hsdR* marker with the wild-type *dnaC* gene. One of these isolates was named strain KW1. The physical absence of the *gus* operon in strain KW1 was confirmed by Southern hybridization analysis using a 6·2 kb *Eco*RI–*Hin*dIII fragment from pRAJ289 containing *gusABC* as a probe.

Strain KW1 was used as the recipient strain in all subsequent DNA manipulations involving the *gusA* gene (except for manipulations involving pUT/mini-Tn*5* Sm/Sp and derivatives, which had to be carried out in a *λ-pir* lysogen), as presence of a *gusA* insert could be unambiguously detected by formation of blue colonies on media containing 50 μg ml^{-1} X-GlcA.

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

DNA manipulations. Routine DNA manipulations were carried out as described by Ausubel *et al.* (1994). Restriction digestions were performed according to manufacturers' instructions and, where appropriate, sticky ends were blunted using the Klenow fragment of DNA polymerase I or T4 polymerase. Oligonucleotides were from Pharmacia LKB. DNA amplification was done on a Corbett FTS-1 thermocyler. The buffer was 50 mM KCl, 10 mM Tris/HCl, pH 8·4, 200 μM dNTPs, 1·5 mM $MgCl_2$, 1 μM primers. *Taq* polymerase was from Perkin Elmer. The amplification programme used was: (95 °C, 1 min) × 1, (95 °C, 10 s; 55 °C, 20 s; 72 °C, 1 min) × 30.

Construction of general plasmids of use in GUS expression constructs

(i) pRAJ289. To create a plasmid containing the entire *gus* (formerly *uid*) operon under the control of a regulatable vector promoter, *gusABC* plus 1·8 kb of downstream sequence was isolated from pBK*uidA* on two fragments, a 539 bp *Hin*cII–*Bam*HI fragment lacking any promoter sequences but containing the Shine–Dalgarno sequence and part of the *gusA* coding sequence, and a 5·5 kb *Bam*HI–*Hin*dIII fragment containing the rest of the operon and downstream sequences. These two fragments were combined in *Sma*I/*Hin*dIII–digested pTTQ18 placing the operon under control of the *tac* promoter in the vector. The resulting construct was digested with *Eco*RI and *Kpn*I, blunt-ended and re-closed, thus regenerating the *Eco*RI site but eliminating the *Kpn*I site to form pRAJ289.

(ii) pRAJ294. To create a derivative containing *gusA* on its own, pRAJ289 was digested with *Aat*II which cleaves 49 bp downstream of the *gusA* terminator codon (inside *gusB*), blunted and a *Hin*dIII linker (CAAGCTTG, New England Biolabs) was added. The remaining *gusBC* and downstream sequences were then eliminated by digestion with *Hin*dIII and ligation of the linker and 3′ polylinker *Hin*dIII sites to form pRAJ294.

(iii) pTacter. A 350 bp *Eco*RI–*Bam*HI fragment containing the *tac* promoter from pDR540 was inserted into pUC8 to create pUCTac. The *trpA* terminator was then added by attaching *Nsi*I linkers (AGATGCATCT, New England Biolabs) to the *trp*A transcription terminator GenBlock (AGCCCGCCTAATGAGCGGGCTTTTTTTT, Pharmacia), cleaving with *Nsi*I and then inserting this fragment into the *Pst*I site of pUCTac to create pTacter.

(Iv) pKW117, pKW118 and pKW119. The upstream polylinker and *gusA* gene (without the 3′ *nos* polyadenylation site) was removed from the *gusA* translational fusion vectors pBI101.1, pBI101.2 and pBI101.3, respectively, as 1·9 kb *Pst*I–*Sst*I fragments which were blunted and inserted into the blunt-ended *Sal*I site of pTacter.

Construction of plasmids and transposons with constitutive Paph–gusA fusions.

These constructs contain the promoter sequences from the *aph* gene from Tn*5* driving an *aph–gusA* translational fusion.

(i) pKW106. To construct this fusion, the *aph* gene was first isolated as a 1·8 kb *Hin*dIII–*Xho*I fragment from pKW28 and inserted into *Hin*dIII/ *Sal*I-digested pUC13 to give pKW101. To create a fusion to *gusA*, pKW101 was digested with *Eag*I which cleaves at nucleotide 35 of the *aph* gene, blunted, and subsequently digested with *Sst*I prior to inserting a 1·9 kb *Sma*I–*Sst*I *gusA* fragment from pBI101.3 to make a translational fusion with *aph* in pKW102. The *aph–gusA* fusion from pKW102 was then inserted as a blunt-ended 2·3 kb *Hin*dIII–*Sal*I fragment into the blunt-ended *Sal*I site of pTacter. In the resultant plasmid, pKW103, a *Hin*dIII site was regenerated at the 5′ end and a *Sal*I site at the 3′ end of the insert, with the *trpA* transcriptional terminator downstream of *gusA*. To separate this P*aph–gusA*–ter cassette from the *tac* promoter in pTacter, the whole cassette was inserted into pUC13 as a 2·3 kb *Hin*dIII fragment to create pKW106.

(ii) mTn*5*SS*gusA20*. The 2·3 kb *aph–gusA*–ter *Hin*dIII cassette from pKW106 was inserted into pUC18Not to create pJC67, and the resulting 2·4 kb *Not*I cassette was inserted into pUT/mini-Tn*5* Sm/Sp to create pCAM120 containing mTn*5*SS*gusA20*.

(iii) mTn*5*SS*gusA21*. This transposon was created to contain a unique *Spe*I site. The 2·3 kb *Hin*dIII cassette from pKW106 was inserted into the pBluescript SKII(+) (Stratagene) polylinker, which contains an adjacent *Spe*I site, to create pKW111. This was digested with *Xho*I and *Sst*I and the 2·4 kb P*aph–gus*–ter cassette plus adjacent *Spe*I site was inserted into *Sal*I–*Sst*I digested pUC18Not, creating pKW121. Finally, pCAM121 containing mTn*5*SS*gusA21* was constructed by cloning the *Not*I cassette from pKW121 into *Not*I-digested pUT/mini-Tn*5* Sm/Sp. It should be noted that there are three *Not*I sites in pKW121, and that one of the sites bounding the *Not*I cassette in mTn*5*SS*gusA21* therefore derives from the pBluescript SKII(+) polylinker.

Construction of *gusA* transposons using the regulatable tac promoter

(i) mTn*5*SS*gusA10*. The promoterless *gusA* gene from pRAJ294 was inserted as a 1·9 kb blunt-ended *Eco*RI–*Hin*dIII fragment into the *Hin*cII site of pTacter to create pKW104. The resulting 2 kb *Hin*dIII cassette (P*tac–gusA*–ter) was inserted into the *Hin*dIII site of pJC63, which contains the *lacI*^{q} gene from pWM74 as a 1·2 kb *Eco*RI fragment in pUC18Not, to create pJC64. P*tac–gusA*–ter plus *lacI*^{q} was then moved as a 3·3 kb *Not*I fragment into pUT/mini-Tn*5* Sm/Sp to create pCAM110 containing mTn*5*SS*gusA10*.

(ii) mTn*5*SS*gusA11*. A transposon with *gusA* driven consti-

tutively from P*tac* was also created by deleting the *lacI*^{q}-containing *Eco*RI cassette from pJC64 to create pJC66, and inserting the P*tac*–*gusA*–ter cassette without *lacI*^{q} as a *Not*I fragment into pUT/mini-Tn*5* Sm/Sp, forming mTn*5*SS*gusA11*.

Construction of symbiotically active *gusA* transposons

(i) mTn*5*SS*gusA30*. To create a *Rhizobium nifH–gusA* fusion, the *Rhizobium etli* CFN42 *nifH* gene and about 1 kb of flanking sequences on either side was first isolated as a 2·7 kb *Eco*RI–*Hin*dIII fragment from pCQ15 and inserted into pBluescript SKII(+) to create pKW112. About 1·1 kb of upstream sequence and the first 29 codons (i.e. 87 bp) of the *nifH* coding sequence was then subcloned as a 1·2 kb *Eco*RI–*Sal*I fragment into pUC18Not to create pAS11. To make a translational fusion to *gusA*, pAS11 was digested with *Hin*cII and a 2·0 kb *Sma*I, *Hin*dIII-digested, blunt-ended fragment from pKW119 containing *gusA* with the *trpA* terminator was inserted, creating pAS12. This *nifH–gusA* fusion was cloned as a 3·3 kb *Not*I fragment into pUT/mini-Tn*5* Sm/Sp to create pCAM130 containing mTn*5*SS*gusA30*.

(ii) mTn*5*SS*gusA31*. To create a *Bradyrhizobium nifH–gusA* fusion, the upstream 190 bp and the first 22 codons (66 bp) of *Bradyrhizobium* sp. (*Parasponia*) *nifH* were cloned from pBN370 into pUC18Not as a 256 bp *Hin*dIII–*Sal*I fragment to create pAS21. To make a translational fusion to *gusA*, pAS21 was digested with *Sal*I, blunt-ended, and a 2·0 kb *Sma*I–*Hin*dIII-digested, blunt-ended fragment from pKW118 was inserted, creating pAS22. The resulting *nifH–gusA* fusion was cloned as a 2·3 kb *Not*I fragment into pUT/mini-Tn*5* Sm/Sp to create pCAM131 containing mTn*5*SS*gusA31*.

Construction of a promoter–probe transposon

pUT/mTn*5*SS*gusA40*. The promoterless *gusA* gene from pRAJ294 was inserted as a 1·9 kb *Eco*RI–*Hin*dIII fragment into pUC18Not forming pKW120. The resulting 2 kb *Not*I fragment was inserted into pUT/mini-Tn*5* Sm/Sp. A clone in which the *gusA* gene was oriented so that it would be transcribed from the outside end of pUT/mini-Tn*5* Sm/Sp was designated pCAM140, containing mTn*5*SS*gusA40*.

Determination of orientation of *gusA* in mini-transposons. The orientation of *gusA* inserts in the transposons was determined by PCR using the following primers: WIL1 (homologous to the right hand, outside (O) end of mini-Tn*5*, plus an additional 2 bp from the adjacent *Not*I site) 5′ CTGACTCTTATACACAAGTGC 3′; WIL2 (homologous to the region between the *Bam*HI and *Hin*dIII sites flanking the interposon) 5′ GCTCAATCAATCACCGGATCC 3′; and WIL3 (homologous to the non-coding strand of *gusA*, 40 nt downstream of the ATG codon) 5′ GAATGCCCACAG-GCCGTCGAG 3′. A DNA amplification product with WIL1 + WIL3 indicated that the *gusA* gene was orientated such that *gusA* was transcribed into the transposon from the O-end, as in mTn*5*SS*gusA40*. Conversely, an amplification product with WIL2 + WIL3 indicated the reverse orientation. The WIL3 primer was also used to confirm junctions of translational fusions by DNA sequencing.

Quantitative GUS assays. GUS-marked bacteria were grown to mid-exponential phase in YM. Where *gusA* expression was inducible by IPTG (strains marked with mTn*5*SS*gusA10*), duplicate cultures were set up, one containing 2 mM IPTG. For the assays, 1·5 ml of a mid-exponential phase culture was centrifuged and the pellet resuspended in 1 ml 50 mM $NaPO_4$, pH 7·0, 1 mM EDTA. Serial dilutions were made for viable cell counts using the Miles & Misra (Collins & Lyne, 1985) drop count method, prior to carrying out quantitative GUS assays using *p*-nitrophenyl glucuronide (pNPG) as described by Wilson *et al.* (1992).

Growth and inoculation of plants. Frozen rhizobial inoculum was prepared, and plants were grown in 1:1 sand:vermiculite and watered with nitrogen-free Bergersen's medium as described by Wilson *et al.* (1987). Siratro (*Macroptilium atropurpureum*) seedlings were surface-sterilized and scarified by treatment in concentrated H_2SO_4 for 12 min prior to extensive rinsing in sterile water; pigeonpea (*Cajanus cajan*, cultivar Quantum) and common bean (*P. vulgaris*) were surface-sterilized by immersion in 0·1% $HgCl_2$ for 3 min, prior to rinsing in sterile water. Seeds were inoculated with approximately 10^6–10^7 rhizobial cells per seed.

Staining for GUS activity. GUS assay buffers were based on the standard phosphate buffer (Jefferson, 1987) containing 50 mM $NaPO_4$, pH 7·0, 1 mM EDTA, 0·1% Sarkosyl, 0·1% Triton X-100. X-GlcA (generally used at 100 μg ml^{-1}) was from Biosynth. Following staining, roots were cleared using 50% (v/v) household bleach (2·5% final concentration hypochlorite) for 30 min, followed by extensive washing with deionized water.

Results

A restriction$^-$ GUS$^-$ *E. coli* K12 host strain

The *gusA* gene is derived from *E. coli*, and *E. coli* K12 isolates routinely used in the laboratory exhibit low-level GUS activity. This can cause problems when screening for GUS-expressing plasmids. An *E. coli* K12 strain deleted for the entire *gus* operon, SØ200 (Jochimsen *et al.*, 1975), was available, but it retained the EcoK restriction and modification activities (hsdR$^+$, hsdM$^+$). We therefore converted it to a restriction$^-$ genotype by phage P1 transduction (Miller, 1972), generating strain KW1. Plasmids expressing even low-level GUS activity can be unambigously detected in strain KW1 by formation of blue colonies on medium containing 50 μg ml^{-1} X-GlcA.

Plasmids useful for genetic manipulation of GUS in bacteria

Promoterless GUS constructs. For ease of construction of *gus* cassettes under the control of different promoters, two plasmids containing *gusABC* (pRAJ289) and *gusA* only (pRAJ294) were constructed (Fig. 1a, b). In these plasmids the inserts contain the original strong Shine–Dalgarno sequence from the *gus* operon, but are under control of the vector promoter. pRAJ294 is therefore an excellent plasmid for high-level expression of GUS.

Translational fusion vectors. pKW117, pKW118 and pKW119 allow construction of translational fusions to *gusA* in all three reading frames (Fig. 1c), with a strong bacterial transcriptional terminator downstream, followed by a convenient *Hin*dIII site. They parallel the widely-used translational fusion vectors pBI101.1, pBI101.2 and pBI101.3, which contain the eukaryotic *nos* polyadenylation site 3′ to *gusA*.

pTacter. pTacter enables cloning of promoterless genes under the control of the *tac* promoter and with a strong transcriptional terminator downstream, and the subsequent removal of the entire expression cassette as a single *Hin*dIII fragment (Fig. 1d).

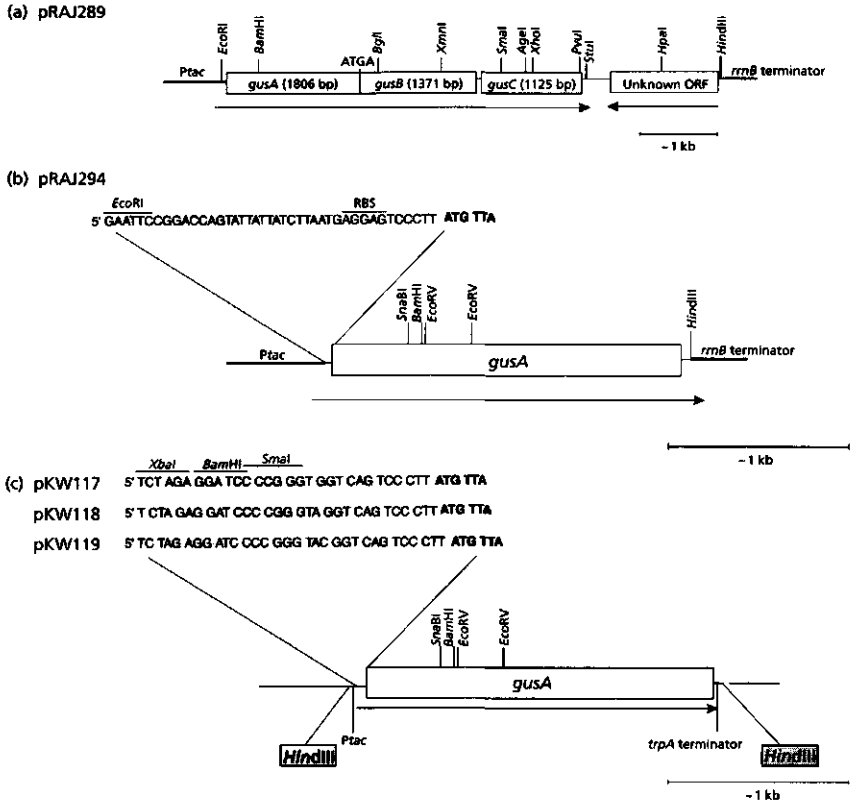

Fig. 1. Plasmids useful for genetic manipulation of *gusA* in bacteria. (a) pRAJ289 contains the *gus* operon from *E. coli* K12 under the control of the *tac* promoter in pTTQ18. *gusA* encodes β-glucuronidase, *gusB* encodes the glucuronide permease and *gusC* is a membrane-associated protein of unknown function (Jefferson *et al.*, 1986; Liang 1992; Wilson *et al.*, 1992). The construct also includes an ORF of unknown function which is oriented in the opposite direction (Liang, 1992). The GenBank accession number for the entire insert is M14641. (b) pRAJ294 contains *gusA* under the control of the *tac* promoter in pTTQ18. The upstream sequence containing the original strong Shine–Dalgarno ribosome-binding site (RBS) from the *gus* operon is indicated on pRAJ294. (c)Translational fusion vectors. pKW117, pKW118 and pKW119 allow construction of translational fusions to *gusA* with the *trpA* bacterial transcriptional terminator downstream. All three sites indicated can be used to construct translational fusions to *gusA* by choosing the vector with the appropriate reading frame as indicated. (d) pTacter. The sequence of the cassette containing the *tac* promoter and the *trpA* transcriptional terminator is shown. The entire cassette is bounded by *Hin*dIII sites as indicated and is in a pUC8 backbone.

Construction of *gusA*-expressing transposons

Transposons with constitutive GUS activity. A key aim was to construct a set of transposons that would be active in as wide a variety of Gram-negative bacteria as possible. It was therefore necessary to identify promoters that could be used to direct transcription of *gusA* in diverse bacterial species. The first promoter selected was the *aph* promoter that drives transcription of the kanamycin resistance gene in Tn*5*. Tn*5* mutagenesis has been shown to work in a

diversity of Gram-negative bacteria using kanamycin selection (de Bruijn & Lupski, 1984), and therefore the *aph* promoter must be active in all these bacterial species. This promoter has not been precisely mapped, but evidence indicates that it is influenced by sequences which lie 110 bp upstream of the translational start of the *aph* gene within IS*50L* (Rothstein & Reznikoff, 1981). Thus, a region of Tn*5* encompassing these upstream sequences and 33 bp of *aph* coding sequence was used to make a translational fusion of the *aph* gene with *gusA*. This fusion was inserted into pUT/mini-Tn*5* Sm/Sp to create mTn*5*SS*gusA20* (Fig. 2a) which gives high-level constitutive expression of GUS in *Rhizobium* (Table 3). mTn*5*SS*gusA21* is similar to mTn*5*SS*gusA20*, except that a unique *Spe*I site was incorporated adjacent to the *gusA* gene (Fig. 2b).

Transposons with regulated *gusA* expression. mTn*5*SS*gusA10* was constructed to enable regulation of *gusA* so that it is only expressed at high levels at the time of assay. This should reduce any metabolic load imposed by GUS expression. mTn*5*SS*gusA10* contains the *lacI*^{q}gene and *gusA* under control of the *tac* promoter (Russell & Bennett, 1982), and therefore expression is repressed until the gratuitous inducer of the *lac* operon, IPTG, is added (Fig. 2c). In liquid culture, on addition of IPTG, expression of *gusA* from mTn*5*SS*gusA10* was induced approximately 30-fold in *Rhizobium* sp. NGR234, and about 20-fold in *R. tropici* CIAT899 (Table 3). Derivatives of strains NGR234 and CIAT899 marked with mTn*5*SS*gusA11*, which contains the same P*tac*–*gusA*–ter cassette, but without the *lacI*^{q} gene, showed constitutive GUS activity slightly higher than that of induced mTn*5*SS*gusA10* (Fig. 2d, Table 3).

Construction of symbiotically expressed *gusA* cassettes. To ensure strong expression of *gusA* under symbiotic conditions, translational fusions of *gusA* to *nifH* genes from both a *Rhizobium* and a *Bradyrhizobium* strain were made: *nifH* encodes the Fe-component of nitrogenase, and is expressed only in symbiotic or microaerobic conditions (Fischer, 1994). mTn*5*SS*gusA30* contains more than 1 kb of upstream sequence from the *nifH* promoter of *R. etli* strain CFN42 (Fig. 2e), and thus includes both the RNA polymerase σ^{54}-dependent promoter sequences, and the upstream activating sequence (UAS) that is typically located about 80–150 bp upstream of *nifH* start sites (Fischer, 1994). By contrast, the *nifH–gusA* fusion in mTn*5*SS*gusA31* contains only about 50 bp of sequence upstream from the deduced *nifH* start site of *Bradyrhizobium* sp. (*Parasponia*) strain Rp501, and does not contain the UAS (Fig. 2f).

Promoter–probe transposon. Finally, a promoter–probe transposon, mTn*5*SS*gusA40*, was constructed (Fig. 2g). In this transposon *gusA* lacks a promoter and is orientated such that it can be transcribed off adjacent promoters in the genomic DNA. In a test mating of mTn*5*SS*gusA40* into *Rhizobium* sp. strain NGR234, expression of the *gusA* gene was found to vary widely from no activity, to activity as high as 190 nmol pNPG hydrolysed per min per 10^{8} cells. This was reflected in the appearance of transconjugant colonies on plates containing 50 μg X-GlcA ml^{-1} which varied from white to deep blue.

Optimization of assay conditions for studying rhizobial infection and nodule occupancy

To determine the optimal staining conditions for studying root colonization and nodule occupancy with regard to sensitivity and cost, *Rhizobium* sp. strain NGR234, marked with all the GUS transposons (except for mTn*5*SS*gusA21* and mTn*5*SS*gusA40*), was inoculated onto siratro and pigeonpea; *R. tropici* strain CIAT899 marked with the same set of transposons was inoculated onto common bean. A basic histochemical GUS assay consists of immersing tissue in buffer containing an appropriate substrate, generally X-GlcA, and looking for spatially restricted colour development (Jefferson, 1987). Factors that can be varied include the concentration of substrate, strategies to eliminate possible background activity from endogenous microbes, and the addition of oxidation catalysts.

Concentration of substrate. The efficiency of detection of nodule occupancy was investigated by using 50, 100, 250 and 500 μg X-GlcA ml^{-1} to stain nodules induced by strain NGR234::*gusA10* on 41-d-old siratro plants. After overnight incubation, nodules incubated in 500 μg ml^{-1} X-GlcA were well stained. Colour development was apparent in the other treatments as well, but concentration had a pronounced effect: nodules incubated in 50 μg X-GlcA ml^{-1} were only slightly blue, those in 100 μg X-GlcA ml^{-1} were mid-blue and those in 250 μg X-GlcA ml^{-1} were mid- to dark-blue. The plants were then left at room temperature in the substrate. It was apparent that colour development was continuing over several days, and after 1 week colour development was as strong in nodules that had been incubated in 100 μg X-GlcA ml^{-1}, as in those incubated in 500 μg X-GlcA ml^{-1}. It was only in the treatments that used 50 μg X-GlcA ml^{-1} that the nodule staining was less pronounced. We therefore concluded that 100 μg ml^{-1} was a suitable concentration for these assays. No blue colour was observed in the nodules of plants nodulated by the parental strain NGR234 even after 1 week's incubation in the buffer.

GUS activity from other microbes. Another difference between treatments was the appearance of staining either on the surface or within the root. This staining occurred particularly where the shoot had been cut from the root and at sites of lateral root emergence, and was observed only in the 250 and 500 μg X-GlcA ml^{-1} treatments. Significantly, this staining was also observed on the roots of plants that were inoculated with the unmarked, parental strain NGR234 in which the nodules remained unstained.

In *E. coli*, and at least some other bacteria that possess GUS activity, GUS is induced only in the presence of particular concentrations of glucuronide substrates (Stoeber, 1961; Tör *et al.*, 1992; Wilson *et al.*, 1992). A possible explanation, therefore, was the presence of additional microbes with inducible GUS activity. To test this possibility, basic phosphate buffer containing 500 μg

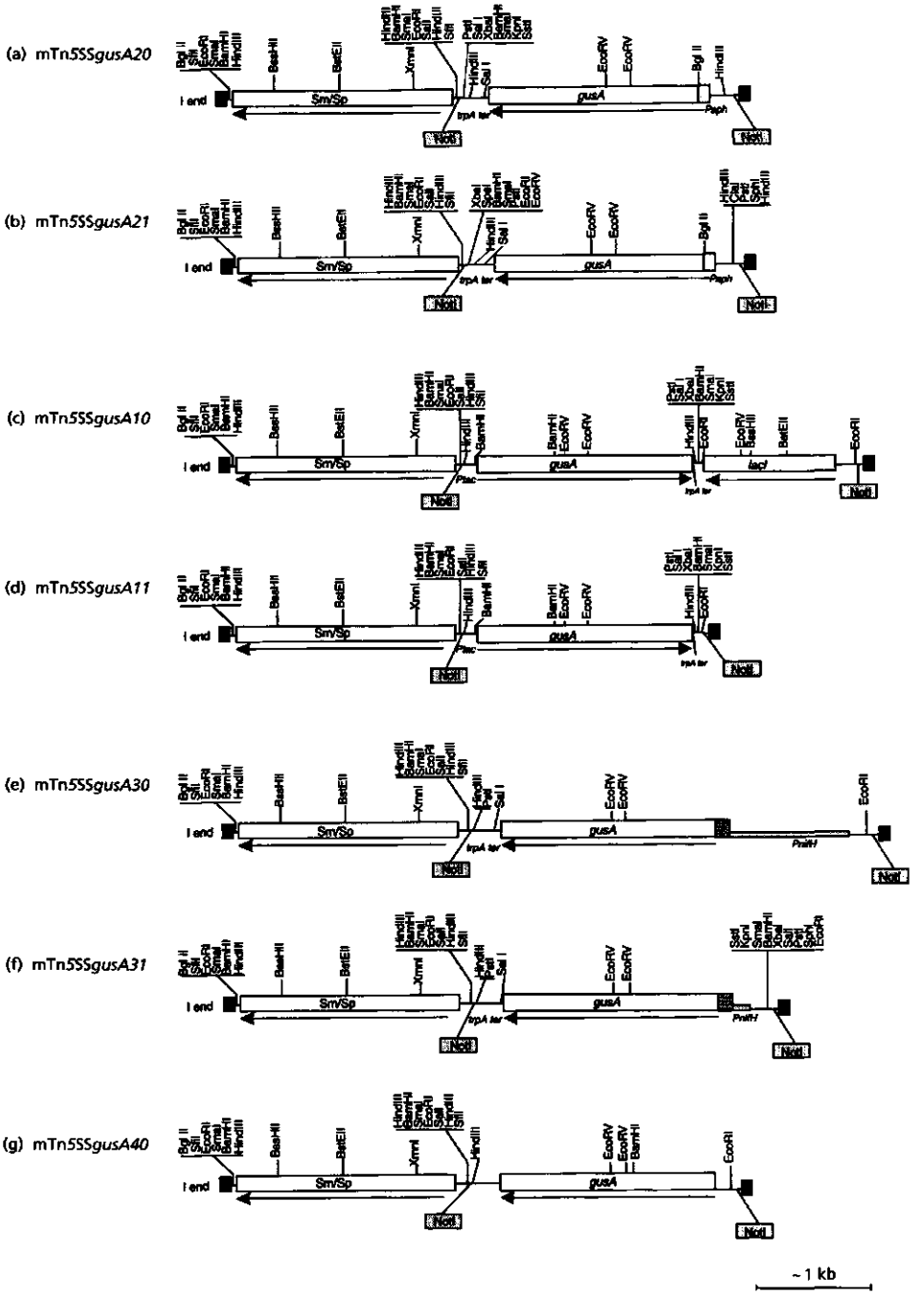

Fig. 2. For legend see facing page.

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

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

Transposon inserted	GUS activity (nmol pNPG hydolysed per min per 10^9 viable cells) in:		
	IPTG	Strain NGR234	Strain CIAT899
None	–	0	0
mTn*5*SS*gusA10*	–	4·45±0·8	6·06±0·8
mTn*5*SS*gusA10*	+	149±20	119±26
mTn*5*SS*gusA11*	–	182±30	214±20
mTn*5*SS*gusA20*	–	121±8	139±50

X-GlcA ml^{-1} was prepared and divided into four aliquots with the addition of respectively: nothing; 100 µg chloramphenicol ml^{-1}; 100 µg cycloheximide ml^{-1}; 100 µg chloramphenicol ml^{-1} plus 100 µg cycloheximide ml^{-1}. Siratro plants (64-d-old, nodulated by parental strain NGR234, and by strain NGR234::*gusA10*) were harvested and incubated in each of these buffers.

Good staining was observed in the nodules of all treatments nodulated by strain NGR234::*gusA10*, and no staining was observed in nodules induced by parental strain NGR234. In chloramphenicol-containing buffers, no staining was observed on the surface of the roots of any plants. By contrast, after overnight incubation at 37 °C, there was substantial staining on the root surface of all plants incubated in the two sets of buffers that did not contain chloramphenicol (no addition, or plus cycloheximide only), including those nodulated by the unmarked parental strain NGR234.

Oxidation of substrate. One of the factors affecting the rate of development of blue product is the oxidative potential. The reaction that produces the indigo precipitate from X-GlcA occurs in two steps, the first step being hydrolysis of the substrate by GUS and the second step being oxidative dimerization of the colourless indoxyl that is released following GUS cleavage (Wilson, 1995). This could be a particular problem in nodules since the ambient oxygen concentration in an active nodule can drop from about 250 µM to less than 1 µM from the outer cortex to the inner bacteroid zone (Witty *et al.*, 1987). We therefore examined the effect of adding oxidation catalysts to the buffer to see whether this would aid the development of blue colour. In fact the opposite effect was observed: the inclusion of 1 mM potassium ferricyanide or 1 mM potassium ferrocyanide, either separately or together, slightly decreased colour development in intact nodules.

Clearing of the tissue. The brown pigmentation present in roots and the red colour of leghaemoglobin in mature nodules can hinder the visualization of blue colour in nodules. We therefore tested various root-clearing protocols (Bevege, 1968; O'Brien & von Teichman, 1974), but found that simple room temperature treatment in bleach was equally effective and greatly facilitated visualization of blue-stained nodules.

Use of the transposons to study the *Rhizobium*–legume interaction

Detection of marked bacteria in the rhizosphere. A subset of the transposons, mTn*5*SS*gusA10*, mTn*5*SS*gusA11*, mTn*5*SS*gusA20* and mTn*5*SS*gusA21* enable expression of GUS in rhizobia in the free-living state, as well as in nodules. Following a histochemical GUS assay, dense areas of colonizing bacteria were visible as blue patches on the root surface and early stages of infection, including root hair colonization and infection and penetration of the root cortex could be readily visualized (Fig. 3a). To examine these early stages of infection, higher concentrations (150 µg ml^{-1}) of substrate were used and 1 mM potassium ferricyanide was included in the buffer.

Young nodules, including incipient nodules just emerging from the root cortex, could also be readily detected using these transposons (Fig. 3b). However, we found that older nodules induced by strains marked with these transposons could not be reliably identified using the X-GlcA assay. For example, siratro plants inoculated with *Rhizobium* strain NGR234 marked with mTn*5*SS*gusA11* or mTn*5*SS*gusA20*, examined for nodule occupancy 42 d after inoculation, showed very variable results. On some plants all the nodules stained blue, whereas in others less than 50% of the nodules stained blue. The unstained nodules were almost certainly not due to cross-contamination as there were no nodules on any of the uninoculated plants.

The behaviour of mTn*5*SS*gusA10*, in which *gusA* expression is regulated by the product of the *lacI*^{q} gene, differs from that of the transposons with constitutive *gusA* expression in free-living bacteria. On solid medium containing X-GlcA, blue colonies were formed by *Rhizobium* strains containing this transposon only in the presence of IPTG (1 mM), in contrast to strains marked with the other transposons in this group which did not require any inducer to form blue colonies on solid medium. When used to infect plants, minimal surface-

Fig. 2. **Restriction maps of GUS transposons. mTn*5*SS*gusA10* expresses GUS in a regulated manner, dependent on induction by IPTG. mTn*5*SS*gusA11*, mTn*5*SS*gusA20* and mTn*5*SS*gusA21* express GUS constitutively. mTn*5*SS*gusA21* is designed to be useful for mapping the site of transposon insertions using PFGE. mTn*5*SS*gusA30* and mTn*5*SS*gusA31* both have GUS expressed from a symbiotically active promoter. mTn*5*SS*gusA40* is a promoter–probe transposon. The antibiotic resistance cassette in each transposon is flanked by transcriptional and translational terminators. Note that there is an internal *Bam*HI site as indicated in the *gusA* gene in a number of the transposons. The restriction maps refer specifically to the transposons, and do not take into account restriction sites present in the delivery plasmid, pUT.**

Fig. 3. Examples of assays carried out using GUS-marked strains. (a) Early stages of infection of a siratro root infected with *Rhizobium* sp. NGR234 marked with mTn*5SSgusA20*. The photograph shows a root harvested 20 d after inoculation. For this type of localization, the roots were vacuum-infiltrated in buffer containing 150 μg X-GlcA ml^{-1} and 1 mM potassium ferricyanide, and then incubated at 37 °C overnight. Roots were cleared using 2·5 % hypochlorite prior to photography. (b) Nodules induced on a siratro root (20 d after inoculation) infected with *Rhizobium* sp. NGR234 marked with mTn*5SSgusA11*. Note surface staining and detection of very young nodules. (c) Adjacent pigeonpea nodules induced by *Rhizobium* sp. NGR234 and *Rhizobium* sp. NGR234:*gusA31*. Note the precise discrimination between nodules induced by a marked and an unmarked strain, and the absence of surface staining on the root. (d) Hand section through a pigeonpea nodule induced by *Rhizobium* sp. NGR234 marked with mTn*5SSgusA11* showing expression is limited to the outer regions of the nodule. (e) Hand section through a pigeonpea nodule induced by *Rhizobium* sp. NGR234 marked with mTn*5SSgusA31* showing expression is maximal in the central regions of the nodule. (f) Pigeonpea plant inoculated with *Rhizobium* sp. NGR234 and *Rhizobium* sp. NGR234:*gusA31* in a ratio of 7:1. (g) Pigeonpea plant inoculated with *Rhizobium* sp. NGR234 and *Rhizobium* sp. NGR234:*gusA31* in a ratio of 1:15.

staining was observed on roots inoculated with strains marked with mTn*5SSgusA10*, and incubation in 1 mM IPTG for 5 h at 30 °C prior to the GUS assay had no effect on the degree of staining using this transposon. Surprisingly, siratro nodules occupied by strains marked with this transposon could be reliably detected at a later stage than those induced by strains marked with either mTn*5SSgusA11* or mTn*5SSgusA20*; on siratro plants 42-d post-inoculation, all nodules induced by two independent isolates of strain NGR234:*gusA10* stained blue in contrast to the results discussed above for mTn*5SSgusA11* or mTn*5SSgusA20*.

Use of transposons to determine nodule occupancy

To obtain efficient staining in mature nitrogen-fixing nodules, two transposons with translational fusions of *gusA* to the *nifH* gene of *R. etli* strain CFN42 (mTn*5SSgusA30*) and to that of *Bradyrhizobium* sp. (*Parasponia*) strain Rp501 (mTn*5SSgusA31*) were used. In separate experiments, *Rhizobium* sp. strain NGR234 marked with mTn*5SSgusA31* was inoculated onto siratro and pigeonpea, and *R. tropici* strain CIAT899 marked with both transposons was inoculated onto *Phaseolus* plants. Very deep blue staining was observed in all nodules induced by CIAT899::*gusA30* and CIAT899::*gusA31* on *P. vulgaris* up to 37 d after inoculation (date of final harvest). No difference in intensity of staining was observed between strains marked with the two transposons. Likewise, pigeonpea and siratro nodules induced by NGR234::*gusA31* stained deeply even at a harvest date of 70 d after planting. No staining was observed on the root surface when using these transposons (Fig. 3c). Fig. 3(c) also indicates how precise the discrimination is between adjacent nodules occupied by a marked and an unmarked strain, even using X-GlcA buffer without inclusion of potassium ferricyanide or potassium ferrocyanide, and leaving the nodules in the staining buffer for 3 d prior to photography.

Pattern of expression of transposons within nodules. The different promoters used to drive *gusA* expression in the transposons might be expected to give different spatial patterns of activity in the nodule. This was examined by hand-sectioning pigeonpea nodules from roots harvested 26 d after planting and inoculation. Nodules induced by *Rhizobium* sp. NGR234:*gusA31* showed strong GUS activity in the central, nitrogen-fixing zone of the nodule (Fig. 3d), as did nodules induced on *P. vulgaris* by CIAT899::*gusA30*. By contrast, nodules induced by *Rhizobium* sp. NGR234:*gusA11* showed maximal expression in the peripheral area of the nodule, presumably where new infections are taking place (pigeonpea forms indeterminate nodules) (Fig. 3e). The latter pattern of expression is also observed in nodules induced by isolates marked with transposons mTn*5SSgusA10* and mTn*5SSgusA20*.

Time delay between harvest and assay. If this assay is to be of practical use in field analysis it is important that initiation of the GUS assay can be delayed for several hours post-harvest to allow transport of nodulated root systems from the field to the laboratory. To examine this, staining of 42-d-old siratro plants inoculated with strains NGR234::*gusA10*, NGR234::*gusA11* and NGR234::*gusA20* was commenced at three different times after harvest. The first group were stained within 1 h of harvest, the second set 6 h after harvest, having been kept at ambient temperature (about 20 °C), and the final set was stained the following day after being kept at ambient temperature for 6 h, and then stored at 4 °C overnight. No correlation was observed between the time delay before staining and the percentage of nodules stained.

Effect on symbiotic properties. Dry shoot weights were measured at all harvest dates and no significant differences were observed between plants inoculated with the parental strains NGR234 or CIAT899 and the GUS-marked derivatives. This included pigeonpea and siratro plants harvested 70 d after planting. By contrast, non-nodulated plants showed yellow leaves and significantly reduced shoot weight compared to plants inoculated with the wild-type strains.

Use of GUS as a marker in nodule occupancy competition assays. A key aim is to use GUS as a marker in rhizobial competition studies. An experiment was set up in which the parental strain NGR234 was co-inoculated with NGR234::*gusA31* in about 10:1, 1:1 or 1:10 ratios to demonstrate the principle of using these markers in competition assays. Independent nodules induced by marked versus unmarked strains could be discriminated very precisely (Fig. 3c), and the proportion of blue nodules increased with an increasing proportion of the GUS-marked strain in the inoculum. Following viable cell number counts at the time of inoculation, the actual ratios of inoculation of wild-type to GUS^+ strains were found to be closer to 7:1, 1:1·5, and 1:15, and these gave rise respectively to 19, 80 and 86% blue nodules on pigeonpea, and to 0, 82 and 91% blue nodules on siratro (average of two plants assayed at final harvest) (Fig. 3f, g).

DISCUSSION

We describe here additional vectors useful for the molecular manipulation of the *gusA* gene (Fig. 1), and a series of transposons that express *gusA* from different promoters (Fig. 2). The transposons are designed primarily for use by microbial ecologists for measuring population changes in soil and in the rhizosphere and, in particular, as a tool for determining nodule occupancy in rhizobial competition studies.

Each of the different transposons is designed for a specific purpose. mTn*5SSgusA10* is primarily for studying populations of free-living bacteria, as *gusA* expression remains at a basal level until the addition of IPTG, when it shows strong induction both in liquid culture (Table 3) and as colonies on agar plates. The efficiency of regulation of the *tac* promoter by the *lacI* product varies in different Gram-negative bacteria (Fürste *et al.*, 1986), and the extent of induction observed in *Rhizobium* sp. NGR234 and *R. tropici* CIAT899 (about 30-fold and 20-fold, respectively)

falls within the range observed in other species (10–200-fold induction; Fürste *et al.*, 1986). This regulation should reduce possible effects on ecological fitness as high-level expression of the marker gene is not induced until the experimental assay is initiated. mTn*5*SS*gusA10* can also be used in nodule occupancy studies, but this may be due to basal expression as there is no obvious effect of addition of IPTG on the development of blue colour in nodules. This is unlikely to be a problem of IPTG penetration, as galactosides are not charged, in contrast to glucuronides, and therefore should pass through membranes more readily than the accompanying GUS substrates. The lack of apparent induction could be because the bacteria are not multiplying as rapidly as in free-living culture in rich medium, and are therefore unable to initiate high-level synthesis of new proteins as efficiently. Quantitative assays on nodule tissue using pNPG as substrate would be required to analyse this further.

The transposons mTn*5*SS*gusA11* and mTn*5*SS*gusA20* both give strong constitutive GUS expression in the free-living state (Table 3), and are optimal for studies of rhizosphere colonization (Fig. 3a). They can also be used for nodule occupancy studies in young plants (Fig. 3b). However, expression from these transposons declines in older nodules, and thus they are not optimal for longer-term nodule occupancy experiments. This decline in expression in older nodules may be due in part to the temporal and spatial patterns of GUS expression conferred by these promoters, which appears strongest in the outer zones of pigeonpea nodules where undifferentiated bacteria may still be present, and reduced in the central nitrogen-fixing zone of the nodule (Fig. 3d). Similar spatial restriction of GUS expression has been obtained using an R. *leguminosarum* bv. *trifolii* strain marked with mTn*5*SS*gusA20* to infect subterranean clover (de Boer & Djordjevic, 1995). It is known that expression of some genes is specifically repressed in bacteroids (e.g. de Maagd *et al.*, 1994). However, further work would be needed to clarify whether this is the case here.

mTn*5*SS*gusA21* is similar to mTn*5*SS*gusA20*, except that a unique *Spe*I site was incorporated adjacent to the *gusA* gene, to facilitate chromosomal mapping of insertions as *Spe*I is a rare-cutting enzyme in bacteria with high G + C contents, including rhizobia (Sobral *et al.*, 1991). For example, *Spe*I was used in PFGE to analyse the symbiotic plasmid and facilitate ordering of an overlapping cosmid library in *Rhizobium* strain NGR234 (Perret *et al.*, 1991). Since mTn*5*SS*gusA21* (and all the other mini-Tn*5*-based GUS transposons) also contains *Not*I sites, which are rare cutters in other species, the transposons could alternatively be used for chromosomal mapping of insertions with PFGE using this enzyme.

For longer-term nodule occupancy experiments, either of the two transposons, mTn*5*SS*gusA30* or mTn*5*SS*gusA31*, containing *gusA* expressed from a symbiotic promoter, are recommended. Although these two transposons differ both in the origin of the *nifH* promoter and in the presence versus the absence of the NifA-dependent upstream activating sequence, no differences in GUS expression were apparent in symbiotic conditions using the histochemical assay on nodulated *P. vulgaris* plants. Hand sections revealed maximal expression of GUS in the central, nitrogen-fixing zone of active nodules as expected. The importance of the UASs on symbiotic, as opposed to microaerobic, activity of *nif* promoters is uncertain. In *B. japonicum*, deletion of UAS sequences from the *nifD* promoter reduced its activity to about 10% of that of the wild-type promoter in nodules (Alvarez-Morales *et al.*, 1986), whereas in R. *meliloti*, a *nifH* promoter lacking the UAS still retained 50% of the wild-type symbiotic activity (Wang *et al.*, 1991). Quantitative GUS assays on individual nodules are necessary to examine this further.

Finally we describe a *gusA* promoter–probe transposon, mTn*5*SS*gusA40*. The utility of this transposon has not been compared directly to earlier promoter–probe *gusA* transposons described by Sharma & Signet (1990). mTn*5*SS*gusA40*, like the transposons described by Sharma & Signer (1990) should be of use both for molecular genetic studies, and for screening bacteria for promoters which respond to specific environmental signals, such as components of root exudate, or for promoters which are of utility in other experimental situations. For example, to optimize rhizobial competition assays it would be possible to screen individual isolates of a *Rhizobium* strain marked with this transposon for isolates which gave the maximal longevity of expression in mature legume nodules.

The assay conditions developed here for studying nodule occupancy differ in a number of parameters from those routinely used in plant molecular biology (Jefferson, 1987; De Block & Debrouwer, 1992). In plant molecular biology, where absolutely precise cellular or sub-cellular localization of GUS activity is required, recommended conditions are 1–3 mM X-GlcA, with the inclusion of agents to promote the oxidative dimerization of the colourless product of X-GlcA cleavage, to give the blue precipitate. Unfortunately, X-GlcA is an expensive substrate, and while such conditions would be perfectly suitable for nodule occupancy studies, they would lead to the assay being very costly. By keeping the substrate concentration low (100 $\mu g\ ml^{-1}$), the cost of the assay is reduced by at least 10-fold.

To maximize sensitivity of the assay with a low substrate concentration, potassium ferricyanide and potassium ferrocyanide are omitted from the buffer as these compounds, while enhancing the precision of spatial localization of GUS activity, also reduce the degree of blue staining (our results, and De Block & Debrouwer, 1992). Finally, as GUS is stable over several days, it is possible to allow reactions to proceed for much longer time periods if necessary. As long as controls inoculated with unmarked bacteria are included, these reaction conditions work well to distinguish nodules occupied by GUS-marked bacteria from those occupied by unmarked strains. For example Fig. 3(c) shows the precision of discrimination between two adjacent nodules following incubation of the roots in buffer without any oxidation catalyst over a period of 3 d. However, if more precise

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

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

This set of transposons is designed to be of use in a diverse range of Gram-negative bacteria. Although their use in species other than *Rhizobium* is not described here, Tn*5* is known to have an extremely broad host-range (de Bruijn & Lupski, 1984), and there is no reason to suppose that inclusion of the *gusA* gene will affect the host range of these transposons. Indeed, Tn*5*KW107 (Wilson *et al.*, 1991) and mTn*5SSgusA40* have both been used in *Pseudomonas* spp. (Wilson *et al.*, 1994; L. de Weger, personal communication), and mTn*5SSgusA11* and mTn*5SSgusA20* have both been used in *Azospirillum* spp. (C. Christiansen-Weniger, C. Nirmala & S. Katupitya, personal communications).

The transposons described here all confer streptomycin/spectinomycin resistance and we have found that spectinomycin is an excellent marker in all the strains that we have tested (unpublished data). This is true even in strains with endogenous streptomycin resistance because, although both streptomycin and spectinomycin can be inactivated by the same enzyme, the two antibiotics actually target different components of the ribosome. Streptomycin affects the S12 protein of the 30S ribosomal subunit, whereas spectinomycin acts on the S5 protein of the same subunit (Bryan, 1982). Thus, a strain with endogenous or acquired resistance to streptomycin will often still be sensitive to spectinomycin. Additionally, as mini-transposons are available with a wide range of selectable markers, and with unique *Not*I sites for cloning, it is a matter of a single-step cloning using precursor plasmids described in this paper to construct GUS transposons with the same expression cassettes but with different selectable markers, conferring either antibiotic (de Lorenzo *et al.*, 1990) or non-antibiotic resistance (Herrero *et al.*, 1990).

The key advantage of using GUS-marked strains for rhizobial competition studies is that whole root systems – and hence extremely high numbers of nodules – can be analysed for nodule occupancy in a one-step assay. It is also practical for future field use as initiation of the assay can be delayed for at least 24 h after harvest to allow transport of harvested roots from the field to the laboratory. It is relatively inexpensive, despite the high cost of the substrate, costing no more than one or two dollars to assay nodule occupancy on a whole root system and hence comparing favourably with the costs of labour involved in the alternative methods available. Additionally, there is the strong advantage that this assay makes it easy to record types of information that are usually too laborious to gather. For example, information regarding the position of nodules induced by the inoculum strain down the root is preserved.

As GUS-marked bacteria can be localized on the root surface or in infection threads, this opens the possibility of studying the early stages of infection and relating this to ultimate success in competition for nodule occupancy. For instance, if relationships between rhizosphere colonization, root hair infection and nodulation could be assessed, this would provide a means of studying the interaction between different strains and the plant root at a resolution not previously possible. These transposons have already been used to study the point at which nodule development fails in strain–cultivar specific interactions between different *R. leguminosarum* bv. *trifolii* strains and subterranean clover cultivars (de Boer & Djordjevic, 1995).

The question of the effect on symbiotic or other ecological properties is also an important one. It is clear that some preliminary screening of marked strains is necessary to ensure that there are no major changes in these properties. However, our initial results (unpublished data) and those of others (Streit *et al.*, 1995) indicate that it is easy to identify marked derivatives which do not differ from the parent in competitive ability for use in ecological studies. The dramatic increase in throughput of nodule typing, and the consequent increase in statistical accuracy (see Wilson, 1995), are more than sufficient to compensate for the work involved in the initial screening step. The throughput of analysis is also far greater, and the iterative cost far lower, than with DNA-based methods which are currently under development (e.g. Richardson *et al.*, 1995), although the latter have a significant role to play in assessment of general population structure (see Wilson, 1995).

The efficacy of these marker genes now needs to be rigorously tested in greenhouse and field experiments (under authority of the appropriate regulatory bodies). For example, questions that need to be answered include (i) over what period of the plant's lifespan will the GUS assay be efficient, and (ii) how successful will the assay be in field-grown plants? If successful in field situations, such methods which allow rapid, cost-effective screening of the field performance of beneficial microbes could become standard tools for analysing rhizobial competition and many other aspects of microbial ecology.

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CHAPTER 3

Measurement of the competitiveness index of *Rhizobium tropici* strain CIAT899 derivatives marked with the *gusA* gene

A. Sessitsch, P. K. Jjemba, G. Hardarson, A. D. L. Akkermans and K. J. Wilson

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Measurement of the competitiveness index of *Rhizobium tropici* strain CIAT899 derivatives marked with the *gusA* gene

Angela Sessitsch[1], Patrick J. Jjemba[1], Gudni Hardarson[1], Antoon D. L. Akkermans[2] and Kate J. Wilson[3]

1. Soil Science Unit, FAO/IAEA Agriculture and Biotechnology Laboratories, A-2444 Seibersdorf, Austria
2. Department of Microbiology, Wageningen Agricultural University, Wageningen 6703 CT, The Netherlands
3. Center for the Application of Molecular Biology to International Agriculture (CAMBIA), GPO Box 3200, Canberra, ACT 2601, Australia[1]

Summary

The *gusA* gene encoding ß-glucuronidase has been adapted for use as a marker gene for ecological analysis of Gram-negative bacteria. A key requirement for such marker genes is that they should not directly affect the ecological behaviour being studied. In this paper we examine the impact of introduction of the *gusA* gene on a representative and easily studied ecological phenotype, rhizobial nodulation competitiveness. Five independent isolates of *Rhizobium tropici* strain CIAT899 marked with the *gusA* gene on mini-transposon mTn*5*SS*gusA*10 were characterized for nodulation characteristics and competitive abilities on common bean (*Phaseolus vulgaris)*. Insertion of mTn*5*SS*gusA*10 did not significantly change the nodulation or nitrogen fixation behaviour of *R. tropici* CIAT899. However, the competitiveness index of different mTn*5*SS*gusA*10-marked derivatives varied relative to the parental strain, both between three independent experiments and between isolates. One isolate was less competitive than the wild-type in all three experiments. The competitiveness indices of the other four *gusA*-marked strains varied between experiments, but in each case they appeared either equally competitive or more competitive than the parental strain. The data obtained enabled highly statistically significant calculations of competitiveness indices, as all the nodules on each plant could be analysed for nodule occupancy. These experiments indicate that although primary selection of marked strains is essential, *gusA*-marked *Rhizobium* derivatives with competitive abilities indistinguishable from the parental strain can readily be obtained.

INTRODUCTION

The *gusA* marker gene, encoding the enzyme ß-glucuronidase (GUS), has been adapted for use as a marker for ecological studies in Gram-negative bacteria (Wilson *et al.*, 1995), with particular emphasis on its use as a marker for *Rhizobium* competition studies (Streit *et al.*, 1992; Streit *et al.*, 1995; Wilson *et al.*, 1995). Introduction of the *gusA* gene into a *Rhizobium* strain enables detection of marked bacteria through a simple colour assay, the development of blue colour following incubation with X-GlcA (5-bromo-4-chloro-3-indolyl ß-D-glucuronide). For strain identification this has a number of advantages over previous techniques such as intrinsic (Josey *et al.*,1979) or induced antibiotic resistance markers (Bushby, 1981; Turco *et al.*, 1986), fluorescently labelled antibodies (Schmidt *et al.*, 1968), immunodiffusion (Dudman, 1971) or ELISA (Berger *et al.*, 1979). These include a high degree of specificity due to the absence of GUS activity in both rhizobia and their plant hosts, and the fact that the assay can be carried out on intact nodulated root systems (Wilson *et al.*, 1995), thus obviating the need for picking of nodules for individual analysis of their contents which has been a major impediment in competition studies. The latter is also a substantial advantage in terms of cost and speed of throughput compared to other molecular biological techniques such as analysis of plasmid profiles (Shishido and Pepper, 1990), or discrimination of strains using polymerase chain reaction (PCR) based techniques, including amplification of targeted and random DNA sequences (de Bruijn, 1992; Pillai *et al.*, 1992; Richardson *et al.*, 1995).

A number of mini-transposons have been constructed that express *gusA* under different promoters for use in various ecological situations (Wilson *et al.*, 1995). These "mini-transposons" are disarmed to prevent autonomous transposition following insertion in the host genome, rendering them highly suitable for ecological studies involving microbial releases (Herrero *et al.*, 1990; de Lorenzo *et al.*, 1990). However, to use any marker gene system for ecological studies, the effect on fitness of the host organism must first be rigorously evaluated. There are at least three potential impacts that could be associated with use of the GUS mini-transposons. The first is the effect of expression of the *gusA* gene; the second the effect of expression of the linked selectable marker in the mini-transposon, the *aadA* gene encoding spectinomycin/streptomycin resistance; and the third is the mutagenic impact of insertion of the transposon at random in the host genome.

To date the impact of introduced genes on the ecological fitness of the host organism has only rarely been evaluated (Doyle *et al.*, 1995). Initially it was widely

assumed that genetically engineered microorganisms (GEMs) would always be impaired in fitness compared to parental strains, due to the additional metabolic load imposed by expression of the introduced DNA. In practice, this has proven not to be the case, with a number of studies demonstrating equal survival of GEMs and their parents. For example, a strain of *Erwinia carotovora*, engineered to contain a chromosomal kanamycin resistance gene, showed equivalent survival capabilities to its parental strain in soil microcosms (Orvos *et al.*, 1990). Likewise *Pseudomonas aeroginosa* and *P. putida* growth rates were unaffected by introduced plasmids, although survival capabilities may have declined slightly (Yeung *et al.*, 1989). In other examples, fitness was compromised. For example, strains of *P. fluorescens* marked with a *Bacillus* δ-endotoxin gene had slightly decreased growth and survival capabilities compared to the parental strain (van Elsas *et al.*, 1991). Further, effects on fitness may be dependent on the nature of the host strain rather than the nature of the foreign DNA (Devanas *et al.*,1986), and the host genome may even evolve to become adapted to introduced DNA such that loss of that DNA subsequently reduces fitness (Bouma and Lenski, 1988).

There are few examples where fitness parameters other than growth or survival have been measured. Lam *et al.*,(1990) analysed over 1,200 mutants of *P. putida* containing a promoterless *lacZ* gene on a transposon Tn*5* derivative for their ability to colonize roots, and found isolates with both increased colonization ability and severely decreased colonization ability. However, the majority of isolates showed a colonization ability that differed little from the wild type strain. A few studies have examined the effect on competition of marking rhizobia with the intact transposon Tn*5*. Sharma *et al.*,(1991) found that Tn*5* insertion did not affect the competitive ability of two strains of chickpea rhizobia which had wild-type fixation abilities. Brockman *et al.*,(1991) studied the symbiotic effectiveness and competitive ability of *R. leguminosarum* bv. *viciae* and *R. leguminosarum* bv. *phaseoli* strains marked variously with spontaneous antibiotic resistances and/or transposon Tn*5* insertions or Tn*5* plus vector sequences. The two *R. leguminosarum* bv *viceae* isolates that contained only Tn*5* insertions were not affected in either symbiotic effectiveness or competitive ability. Of three Tn*5*-containing isolates of *R. leguminosarum* bv. *phaseoli*, one was unaffected in these properties, and two showed reduced nodule occupancy, with one of these exhibiting apparently increased symbiotic effectiveness. Rynne *et al.*,(1994) used transposon Tn*5-233* to isolate three mutants of *R. leguminosarum* bv *trifolii* which were defective in aromatic compound degradation, but unaltered in competitive ability. These examples imply that effects of introduced transposons on ecological behaviour are due to position effects rather than inherent properties of the transposons themselves.

The ability of rhizobia to compete for occupancy of nodules on the legume host can be measured in a number of ways. Most commonly, "percentage nodule occupancy" is quoted as reflecting competitive ability, which is generally determined by co-inoculating two strains at a 1:1 ratio in sterile conditions in the greenhouse (as in the above examples). In fact it is the relationship between representation in the nodules (percentage nodule occupancy) and representation in the inoculum, that accurately defines competitiveness. A number of mathematical models have been devised to describe this relationship and to calculate the "competitiveness index" or $C_{X:Y}$, a constant derived from a regression of the log of the ratio of strains in the inoculum against the log of the ratio of nodules occupied by the inoculum strain (Marques Pinto *et al.*,1974; Weaver and Frederick, 1974; Amarger and Lobreau, 1982; Beattie *et al.*, 1989).

In the present study, we have examined the impact of insertion of the *gusA* marker gene on the competitive ability of *R. tropici* strain CIAT899 by determining the competitiveness indices of five *gusA*-marked derivatives of strain CIAT899 relative to that of the parental strain. For the first time, competitiveness indices could be calculated using analysis of all the nodules on each host plant, rather than the sampling strategy that was necessary in earlier work.

MATERIALS AND METHODS

Bacterial strains and media. The bacteria used are listed in Table 1. *R. tropici* strain CIAT899 and its derivatives were grown in a modified yeast extract mannitol (YM) medium (Danso and Alexander, 1974) at 28°C and *E. coli* was grown in LB (Ausubel *et al.*, 1995) at 37°C. Derivatives of strain CIAT899 containing mTn*5*SS*gusA*10 insertions were obtained by plate matings between CIAT899 and the *mob*$^+$ *E. coli* donor strain S17-1(λ-pir) harbouring pCAM110 as previously described by Wilson *et al.*, (1995). Transconjugants were selected on agar plates containing minimal medium (Brown and Dilworth, 1975) amended with 50 μg spectinomycin ml^{-1} and were obtained at a frequency of one per 10^4 recipient cells. Eight transconjugants were purified on the selection medium amended with 40 μg ml^{-1} of the inducer isopropyl-β-D-galactopyranoside (IPTG) and 50 μg ml^{-1} of the chromogenic substrate 5-bromo-4-chloro-3-indolyl-β-D-glucuronic acid (X-GlcA). These transconjugants were separately inoculated onto *Phaseolus vulgaris* plants grown as described below in N-free conditions (three plants per isolate), and after 3 weeks growth the nodules were checked for development of blue colour in buffer containing X-GlcA as described below. Five isolates (A, B, C, D, E), which gave

deep blue nodules, were chosen for further study. The growth rates of CIAT899 and its derivatives were determined by inoculating single colonies into Brown and Dilworth (1975) minimal medium with sucrose (0.2% w/v) as carbon source and ammonium chloride (0.7 g l^{-1}) as nitrogen source and measuring the optical density at 600 nm (OD_{600}) of a growing culture over 12 h.

DNA isolation and Southern analysis. Total genomic DNA from CIAT899 and its mTn*5*SS*gusA*10-marked derivatives was isolated as described (Ausubel *et al.*, 1995). Total DNA of CIAT899 and CIAT899::*gusA*10 A, B, C, D and E was digested separately with *Eco*RV and *Not*I and 2 µg of DNA was separated by gel electrophoresis (1.2% and 1% agarose, w/v, respectively) and blotted onto nylon membrane (Nylon 66, Sartorius). For the probe, the 3.3 kb *Not*I fragment containing the *gusA* and *lacI* genes from mTn*5*SS*gusA*10 (Fig. 1a) was isolated from a low melting point agarose gel (Ausubel *et al.*, 1995) and labeled with ^{32}P by random priming (Ausubel *et al.*, 1995) to high specific activity (10^8 counts min^{-1} μg^{-1} DNA). The membrane was prehybridized for 6 h at 65°C in 5 x SSC (1 x SSC is 150 mM NaCl, 15 mM sodium citrate), 0.5% sodium dodecyl sulfate (SDS), 0.1% Ficoll, 0.1% bovine serum albumin and 0.1% polyvinylpyrrolidone. Hybridization was performed in the same solution for 16 h at 65°C with the addition of 1.4 x 10^6 counts min^{-1} of probe. The filters were then washed twice with 2 x SSC, 0.1% SDS for 10 min at 65°C, once with 1 x SSC, 0.1% SDS for 10 min at 65°C and once with 0.5 x SSC, 0.1% SDS at 65°C. Autoradiography was carried out at -70°C for 6 h in the presence of intensifying screens.

GUS-activity. GUS-activity was quantified using a spectrophotometric assay in which the amount of p-nitrophenol (pNP) produced by β-glucuronidase activity from p-nitrophenyl glucuronide (pNPG) was measured (Wilson *et al.*, 1995). GUS activity was normalized to the number of viable cells as determined using a Miles and Misra drop count assay (Collins and Lyne, 1985) and was expressed in nmol pNP produced min^{-1} 10^9 $cells^{-1}$ (nmol pNP 10^9 $cells^{-1}$ min^{-1}). Three replicate cultures were used for each strain. IPTG-inducible GUS activity was measured following growth of the cultures in YM broth containing 5 m*M* IPTG for 16 h.

Competition experiments. Three competition experiments were carried out between the parental strain and the five independent marked derivatives. Surface-sterilized seeds of *P. vulgaris L.* cv. Riz 44 (obtained from the Centro Internacional de Agricultura Tropical (CIAT), Colombia) were germinated on 1.5% (w/v) water-agar plates and the seedlings transplanted into sterile modified Leonard jars (Vincent, 1970) containing sand and N-free nutrient solution (Somasegaran and Hoben, 1985). Each seedling was inoculated with a 1 ml cell suspension of CIAT899 and its derivatives either individually or in combination at five different ratios (levels I to V).

Table 1. Bacteria and plasmids used in the present study

Species or plasmid	Description	Reference or Source
R. tropici		
CIAT899	Wild-type strain	Martínez-Romero *et al.*, 1991
CIAT899::*gusA*10 A	Sm^r, Sp^r, GUS^+; mTn*5*SS*gusA*10 integrated in genome	This study
CIAT899::*gusA*10 B	-" -	This study
CIAT899::*gusA*10 C	-" -	This study
CIAT899::*gusA*10 D	-" -	This study
CIAT899::*gusA*10 E	-" -	This study
E. coli		
S17-1(λ-pir)	RP4-2 (Tc^S::Mu) (Km^S::Tn7) Tp^r Sm^r recA λ-pir	V. de Lorenzo, University of Madrid
Plasmids		
pCAM110	Ap^r, Sm^r, Sp^r; mobilizable plasmid carrying mTn*5*SS*gusA*10 transposon	Wilson *et al.*, 1995

Level I contained the highest proportion of the marked strain and level V had the lowest proportion of CIAT899::*gusA*10 strains. Inoculum was applied at approximately 2 x 10^8 to 10^9 cfu per seedling for the first experiment and at 10^6 cfu per seedling for the subsequent experiments; the exact numbers of each strain were verified at the time of inoculation using the Miles and Misra drop count method (Collins and Lyne, 1985). Jars containing single plants were arranged in a complete randomized block design (five replicates in experiments one and two, three replicates in experiment three). Controls included uninoculated plants and plants grown with the addition of 0.05% w/v KNO_3 i.e. 70 μg ml^{-1} N. Plants were grown at 20-25°C with 16 h days and were harvested 30-35 days after planting, at early vegetative stage. Shoot dry weight and nodule numbers were determined.

Staining of nodules. Nodulated roots were harvested, washed with water and immersed in 40 ml GUS-extraction buffer (50 m*M* sodium phosphate buffer pH 7.0, 0.1% (v/v) Triton X-100, 0.1% (w/v) Sarcosyl, 0.05% (w/v) SDS, 1 m*M* EDTA) amended with 40 μg IPTG ml^{-1} and 100 μg X-GlcA ml^{-1}. A vacuum was applied for 10-15 min to facilitate penetration of the substrate. Afterwards, the roots were incubated for 24 h at 37°C in the substrate-containing buffer. The roots were then transferred to fresh buffer amended with IPTG and X-GlcA and vacuum was again applied for 10 - 15 min followed by incubation at 37°C overnight.

Statistical analysis. The mean value for log of the proportion of nodules occupied by the GUS-marked strain was calculated for each inoculation ratio (I through V) and competitiveness indices were then calculated by linear regression using the equation of Beattie *et al.*,(1989) as described in results. The probability that the intercept (i.e. $C_{X:Y}$) was equal to zero was calculated, as were the 95% confidence limits for both intercept and slope. The coefficient of determination and the probability that the slope of the regression line is zero were also calculated for each data set to verify that a linear relationship did exist between the log of inoculation ratio and the log of the proportion of nodule occupied by a strain. Data on growth rates and GUS activities of the different strains, and on shoot dry weight and nodule number for the plants, were first subjected to analysis of variance. If this showed there to be significant differences between treatments, the means were separated using Duncan's Multiple Range Test (Gomez and Gomez, 1984).

RESULTS

Southern analysis. To confirm that five independent mTn*5*SS*gusA*10-marked derivatives were being studied, the position of insertion of the transposon in the CIAT899 genome was determined for each derivative using a Southern blot analysis. Total DNA of the wild type strain CIAT899 and the transconjugants used in this study, digested with *Eco*RV, was probed with the 3.3 kb *Not*I fragment containing the *gusA* and *lacI* genes from mTn*5*SS*gusA*10 (Fig. 1a). No hybridization signal was obtained with the parental strain CIAT899. Two *Eco*RV fragments of 1.4 and 0.2 kb, both of which are internal to mTn*5*SS*gusA*10, hybridized in all transconjugants except isolate E; DNA from isolate E showed hybridization of a 0.2 kb fragment, but not of a 1.4 kb fragment (Fig. 1b). Additional hybridizing *Eco*RV fragments were apparent in the different isolates. These correspond to restriction fragments containing the ends of the transposon and the adjacent genomic DNA, and vary in size depending on the insertion

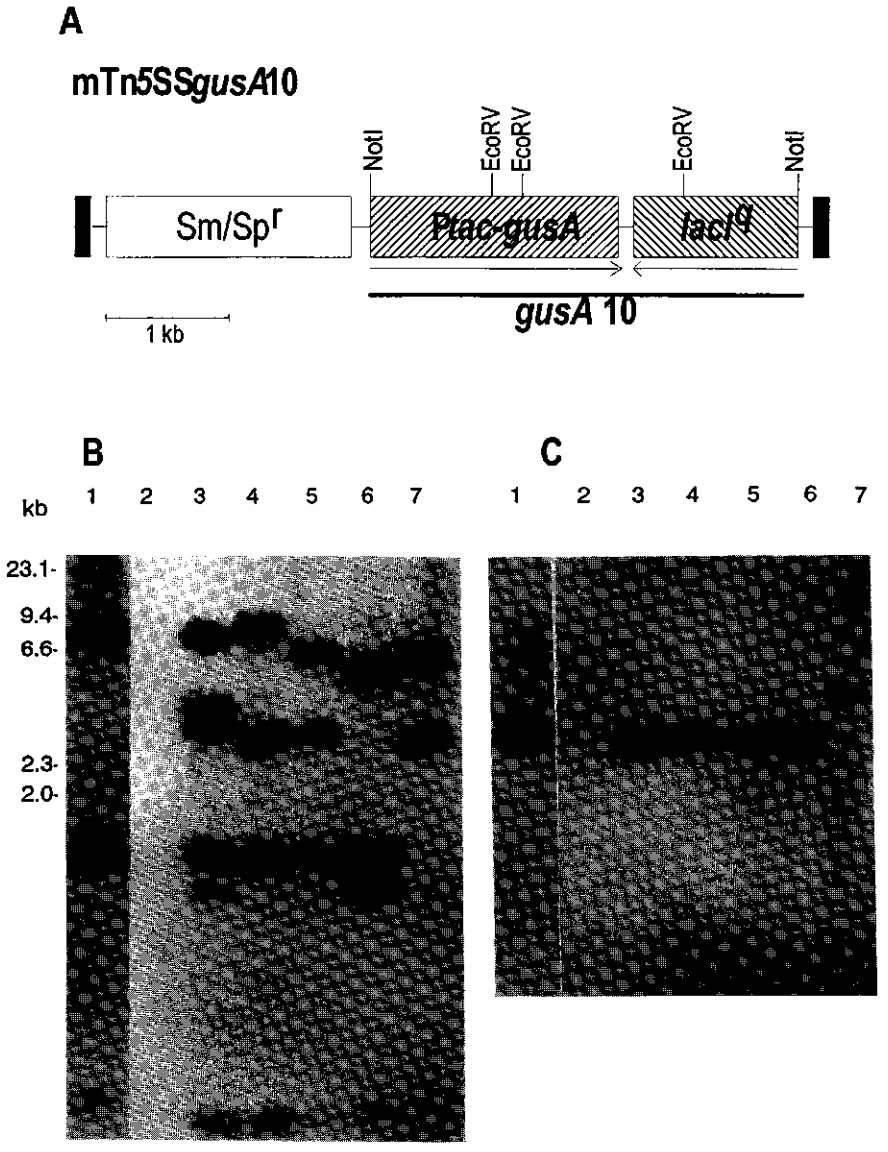

Fig 1: Southern analysis of *gusA*-marked *R. tropici* CIAT899 transconjugants.
(a) Structure of mTn*5*SS*gusA*10. (b, c) Autoradiograms of total DNA and plasmid control DNA, digested with *EcoRV* (b) and *NotI* (c), respectively. Hybridization was performed using the ^{32}P-labelled 3.3 kb *NotI* fragment of mTn*5*SS*gusA*10 (hatched area in Fig. 1a) as a probe. Plasmid pCAM110 which contains mTn*5*SS*gusA*10 (lane 1) and total DNAs from strains CIAT899, CIAT899::*gusA*10 A, B,C, D and E (lanes 2 - 7) are shown.

site of the marker gene in the genome. Two such border fragments were apparent in isolates B, C, D and E, indicating that mTn*5*SS*gusA*10 was introduced in single copy and was inserted randomly in the genome. However, CIAT899::*gusA*10 isolate A showed four variable fragments suggesting that two copies of mTn*5*SS*gusA*10 had been integrated (Fig. 1b).

The absence of a 1.4 kb *Eco*RV fragment hybridizing to mTn*5*SS*gusA*10 in isolate E suggested that the *Eco*RV restriction site in the *lacI* gene was lost (Fig. 1a, 1b). This was further supported by hybridization of genomic DNA of all strains digested with *Not*I with the same probe. The genomic fragment which hybridized in isolate E was of a different size from the predicted 3.3 kb observed in the other isolates (Fig. 1c), indicating that one of the two *Not*I sites had been lost. Most probably a deletion of the "right-hand" side of the transposon had occurred, leading to loss of most of the *lacI* gene, including the *Eco*RV site and adjacent *Not*I site (Fig. 1a). Consistent with this hypothesis, *gusA* in isolate E was constitutively expressed rather than being inducible with IPTG (Table 2).

Growth analysis. CIAT899 and the five CIAT899::*gusA*10 isolates showed similar growth behaviour in minimal medium (Fig. 2). Mean generation times, which were calculated over the logarithmic period of growth (88 to 482 min), were very similar between strains (Table 2).

GUS-activity. The GUS activity of CIAT899 and the five *gusA*-marked isolates was quantified both with and without induction by IPTG (Table 2). Strain CIAT899 had no detectable GUS activity. GUS-marked isolates A, B, C and D showed 30-60 fold induction by IPTG. In the absence of IPTG there was no detectable difference between the basal GUS activities of these four strains. Following induction, differences up to two-fold were detectable, but these differences were significant only at the $p = 0.05$ level, and not at the $p = 0.01$ level. By contrast, GUS activity for isolate E was high both in the presence and absence of IPTG, and no significant difference could be detected between the values of induced and uninduced GUS activity for isolate E (Table 2).

Symbiotic characteristics and competitive ability of GUS-marked isolates. The central aim of this work was to compare the symbiotic and competitive characteristics of the GUS-marked isolates with the parental strain, and this was done by evaluating their performance in symbiosis with the host legume species *P. vulgaris* L., cv Riz44, in three separate experiments. In all three experiments, no significant differences were observed between shoot dry weight or nodule number (although there was considerable plant to plant variation in nodule number) for all five isolates compared to the parental strain CIAT899. Data from one experiment are shown in Table 3. Similar values were obtained in the other two experiments (data not shown).

Table 2. Growth rates and GUS-activities of wild-type strain CIAT899 and five isolates marked with *gusA*

Strain	Mean generation time (min)	GUS-activity (no IPTG)	GUS-activity (plus 5 m*M* IPTG)
CIAT899	137	0	0
CIAT899::*gusA*10 A	137	2.3[a]	140.0[a]
CIAT899::*gusA*10 B	150	4.5[a]	150.0[a]
CIAT899::*gusA*10 C	137	1.6[a]	66.0[b]
CIAT899::*gusA*10 D	137	2.1[a]	100.0[ab]
CIAT899::*gusA*10 E	131	56.0[b]	79.0[b]

Growth rates are presented as mean generation times and are calculated from values of OD_{600} obtained between 88 min and 482 min, when growth was logarithmic (see Fig. 2).

GUS activity is reported as nmol pNPG hydrolysed 10^9 $cells^{-1}$ min^{-1}. Values given are the mean of three replicates. Means within one column which are not significantly different from each other at p = 0.05 share the same letters as superscripts.

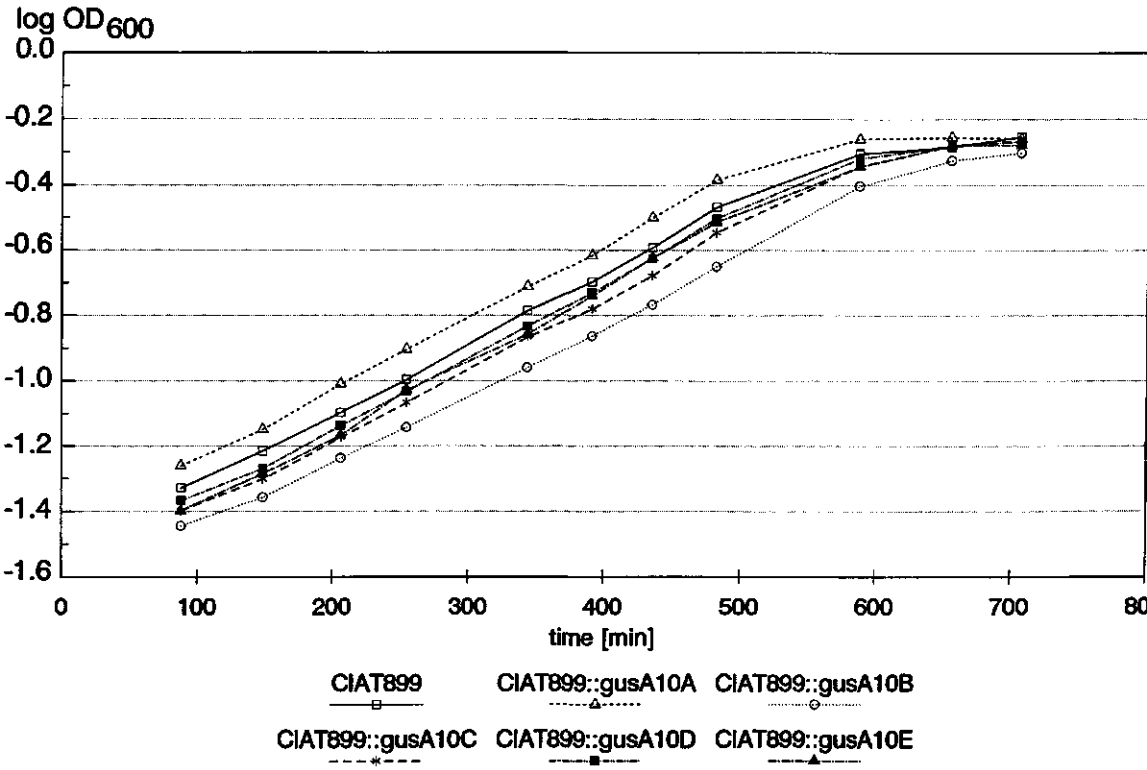

Fig. 2. Growth curves of strain CIAT899 and five different mTn*5*SS*gusA*10-marked derivatives. Single colonies were inoculated into minimal medium and growth was monitored over a 12 h period.

Table 3: Shoot dry weight, nodule numbers and nodule occupancy of plants inoculated with different ratios of wild-type strain CIAT899 and five GUS-marked derivatives

Isolate	Ratio (GUS-marked strain : wildtype)	Shoot dry weight (g)	Number of nodules	% GUS-marked nodules	% CIAT899 nodules	% mixed nodules
CIAT899::gusA10A	single strain	0.9	19	100	0	0
	9	0.8	16	84	13	3
	4	0.8	14	74	21	5
	1	0.7	15	59	32	9
	0.25	1.1	19	38	55	8
	0.1	0.9	15	26	65	9
CIAT899::gusA10B	single strain	0.7	18	100	0	0
	9	0.8	14	53	36	11
	4	1.0	16	30	59	11
	1	0.8	18	16	76	8
	0.25	1.0	17	5	90	5
	0.1	1.1	15	2	95	3
CIAT899::gusA10C	single strain	0.7	16	100	0	0
	9	0.7	16	82	13	6
	4	0.9	16	67	24	9
	1	0.7	13	47	46	7
	0.25	0.7	14	20	68	12
	0.1	0.9	11	20	69	11
CIAT899::gusA10D	single strain	0.8	17	100	0	0
	9	0.8	24	86	9	6
	4	0.9	17	79	16	5
	1	0.9	17	48	44	8
	0.25	1.0	15	23	68	9
	0.1	0.9	18	11	84	5
CIAT899::gusA10E	single strain	0.8	19	100	0	0
	6.4	0.8	22	86	9	5
	2.8	1.0	23	80	13	6
	0.8	0.9	17	59	33	8
	0.2	0.6	14	38	55	7
	0.1	0.8	17	25	68	8
CIAT899	single strain	0.9	20	0	100	0
no inoculation +N		2.4	0^{f}	n.a.	n.a.	n.a.
no inoculation -N		0.2	0^{f}	n.a.	n.a.	n.a.

Data are from experiment three and are the means of three replicates. Means within one column which are not significantly different from each other at $p = 0.05$ share the same letters as superscripts. The data in the last three columns are an example of the data used to calculate competitiveness indices. These percentages are based on the nodule numbers given in the previous column.

n.a. = not applicable

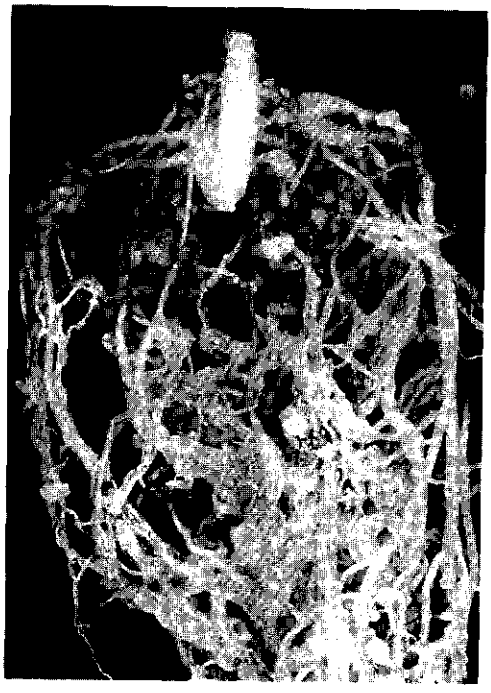

Fig 3: (a) Stained root showing nodule occupancy by CIAT899 (unstained nodules) and a CIAT899 GUS-marked derivative (blue nodules; here black)

Fig 3: (b) Nodules cluster showing nodules occupied either by CIAT899 or by a CIAT899::*gusA*10 derivative (blue nodule; here black), and one nodule occupied by both strains.

To determine the influence of insertion of mTn5SS*gusA*10 on the competitive ability of the five isolates, each GUS-marked strain was co-inoculated with parental strain CIAT899 in five different ratios and nodule occupancy was determined following XGlcA staining of whole roots (Fig. 3a). On plants that were coinoculated with GUS-marked isolates and the unmarked parental strain, a small proportion (0 - 20%) of nodules showed distinct blue and unstained regions (Fig. 3b). The unstained parts of these nodules were considered not to be due to substrate limitation as all nodules of treatments where a mTn*5*SS*gusA*10-marked derivative was used as a single-strain inoculum, developed a deep blue colour with inner and outer layers of the nodule being equally stained, as judged by hand sectioning. The partial staining could therefore have been due to dual occupancy of nodules by marked and unmarked strains.

This was confirmed by carrying out nodule isolations. 10 nodules per plant were selected at random from the mixed inoculation treatments and were surface sterilized, crushed and spread on YM plates containing 50 $\mu g\ ml^{-1}$ X-GlcA. Strains were identified as GUS-marked or wildtype depending on whether the colony turned blue or not. The proportion of mixed infection nodules based on this plating assay ranged from 0% to 30% per plant (i.e. 3 out of 10 nodules sampled), while that estimated based on partial staining of nodules ranged from 0 to 20%. It is difficult to compare the two figures exactly, because the sample sizes were considerably different in each case (10 nodules versus greater than 150 nodules), but the plating data support the idea that partial staining of nodules is due to dual occupancy. As the staining assay is a destructive one, it is not possible to directly confirm that partially stained nodules contain a mixed infection by isolating bacteria from such nodules.

For all three experiments, the competitiveness indices of the GUS-marked derivatives were calculated relative to the unmarked parental strain using the model described by Beattie *et al.*, (1989). In this model, competition between two different strains is described by the relationship:

$$\log [(P_X + P_{both}) / (P_Y + P_{both})] = C_{X:Y} + k [\log (I_X / I_Y)]$$

where strain X and Y are the two competing strains; P_X and P_Y are the proportion of nodules occupied by X and Y, respectively; P_{both} is the proportion of nodules occupied by both strains; and I_X and I_Y represent the concentrations of strains X and Y in the inoculum. The intercept of this equation, $C_{X:Y}$, is defined as the competitiveness index: a statistically significant positive value indicates that strain X is more competitive than strain Y, and a negative value that it is less competitive. The slope, k, gives the rate at which the nodule occupancy ratio changes as the inoculum

ratio changes. This can vary widely, from values as low as 0.038 to as high as 1.218 (Amarger and Lobreau, 1982). Data for nodule number and nodule occupancy from one experiment are shown in Table 3. The data for each isolate are presented in Fig. 4 and the calculated slope (k) and intercept ($C_{X:Y}$) values for all three experiments are given in Table 4. In each case the GUS-marked isolate is strain "X" and the unmarked parent is strain "Y".

In all cases except one (isolate D, experiment one), there was a strong linear relationship between the log of the proportion of nodules occupied by a particular strain and the log of the ratio of that strain in the inoculum, as indicated by the probability that the slope of the linear relationship is zero being substantially less than $p = 0.01$ in the majority of cases (Table 4). Additionally, the coefficients of determination for the relationship were also very high (Table 4). These statistical considerations indicate that these data show a good fit to the model developed by Beattie *et al.*,(1989).

The competitiveness indices ($C_{X:Y}$) showed some variation in the actual calculated value between experiments, but were consistent in general trend across experiments. For example, isolate A had a competitiveness index indistinguishable from that of CIAT899 in experiments one and two, but showed a substantially higher competitiveness index in experiment three ($p < 0.01$, Table 4). Isolate E was indistinguishable from the parental strain in experiment one, but showed a significantly enhanced competitive ability in experiments two and three. The only isolate to show the same result between the three experiments is isolate B, which showed a reduced competitiveness index relative to the parental strain. The calculated k-values also varied considerably, but were all positive (except for isolate D, experiment one, which did not fit the model as discussed above) indicating a positive relationship between representation in the inoculum and nodule occupancy.

DISCUSSION

An important requirement in using genetically-marked microorganisms in ecological studies is that the marked strains should not differ from the wild-type strain in the trait being studied. In the case of *Rhizobium*, it is critical that the marked strain maintains its nodulation behaviour and competitive ability. In this study, insertion of the mTn*5*SS*gusA*10 element did not significantly change the total nodule number produced by strain CIAT899 (Table 3). In addition, the dry shoot weight of plants dependent on the GUS-marked isolates for nitrogen fixation was not significantly different from wild-type inoculated plants, and was significantly higher than that of

Table 4: Competitiveness index and *k*-value for GUS marked strains relative to the parental strain in three separate experiments

Strain	Experiment	Coefficient of determination (R^2)	Probability that slope of regression line = 0	Cx:y	95% confidence interval for Cx:y	Probability that Cx:y = 0	Competitive ability relative to the wildtype[a]	*k*	95% confidence interval for *k*
CIAT899::*gusA*10 A	1	0.918	0.01	-0.12	-0.37 to 0.14	0.24	S	0.60	0.23 to 0.93
	2	0.837	0.03	-0.33	-0.85 to 0.18	0.13	S	0.82	0.16 to 1.48
	3	0.996	<0.01	+0.21	0.17 to 0.26	<0.01	+	0.55	0.49 to 0.62
CIAT899::*gusA*10 B	1	0.927	<0.01	-0.38	-0.72 to -0.04	0.04	-	0.87	0.42 to 1.31
	2	0.990	<0.01	-0.43	-0.56 to -0.30	<0.01	-	0.90	0.73 to 1.01
	3	0.994	<0.01	-0.60	-0.68 to -0.52	<0.01	-	0.73	0.63 to 0.84
CIAT899::*gusA*10 C	1	0.964	<0.01	+0.42	0.22 to 0.63	0.01	+	0.76	0.49 to 1.03
	2	0.966	<0.01	+0.38	0.09 to 0.67	0.02	+	1.07	0.70 to 1.44
	3	0.976	<0.01	+0.05	-0.07 to 0.17	0.29	S	0.60	0.43 to 0.77
CIAT899::*gusA*10 D	1	0.618	0.11	+0.41	-0.17 to 0.98	0.11	S	0.51	-0.23 to 1.25
	2	0.996	<0.01	+0.42	0.34 to 0.50	<0.01	+	0.95	0.85 to 1.06
	3	0.996	<0.01	+0.07	-0.01 to 0.14	0.07	S	0.82	0.72 to 0.93
CIAT899::*gusA*10 E	1	0.889	0.02	+0.05	-0.25 to 0.34	0.67	S	0.59	0.21 to 0.98
	2	0.996	<0.01	+0.55	0.49 to 0.63	<0.01	+	0.78	0.69 to 0.87
	3	0.998	<0.01	+0.32	0.28 to 0.37	<0.01	+	0.67	0.60 to 0.72

[a] The values here represent the statistically significant position relative to the parent: + = higher competitiveness index; S = same competitiveness index; - = reduced competitiveness index

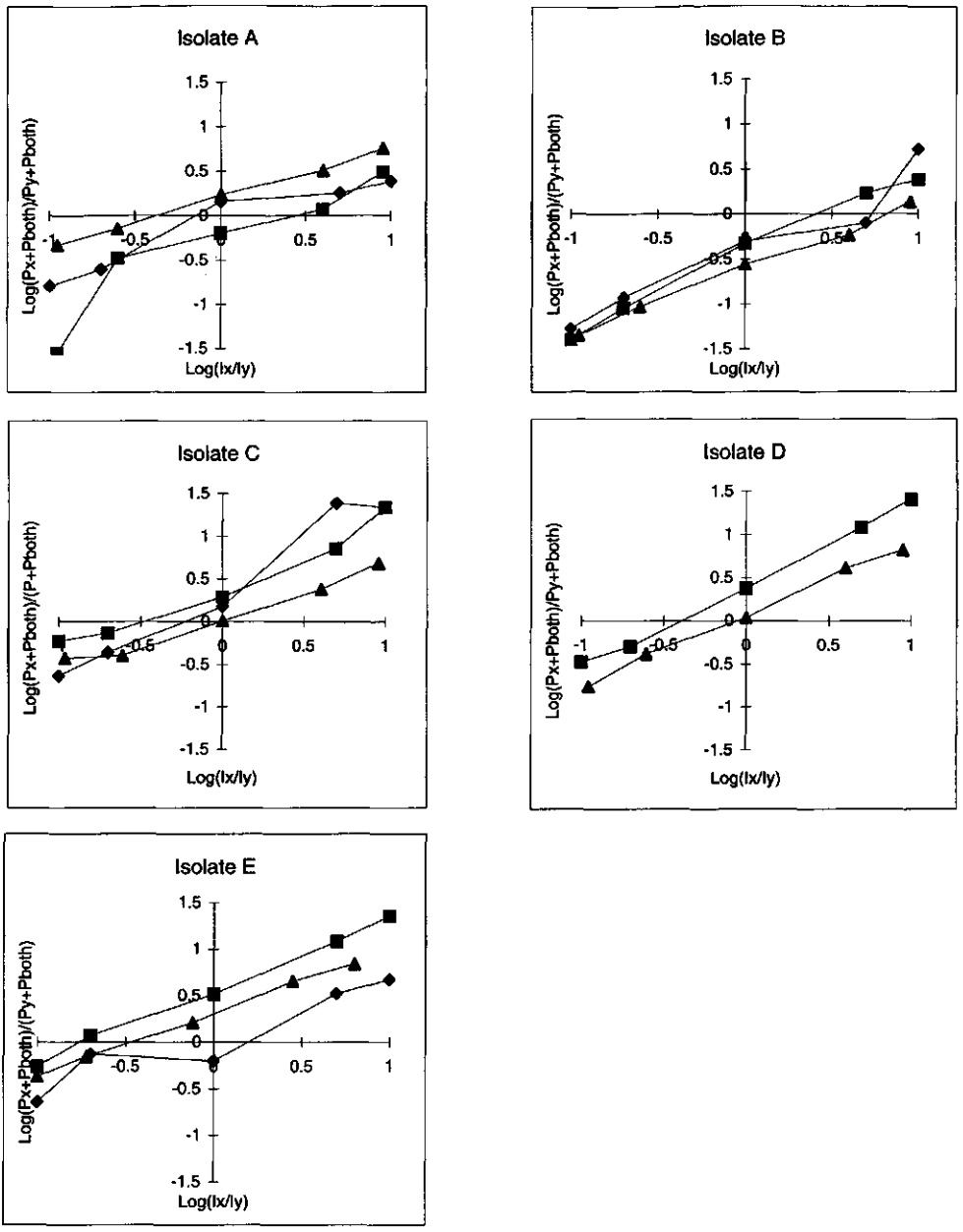

Fig 4: Graphical presentation of the data used to generate the linear regressions of log [$(P_X + P_{both}) / (P_Y + P_{both})$] against [log (I_X / I_Y)] for GUS-marked CIAT899 isolates A to E (strain "X") when competed against the parental strain CIAT899 (strain "Y"). The intercept of the linear regression is $C_{X:Y}$ and the slope is k. For each strain, data from all three experiments are shown: experiment 1 ◆; experiment 2 ■; experiment 3 ▲. Data for isolate D experiment one are not shown as there was not a significant linear relationship between the proportion of nodules occupied by the GUS-marked strain and the proportion in the inoculum (see text).

uninoculated controls (Table 3) indicating no apparent effect on the nitrogen fixation ability of the strains.

The most important trait being studied was the competitive ability of the marked strains. This was measured by determining the competitiveness indices relative to the parental strain. The results, presented in Fig. 4 and Table 4, showed numerical variation between experiments, with four out of the five strains tested exhibiting competitiveness indices either equal to or greater than the parental strain. Only one strain, isolate B, showed a consistent competitiveness index relative to the parental strain in all three experiments, with a significant reduction in competitiveness index compared to wildtype CIAT899.

The apparent variation between experiments is difficult to explain. The individual data sets fit the model used very well, with coefficients of determination (R^2, indicating the proportion of variation in the data which is attributable to the regression line) for the proposed linear relationship between inoculum ratio and nodule occupancy ranging from 0.837 to 0.998 (other than isolate D, experiment one; Table 4), and the probability that there is not a linear relationship between the two parameters being <0.01 in most cases (Table 4). The high statistical significance of these measurements are almost certainly a result of using a very complete and accurate data set. Viable cell numbers of each strain in the inoculum were determined at the time of inoculation, and errors in determination of proportion of nodule occupancy are an absolute minimum as all the nodules were assayed for nodule occupancy, rather than applying a sampling strategy as is required with other means of assessing nodule occupancy.

In fact the variation between experiments may not be as great as it appears at first sight and it is quite possible that this is to be expected when comparing pairs of strains that do in fact have very similar competitive abilities. In no case did a GUS-marked strain appear significantly more competitive that the parental strain in one experiment, and significantly less competitive in another experiment. In the one case, isolate B, where the results were consistent across all three experiments, it is evident that this isolate is affected in competitive ability, most likely due to a positional effect of the site of transposon insertion. However, it remains premature to argue, for example, that isolates C and E are more competitive than the parental strain. The main conclusion to be drawn from these results is that, while there was a clear decrease in competitiveness index in the case of isolate B, there is no inherent detrimental effect of insertion of mTn5SS*gusA*10 on the competitive ability of *R. tropici* CIAT899.

An interesting observation in these experiments was the appearance of partially stained nodules in mixed inoculum treatments but not in single strain treatments. That

these were due to dual occupancy by GUS-marked and wildtype strains was confirmed by nodule isolation and plating experiments. Partially-stained nodules were also observed by Krishnan and Pueppke (1992) when using X-Gal to examine infection by a mutant *lacZ*-marked *R. fredii* strain co-inoculated with a non-marked strain (following fixation of the plant to eliminate background ß-galactosidase activity). This ability to readily detect dual occupancy nodules is an additional advantage of marker gene systems for studying rhizobial competition, and also facilitates more accurate calculation of competitiveness indices.

Regarding other differences between the strains, there was no difference in growth rates between CIAT899 and the different GUS-marked isolates, and hence there was no correlation between growth rate and competitiveness as found by Li and Alexander (1986). The difference in measured GUS activity was only significant at the $p = 0.05$ level between the five isolates when induced with IPTG, and there was no difference in measured GUS activity in uninduced conditions. There was no correlation between GUS activity and measurements of competitiveness index.

One difference which is perhaps important is the pattern of integration of the transposon. In two out of the five strains tested, the transposon had not integrated as an intact single-copy insertion. In isolate E, part of the transposon had been deleted and Southern analysis, and measurement of GUS activity in the presence and absence of the inducer IPTG, indicated that this included part of the repressor-encoding *lacI* gene. In isolate A, there were two copies of the transposon, although the measured GUS activity was not significantly different than that of isolates B or D, both of which contained a single integrated copy. There did not appear to be any relationship between these aberrant insertion events and competitiveness index.

Our results are highly encouraging for future use of the GUS system as a marker in microbial ecology. They imply that specific isolates with impaired competitive ability are due to the site of the location of the transposon, rather than the presence of the GUS transposon *per se*. The fact that an isolate with constitutive GUS activity (isolate E) was unaltered in competitive ability, further emphasizes the lack of inherent effect of *gusA* expression on competitive ability.

It is clear, however, that initial screening is necessary before using strains with marker genes in ecological experiments, to eliminate isolates in which important genes for ecological behaviour may have been affected by insertion of the marker. Although it is difficult to rigorously assess ecological behaviour, the expedient of coinoculating parent and marked derivative in a one-to-one ratio, and ensuring that the proportion of blue nodules does not differ significantly from 50%, appears to be the most reliable test that can be carried out in the case of rhizobial competition (see Streit *et al.*, 1995). In this case, the value of (I_X / I_Y) will be zero, and hence the measured

value of nodule occupancies of both strains can be used to infer an approximate value for $C_{X:Y}$.

The observation that there is no inherent impact of *gusA* insertion on competitive ability also means that these transposons could be used to study the genetic basis of rhizobial competition. The GUS transposons have a number of major advantages over other systems:

1) they allow rapid screening of the competitive ability of potential mutants, opening the possibility of large-scale screening for competition mutants for the first time;
2) competition mutants so identified would primarily be caused by the transposon insertion, facilitating isolation or genomic mapping of important competition genes.
3) mutants can rapidly be screened in a variety of conditions to determine whether the effect is general or is related to a specific factor such as host cultivar or specific environmental conditions;
4) the point at which competition is affected could be determined using a histochemical GUS assay; for example, do infection threads initiate and then abort (de Boer and Djordevic, 1995).

The results presented here support the concept that the ecological fitness of GEMs is not necessarily compromised by the presence of inserted DNA (see references in Doyle *et al.*, 1995). The next step will be to determine whether these *gusA*-marked derivatives, which have been extensively characterized in artificial media in the greenhouse, perform similarly in more natural conditions, including in competition with indigenous *Phaseolus*-nodulating strains in soil.

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CHAPTER 4

Simultaneous detection of different *Rhizobium* strains marked with either the *Escherichia coli gusA* gene or the *Pyrococcus furiosus celB* gene

A. Sessitsch, K. J. Wilson, A. D. L. Akkermans, and W. M. de Vos

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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

ANGELA SESSITSCH,[1,2] KATE J. WILSON,[3] ANTOON D. L. AKKERMANS,[1] AND WILLEM M. DE VOS[1]*

Department of Microbiology, Wageningen Agricultural University, 6703 CT Wageningen, The Netherlands[1]; Soils Unit, FAO/IAEA Agriculture and Biotechnology Laboratory, A-2444 Seibersdorf, Austria[2]; and CAMBIA, ACT 2601, Canberra, Australia[3]

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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

* Corresponding author. Mailing address: Department of Microbiology, Hesselink van Suchtelenweg 4, 6703 CT Wageningen, The Netherlands. Phone: 31-317-483100. Fax: 31-317-483829. Electronic mail address: willem.devos@algemeen.micr.wau.nl.

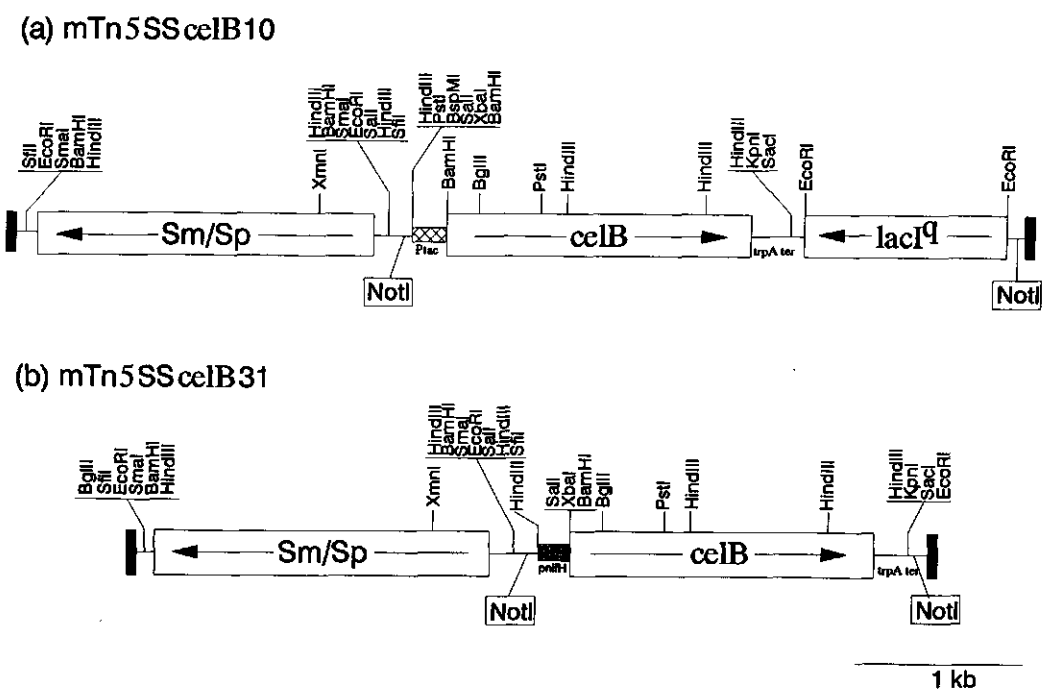


FIG. 1. Genetic and physical maps of *celB* transposons mTn*5*SS*celB10* and mTn*5*SS*celB31*. The spectinomycin-streptomycin cassette, flanked by transcriptional and translational terminators, is indicated, as is the *lacI*^{q} gene in mTn*5*SS*celB10*. Restriction sites of the delivery plasmid pUT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

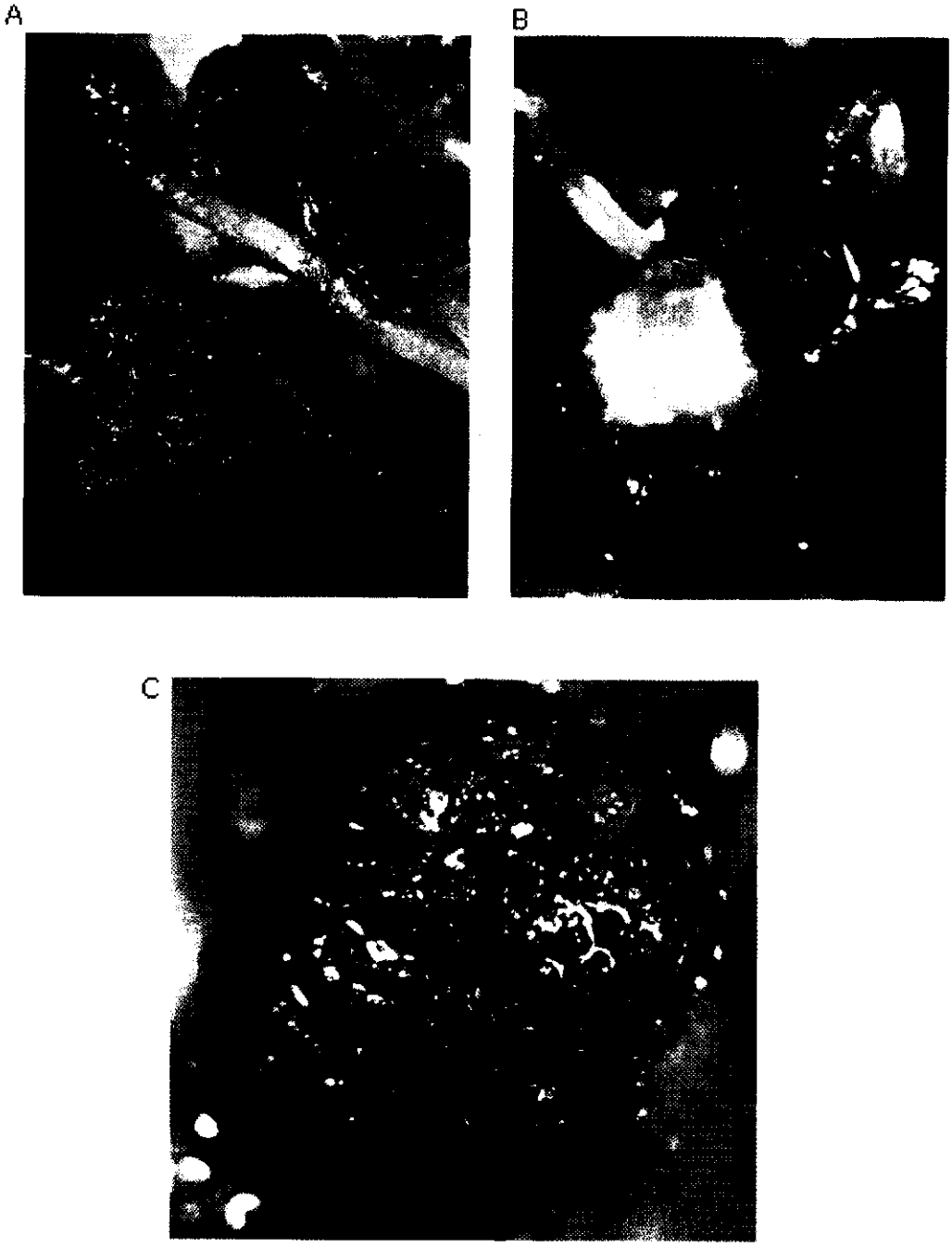
was marked separately with the symbiotic *gusA* transposon mTn*5*SS*gusA30* (19). Transfer frequencies on the order of one per 10^7 recipients were obtained.

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.


A
B
C

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*5SScelB31* minitransposon, which is opposite that of the *gusA* gene in Tn*5SSgusA30* (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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CHAPTER 5

Characterization of *Rhizobium etli* and other *Rhizobium* spp. that nodulate *Phaseolus vulgaris* L. in an Austrian soil

A. Sessitsch, G. Hardarson, A. D. L. Akkermans, and W. M. de Vos

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Characterization of *Rhizobium etli* and other *Rhizobium* spp. that nodulate *Phaseolus vulgaris* L. in an Austrian soil

Angela Sessitsch[1*], Gudni Hardarson[1], Antoon D.L. Akkermans[2] and Willem M. de Vos[2]

1 Soil Science Unit, FAO/IAEA Agriculture and Biotechnology Laboratory, A-2444 Seibersdorf, Austria

2 Department of Microbiology, Wageningen Agricultural University, 6703 CT Wageningen, The Netherlands

Summary

Phaseolus vulgaris L. (common bean) is nodulated by rhizobia present in the fields around the Seibersdorf laboratory although common bean has not been grown for a long time. Using PCR analysis with repetitive primers, plasmid profiles, *nifH* profiles, PCR-RFLP analysis of the 16S rRNA gene and of the 16S rRNA - 23S rRNA intergenic spacer and the nodulation phenotype, two well differentiating groups could be distinguished. One group showed high similarity to *Rhizobium* sp. R602sp, isolated from common bean in France, while the other showed the same characteristics as *R. etli*. We detected little variation in the symbiotic regions but found higher diversity when using approaches targeting the whole genome. Many isolates obtained in this study might have diverged from a limited number of strains, therefore, the Austrian isolates showed high saprophytic and nodulation competence in that particular soil.

INTRODUCTION

Phaseolus vulgaris L. (common bean) has two centers of origin in the Americas, in Mesoamerica and in the Southern Andes, which developed independently (Gebts, 1990). Seeds of common bean were imported to Europe after the discovery of America in 1492 and were grown extensively already 60 years later (Gebts and Bliss, 1988). Rhizobia that can form nitrogen fixing nodules on *Phaseolus vulgaris* were found to be genetically distant and phylogenetically diverse (Piñero et al., 1988; Eardly et al., 1995). Traditionally, common bean nodulating rhizobia have been classified as *R. leguminosarum* bv. *phaseoli* (Jordan, 1984) according to the host plant they nodulate, but later two other species, *R. etli* and *R. tropici* (Martínez-Romero et al., 1991; Segovia et al., 1993), have been defined. Both, *R. leguminosarum* bv. *phaseoli* and *R. etli* maintain multiple copies of the nitrogenase reductase gene (*nifH*) on the symbiotic plasmid but they possess different 16S rRNA gene sequences (Quinto et al., 1982; Martínez et al., 1985; Segovia et al., 1993). *R. tropici* has only one *nifH* gene copy on the symbiotic plasmid (Martínez-Romero et al., 1991). *R. tropici* and *R. etli* nodulate a large number of hosts but their host ranges are different (Martínez-Romero et al., 1991; Hernandez-Lucas et al., 1995). Phylogenetic analysis based on partial 16S rRNA sequences revealed that *R. tropici* is related to *R. leguminosarum*, while *R. etli* showed to be very close to *Rhizobium* sp. Or191 isolated from ineffective alfalfa nodules in Oregon, USA (Eardly et al., 1985) and to strain FL27 isolated from common bean in Mexico (Piñero et al., 1988). In general, strains of *Rhizobium* nodulating common bean are of American origin similar as their host plant. For a long time, *R. leguminosarum* bv. *phaseoli* was believed to be the only microsymbiont of common bean in Europe, but recently *R. tropici* and two new genomic species, *Rhizobium* sp. H152 and *Rhizobium* sp. R602sp, were also found in French soils (Laguerre et al., 1993; Amarger et al., 1994).

In the fields around the Seibersdorf laboratory common bean is well nodulated although this crop has not been cultivated during the last decades. In this study different approaches were used to classify rhizobia nodulating common bean, targeting the nodulation phenotype as well as symbiotic and chromosomal regions of the genome.

MATERIALS AND METHODS

Bacterial strains. Surface-sterilized seeds of common bean (*Phaseolus vulgaris* L. cv. Extender), faba bean (*Vicia faba* cv. Weiselburger), pea (*Pisum sativum* cv. Rheinperle), red clover (*Trifolium repens* cv. Reichersberger) and alfalfa (*Medicago sativa* cv. Saranac) were grown in Leonard jars using soil from fields around the Seibersdorf laboratory. Soil characteristics have been reported by Zapata et al. (1987). Common bean has not been cultivated in the laboratory fields for at least 30 years and is presently a rarely grown plant in this region. Rhizobial strains were isolated from surface-sterilized nodules (Somasegaran and Hoben, 1985) on YM agar plates (Danso and Alexander, 1974). 26 isolates of common bean nodules and 18 isolates, each of faba bean, pea, clover and alfalfa, were used for further characterization. As a control, *Phaseolus vulgaris* L. cv. Extender seeds, with and without surface sterilization, were grown in sterile sand. Reference strains were obtained either from G. Laguerre, France, or from the culture collection at the Seibersdorf Laboratory.

Sample preparation for DNA amplification from cell cultures. Isolates were grown on YM agar plates for 16 h at 28°C. Cells were resuspended in 100 µl TE (Ausubel et al., 1994) and the OD at 600 nm of all samples was adjusted to 2.6. Then, the samples were deep freezed for 4 min at -70°C. Afterwards, the cells were set on ice for 1 min, boiled for 2 min, again left on ice for 1 min and boiled again for 2 min. Finally, the cells were centrifuged for 2 min at 14000 rpm and the supernatant was used for the PCR assay.

PCR using repetitive primers. A slightly modified protocol to that described previously (de Bruijn, 1992) using PCR with repetitive extragenic palindromic (REP) primers was applied to fingerprint the strains of *Rhizobium* isolated from common bean, faba bean, pea, clover and alfalfa. PCR amplifications were performed in a total reaction volume of 25 µl containing 1 x PCR reaction buffer (50 mM KCl; 20 mM Tris.HCl, pH 8.0), 200 µM each of dATP, dCTP, dGTP and dTTP (Pharmacia-LKB), 3 mM $MgCl_2$, 2 µM of each primer, 3 µl of cell extract and 2 U Taq DNA polymerase (Gibco, BRL). All amplifications were performed with a Perkin-Elmer thermocycler (GeneAmp PCR System 9600). The temperature cycle for PCR with REP primers consisted of an initial denaturation step at 95°C for 1 min, followed by 35 cycles of 50 s denaturation at 94°C, 1 min annealing at 40°C and 2 min extension at 72°C and a final extension step for 4 min at 72°C. The total reaction volumes were examined on 1.5% agarose gels.

RP01-PCR was also used to differentiate rhizobial strains isolated from common bean. This method employs a single primer, RPO1 (Richardson et al., 1995; Schofield and Watson, 1985), that works like a random primer. The PCR reaction was carried out as described above in a 25 µl reaction volume using 2.5 µl cell extract and 0.4 µM primer RPO1. The temperature cycle for primer RP01 was: 5 cycles of 30 s denaturation at 94°C, 2 min annealing at 50°C and 90 s extension at 72°C; followed by 35 cycles of 10 s at 94°C, 50 s at 55°C and 90 s at 72°C; followed by a final cycle of 20 s at 94°C, 40 s at 55°C and 5 min at 72°C. Amplification products were examined on 1.5% agarose gels.

For data analysis all fingerprints used for comparison were run on the same gel and all bands were scored. The similarity values were calculated by using the analysis program RFLPscan™ (Scanalytics) and they represent the ratio of shared bands over total bands within two lanes being compared during a matching operation. Dendrograms were generated by using the SAHN (Sequential Agglomerative Hierarchical and Nested; Sneath and Sokal, 1973) analysis of the program NTSYS-pc (Applied Biostatistics, Inc.).

PCR-RFLP analysis of the 16S rRNA gene and of the 16S-23S rRNA intergenic spacer. PCR amplification of the 16S rRNA gene followed by RFLP analysis (Laguerre et al., 1994) was performed with the rhizobial isolates obtained from common bean and with reference strains. PCR conditions were as described above using a 100 µl reaction volume with 8 µl cell extract and 0.1 µM primers rD1 and fD1 (Weisburg et al., 1991). The following temperature cycle was used: an initial denaturation step of 1 min at 95°C followed by 30 cycles of 50 s denaturation at 94°C, 1 min annealing at 48°C and 2 min extension at 72°C and a final extension step of 4 min at 72°C. Aliquots (17 to 19 µl) of PCR products were digested with the following restriction enzymes: *Dde*I, *Msp*I, *Nde*II and *Taq*I (Pharmacia-LKB). The resulting DNA fragments were analysed by horizontal agarose gel electrophoresis in 3% agarose gels.

The PCR-RFLP analysis was carried out with the rhizobial isolates obtained from common bean as described above for the 16S rRNA gene using the primers pHr and p23SROI (Massol-Deya et al., 1995). 17 to 19 µl aliquots of PCR products were digested with the restriction enzymes *Alu*I, *Hae*III, *Hha*I, *Msp*I and *Pal*I (Pharmacia-LKB).

Analysis of plasmids. For the Southern hybridization analysis with a *R. etli nifH* gene probe, genomic DNAs from the isolates nodulating common bean and the reference strains *R. etli* CFN42 and *Rhizobium* sp. R602sp were digested with *Bam*HI and the

resulting fragments were separated by electrophoresis using 0.8% agarose gels. The gels were blotted onto Hybond-N membranes (Amersham). For preparation of the *nifH* gene probe, plasmid pKW112 containing a *nifH* gene of *R. etli* strain CFN42 (Wilson et al., 1995) was digested with *Sal*I. After isolating the 300 bp *nifH* internal fragment from a 1.3% agarose gel utilizing the Geneclean II Kit (Bio 101), the probe was labeled with α-[^{32}P]dATP to high specific activity (10^8 counts min^{-1} μg^{-1} DNA) by using the Multiprime DNA Labeling System (Amersham) according to the manufacturer's protocol. The membranes were hybridized with the *nifH* gene probe in Rapid-hyb buffer (Amersham) at 65°C for 2 h. Washing and autoradiography was carried out as described previously (Sessitsch et al., accepted).

Plasmid profiles of all isolates nodulating common bean were done as described by Hynes et al. (1985).

Nodulation host range. Surface-sterilized seeds of *Phaseolus vulgaris* cv. Extender, *Vicia faba* cv. Weiselburger, *Pisum sativum* cv. Rheinperle, *Trifolium repens* cv. Reichersberger, *Medicago sativa* cv. Saranac, *Glycine max* cv. Clay, *Vigna unguiculata* cv. Red Caloona, *Leucaena leucocephala* cv. Cunningham, *Gliricidia sepium* and *Acacia albida* were germinated on 1.5% (w/v) water-agar plates. The seedlings were transplanted into sterile modified Leonard jars (Vincent, 1970) containing sand and N-free nutrient solution (Somasegaran and Hoben, 1985). Each seedling was inoculated with 10^7 cells of selected common bean isolates and of *R. sp.* R602sp. Plants were grown in the greenhouse at 20-25°C and harvested 28 days after planting.

Control experiments were carried out in order to ensure that the broad host range detected was not due to contamination. Two nodule isolates were prepared from the different nodulated host plants followed by inoculation of sterile *Phaseolus vulgaris, Vigna unguiculata, Leuceana* and *Gliricidia* seedlings with those isolates. In addition, nodule isolates of the various host plants were compared by RPO1-PCR fingerprinting.

RESULTS

PCR using repetitive primers. Fingerprints of 26 rhizobial isolates of common bean and of 18 rhizobial isolates, each of faba bean, pea, clover and alfalfa, were obtained using REP primers. When using REP-PCR, the common bean strains could be divided into two well differentiated groups A and B with respectively 4 and 3 subdivisions

and one strain (CbS-21) with an unique pattern (Fig. 1). Group A represented about 40% and group B around 60% of all common bean nodule isolates (Table 1). The dendrogram showed high similarity between group A isolates and *R.* sp. R602sp, while group B rhizobia did not show high similarity to any of the reference strains used (Fig. 2). Nodule isolates from faba bean, pea, alfalfa and clover showed higher diversity; 7 different profiles were found among 18 faba bean isolates, 10 profiles among 18 pea isolates and 13 profiles among 18 clover and among 18 alfalfa isolates. One identical REP profile was found among isolates from faba bean and pea. Fingerprints of common bean isolates obtained by PCR using the RP01 primer resulted in a similar grouping, but the discrimination level was slightly lower, group A comprised 2 subdivisions, group B could be divided in 3 and again CbS-21 had an unique pattern (Table 1). In this analysis, all group A isolates except CbS-18 showed the same fingerprint as *R.* sp. R602sp, but CbS-18 showed high similarity to *R.* sp. R602sp. Group B isolates showed low similarity to the common bean nodulating reference strains used. Although CbS-21 showed quite distinct profiles in both analyses, it was included in group B as it showed highest similarity to those isolates (Fig. 2). In addition, other, less discriminative, analyses (see below) revealed high similarity to group B isolates.

PCR-RFLP of the 16S rRNA gene and the 16S rRNA - 23S rRNA intergenic spacer. Analysis of the 16S rRNA gene was used to classify the isolated common bean rhizobia into species. The DNA fragment patterns obtained by digesting the 16S rRNA gene with different restriction enzymes were compared with those from reference strains (Table 2). Again, the common bean nodule isolates formed two groups, corresponding to the groups A and B described above. Group A rhizobia were found to be different from *R. leguminosarum.* bv. *phaseoli*, *R. tropici* or *R. etli*, but had the same profiles as *R.* sp. strain FL27, which was isolated in Mexico (Piñero et al., 1988) and *R.* sp. R602sp, a French isolate (Laguerre et. al., 1993, Amarger et al., 1994). Group B isolates showed the same patterns as *R. etli* and the presence of three copies of *nifH*, revealed by Southern hybridization analysis, confirmed their close relationship to *R. etli.*

Higher diversity was found by PCR-RFLP analysis of the 16S rRNA - 23S rRNA intergenic spacer region. Group A and B rhizobia isolated from common bean could be divided into several subgroups which correlated to a certain extent with the results obtained from other comparisons (Table 1).

Table 1. Groupings of Austrian *P. vulgaris* nodule isolates derived from PCR patterns using repetitive primers and PCR-RFLP analysis of the 16S - 23S rRNA intergenic spacer

Isolate	REP-PCR	RPO1-PCR	PCR-RFLP of the 16S-23S rRNA intergenic spacer				
			*Alu*I	*Hae*III	*Hha*I	*Msp*I	*Pal*I
Group A isolates							
CbS-1*	AI	AI	AI	AI	AI	AI	AI
CbS-3	AII	AI	AI	AII	AII	AI	AII
CbS-5	AII	AI	AI	AII	AII	AI	AII
CbS-8	AII	AI	AI	AII	AII	AI	AII
CbS-12	AII	AI	AI	AII	AII	AI	AII
CbS-13	AII	AI	AI	AII	AII	AI	AII
CbS-15	AII	AI	AI	AIII	AI	AI	AIII
CbS-17	AIII	AI	AI	AIII	AI	AI	AIII
CbS-18	AIV	AII	AI	AIV	AIII	AI	AIII
CbS-22	AII	AI	AI	AII	AII	AI	AII
CbS-23	AIII	AI	AI	AIII	AI	AI	AIII
Group B isolates							
CbS-2	BI	BI	BI	BI	BI	BI	BI
CbS-4	BII	BII	BI	BI	BI	BI	BI
CbS-6	BI	BI	BI	BI	BI	BI	BI
CbS-7	BIII	BIII	BII	BII	BII	BII	BII
CbS-9	BII	BI	BI	BI	BI	BI	BI
CbS-10	BII	BII	BI	BI	BI	BI	BI
CbS-11	BI	BI	BI	BI	BI	BI	BI
CbS-14	BII	BI	BI	BI	BI	BI	BI
CbS-16	BI	BI	BI	BI	BI	BI	BI
CbS-19	BI	BI	BI	BI	BI	BI	BI
CbS-20	BI	BI	BI	BI	BI	BI	BI
CbS-24	BII	BI	BI	BI	BI	BI	BI
CbS-25	BII	BII	BI	BI	BI	BI	BI
CbS-26	BI	BI	BI	BI	BI	BI	BI
CbS-21	BIV	BIV	BIII	BIII	BIII	BIII	BIII

* Underlined isolates indicate representative group isolates.

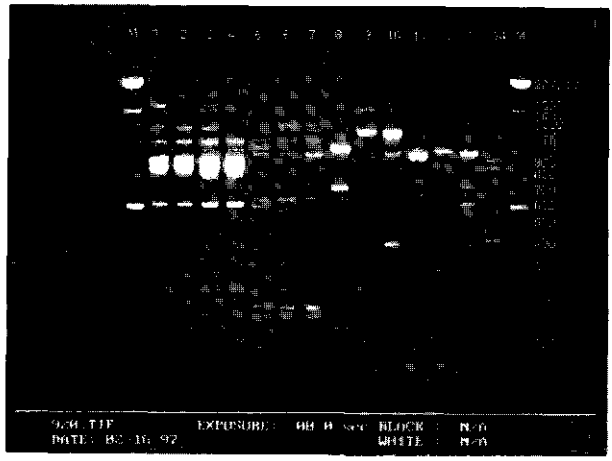

Fig. 1. REP-PCR fingerprint patterns of common bean nodulating isolates and of reference strains. Lanes showing DNA molecular weight standards are labeled with "M". Lanes 1 to 8 show the REP PCR products obtained from represenative group isolates of common bean CbS-1 (lane 1), CbS-3 (lane 2), CbS-17 (lane 3), CbS-18 (lane 4), CbS-2 (lane 5), CbS-4 (lane 6), CbS-7 (lane 7) and CbS-21 (lane 8). Lanes 9 to 14 show the REP PCR products of the common bean reference *Rhizobium* strains *R. tropici* type CIAT899 (lane 9), *R. tropici* type CFN299 (lane 10), *R. etli* CFN42 (lane 11), *R. leguminosarum* bv. *phaseoli* H131 (lane 12), *R.* sp. FL27 (lane 13) and *R.* sp. R602 (lane 14).

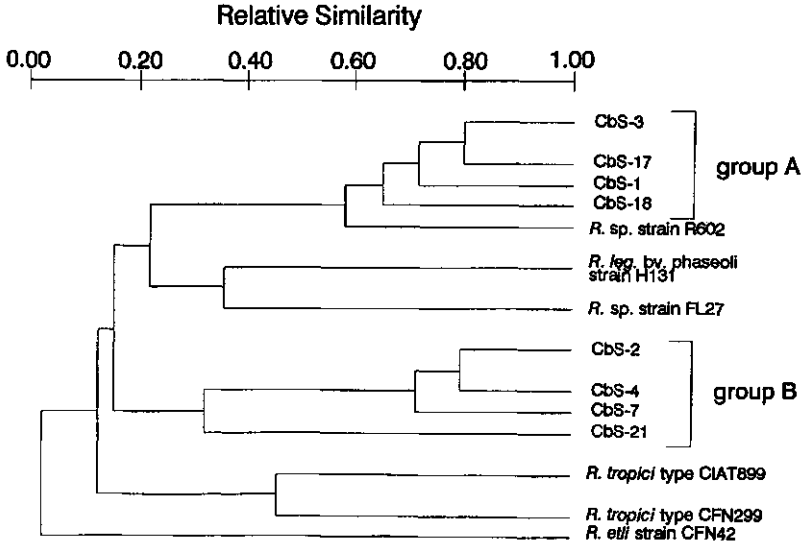

Fig. 2. Dendrogram of common bean nodulating isolates derived from REP PCR fingerprints.

Table 2. PCR-RFLP analysis of the 16S rRNA gene of common bean nodule isolates

Strain, Isolate	Restriction pattern of the amplified 16S rRNA gene			
	*Dde*I	*Msp*I	*Nde*II	*Taq*I
R. l. bv. *viciae* VF39	a	a	a	a
R. l. bv. *phaseoli* H131	a	a	a	a
R. meliloti 2001	a	a	b	a
R. galegae 625	c	f	f	e
R. loti NZP2234	a	j	e	d
R. haukii CCBAU2609T	a	j	e	e
R. fredii MSDJ1536	a	d	d	c
R. spp. (Phaseolus) FL27	a	a	h	e
R. spp. (Phaseolus) R602sp	a	a	h	e
R. spp. (Phaseolus) H152	d	b	g	f
R. etli bv. *phaseoli* CFN42	a	a	b	a
R. tropici type IIA CFN299	b	c	c	b
R. tropici type IIB CIAT899	a	b	a	a
CbS-1, -3, -5, -8, -12, -13, -15, -17, -18, -22, -23	a	a	h	e
CbS-2,- 4, -6, -7, -9, -10, -11, -14, -16, -19, -20, -21, -24, -25, -26	a	a	b	a

Analysis of plasmids. We determined the *nifH* gene hybridization patterns of the common bean isolates. With all group B isolates, except isolate CbS-21, the same pattern was found showing 3 bands, about 9.8, 6.5 and 5.1 kb in size. They shared the 9.8 kb and 6.5 kb bands with *R etli* type strain CFN42. (Martínez et al., 1985). Isolate CbS-21 showed three bands of 13.0, 11.3 and 2.9 kb. Group A rhizobia and *R.* sp. R602sp showed a single band of 8.3 kb. Fig. 3 shows the *nifH* profiles of representative group isolates of the common bean nodulating rhizobia.

Group A and group B strains were also distinguishable by their plasmid profiles. Three plasmids showing the same profile were visualized in group A strains and five plasmids in group B strains, with one exception, CbS-7, that had a different pattern with six plasmids.

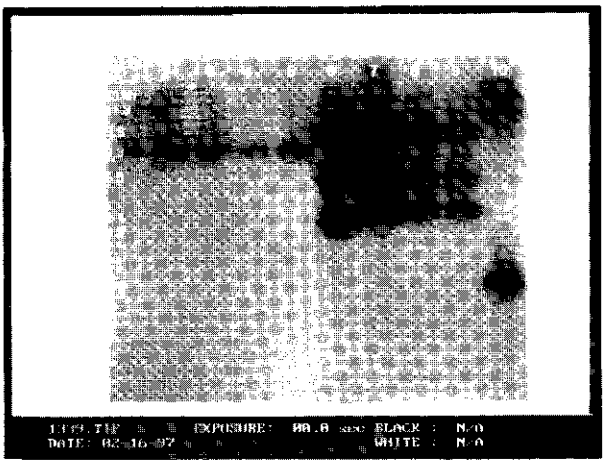

Fig. 3. Autoradiogram of a Southern blot of *BamHI*-digested genomic DNA hybridized with a 300 bp *nifH* fragment of strain CFN42. Strains tested were *R.* sp. R602 (lane 1), CbS-1 (lane 2), CbS-3 (lane 3), CbS-17 (lane 4), CbS-18 (lane 5), *R. etli* CFN42 (lane 6), CbS-2 (lane 7), CbS-4 (lane 8), CbS-7 (lane 9) and CbS-21 (lane 10).

Nodulation host range. Different host ranges were found among isolates of group A and B. The representative isolates of each subgroup were all able to nodulate common bean from which they were isolated initially. They did not nodulate plants commonly grown in that soil as faba bean, pea, alfalfa and clover. Isolates of group A (CbS-1, CbS-3, CbS-17 and CbS-18) and *R.* sp. R602sp showed a broader host range than isolates of group B as they formed nodules on *Vigna unguiculata*, *Leucaena* and *Gliricidia*. Nodule isolates from these plants showed the same host range after re-inoculation and the same RPO1 fingerprints as the original inoculant strain. Isolates of group B (CbS-2, CbS-4, CbS-7 and CbS-21) nodulated only common bean. All nodules formed on the different hosts were red, so we assumed that they contained leghaemoglobin.

In the control experiment, where *P. vulgaris* cv. Extender seeds were grown in sterile sand, no nodules were formed.

DISCUSSION

As common bean had not been cultivated previously in the fields around the Seibersdorf laboratory but is nodulated well without inoculation, the main goal of this study was to characterise *Rhizobium* strains nodulating this crop. Nodulation of common bean was reported to be quite promiscuous (Eardly et al., 1995; van Berkum, 1996), and even strains of *R. meliloti* and *R. fredii* have been found to nodulate common bean (Bromfield and Barran, 1990; Eardly et al., 1985; Eardly et al., 1992; Sadowsky et al, 1988). In order to detect any cross-inoculation, rhizobia were also isolated from other legumes such as faba bean, pea, red clover and alfalfa, all of which nodulate with indigenous strains. Isolates from those legumes showed high diversity when analyzed by REP-PCR. Our analysis did not reveal that bean belonged to the same cross-inoculation group as the other legume plants analyzed. Cross-inoculation was found as expected among rhizobia nodulating pea and faba bean as both are nodulated by *R. leguminosarum* bv. *viciae*. Using a polyphasic approach, two well differentiated groups could be distinguished among the isolates from common bean, which were assigned in this paper as group A and B.

Results obtained from PCR using REP primers have been demonstrated to be in agreement with those obtained from multilocus enzyme electrophoresis (de Bruijn, 1992) and RFLP's (Judd et al., 1993). These and the present findings confirm that this method is an appropiate tool to analyse bacterial communities. However, it has been previously reported that REP-PCR fingerprints reflect the variability of chromosomal DNA regions of *Rhizobium* but not the variability of symbiotic DNA regions (Laguerre et al., 1996). Richardson et al. (1995) showed that *Rhizobium* can be differentiated by their RPO1-PCR amplification pattern at the strain level. In the present study, the classification obtained using this method correlated in general well with that obtained by other methods, although less variation within group A could be detected. As PCR methods using repetitive primers reflect rather differences in the whole genome (chromosome), we applied also other methods targeting specific parts of the genome.

Classification of bacteria has been based to a great extent on the 16S rRNA gene (Willems and Collins, 1993). Laguerre et al. (1994) showed in a recent study that the RFLP analysis of the PCR-amplified 16S rRNA gene was in full agreement with data based on DNA-rRNA hybridizations and sequence analysis of the 16S rRNA gene. Isolates belonging to group A could not be assigned to any of the recognized species nodulating *P. vulgaris*, i. e. *R. leguminosarum* bv. *phaseoli*, *R. etli* or *R. tropici*. However, the RFLP-patterns obtained were the same as from *Rhizobium* sp. R602sp, which was collected in France and classified as a new species (Laguerre et

al., 1993; Laguerre et al., 1994). The partial 16S rRNA gene sequence of R602sp was found to be identical to strain FL27 (Laguerre et al., 1993), a Mexican isolate from common bean which is poor in nitrogen fixation (Piñero et al., 1988). Isolates from group B showed the same 16S rRNA-RFLP pattern as *R. etli* type strain CFN42 (Segovia et al., 1993) that was distinct from other patterns. Southern hybridization with a *nifH* gene probe of strain CFN42 revealed the presence of three *nifH* gene copies in all isolates within family B. This finding furthermore indicated the presence of *R. etli* in this particular soil in Austria as only *R. etli* and *R. leguminosarum* bv. *phaseoli* maintain multiple *nifH* genes. The fact that the fingerprints generated by PCR with repetitive primers did not show high similarity to the *R. etli* reference strain CFN42 could be due to the high genomic instability reported in *R. etli* (Flores et al., 1988; Brom et al., 1991). Furthermore, it has been argumented that repetitive elements change faster than the genome as a whole (Martínez-Romero, 1994) as they might be involved in recombination and amplification events (Flores et al., 1988). The intergenic spacer between the 16S rRNA and the 23S rRNA genes is not well conserved and thus exhibits a large degree of variation (Massol-Deya et al., 1995), that can be used for differentiation at the strain level. Nour et al. (1994) found that RFLP analysis of the 16S rDNA plus intergenic spacer is in accordance with results obtained by MLEE. The different patterns found among isolates belonging to group A and B were in general in good correlation with PCR analysis using repetitive primers. Using plasmid profiles and *nifH* patterns, little variation among the common bean isolates was detected. More discriminative methods would be needed in order to determine the variation of symbiotic genes, which are located on plasmids in most fast-growing strains of *Rhizobium*.

From the results obtained we suggest that the *Phaseolus vulgaris* nodulating strains found in Austria were derived from rhizobia originating in Mesoamerica which might have been imported as seed contaminants. Two analyses indicate that isolates of group B are strains of *R. etli*, a species that has its center of origin in Mesoamerica. Diversity is not very high among the Austrian isolates as the host plant is not native in the area and is not extensively cultivated in Austria. However, it seems that some strains persist well even in the absence of the host plant and at least some of the strains were shown to be very competitive (Sessitsch et al., submitted). It is striking that no differences could be detected among isolates of group A using methods that target symbiotic regions in the genome. Nevertheless they differ in the intergenic region between 16S rRNA and 23S rRNA and variances were detected by PCR with repetitive primers. These isolates might have evolved from one strain and the members of group A might represent divergent lineages selected by this particular environment. In the case of group B, two isolates, isolates CbS-7 and CbS-21, were

very distinct from the majority of the members of this group according to most of the analyses. We presume that these strains carried these differences already when introduced into this soil. Among the other isolates of group B, small differences could be detected by using PCR with repetitive primers and also these isolates might repesent better adapted descendants derived from one strain.

Sequence analysis of the 16S rRNA gene of strains of group A will help to assign the species of this group and to understand phylogenetic relationships to other *Rhizobium* species nodulating common bean. As the isolates obtained in this study possess high saprophytic competence and as some of them appear to be very competitive when nodulating the host plant, studying rhizobial diversity in a particular soil might help to select competitive and adapted inoculant strains.

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CHAPTER 6

Characterization of *Rhizobium pueblae* sp. nov. isolated from *Phaseolus vulgaris* L.

A. Sessitsch, H. Ramírez-Saad, G. Hardarson, A. D. L. Akkermans, and W. M. de Vos

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Characterization of *Rhizobium pueblae* sp. nov. isolated from *Phaseolus vulgaris* L.

Angela Sessitsch[1], Hugo Ramírez-Saad[2,3], Gudni Hardarson[1], Antoon D.L. Akkermans[2] and Willem M. de Vos[2]

1 Soil Science Unit, FAO/IAEA Agriculture and Biotechnology Laboratory, A-2444 Seibersdorf, Austria
2 Department of Microbiology, Wageningen Agricultural University, 6703 CT Wageningen, The Netherlands
3 Departamento de Sistemas Biológicos, Universidad Autónoma Metropolitana-Xochimilco, 04960 México, D.F. Mexico

Summary

A new species of *Rhizobium* obtained from nodules of common bean (*Phaseolus vulgaris* L.) and originating from soils in Mexico, France and Austria is described. The 16S rDNA sequences from six strains showed high levels of homology with a maximum of two nucleotide substitutions. Comparison of the 16S rDNA sequences with those from other bacteria indicated highest similarity to *Rhizobium sp.* OK50, *Rhizobium leguminosarum* strain IAM12609 and *Rhizobium etli*. DNA homology determined by DNA-DNA hybridization was high among the European isolates (45 - 90%) and ranged from 21 to 40% with FL27, but revealed very low homology to the recognized common bean nodulating species, *R. leguminosarum* bv. phaseoli, *R. etli* and *R. tropici*. Ribosomal gene organization was studied by Southern hybridization with the 16S rRNA gene and temperature gradient gel electrophoresis, indicating identical organization and the presence of three identical 16S rRNA copies in the genome of this species. The six strains investigated showed different plasmid profiles according to their geographical origin. We propose that the isolates described are members of a new species, *Rhizobium pueblae*.

INTRODUCTION

Bacteria of the genus *Rhizobium* that are able to nodulate common bean (*Phaseolus vulgaris* L.) have been traditionally classified as *R. leguminosarum* bv. phaseoli (11) according to the host plant they infect. Strains belonging to the other subdivisions of this species, *R. leguminosarum* bv. viciae and bv. trifolii, nodulate peas and clovers, respectively, and their symbiotic plasmids encode different host specificity genes. Nevertheless, rhizobia from common bean have found to be phylogenetically diverse based on different criteria such as protein profiles (30), multilocus enzyme electrophoresis patterns (5, 26), DNA relatedness analysis (14, 35, 42) and differences in the 16S rRNA gene sequence (7, 14, 42). In addition to *R. leguminosarum* bv. phaseoli two new species, *R. etli* (36) and *R. tropici* (17), have been described. Both *R. leguminosarum* bv. phaseoli and *R. etli*, carry multiple copies of the nitrogenase reductase gene (*nifH*) on the symbiotic plasmid but they have different 16S rRNA sequences (15, 28, 36). In contrast, *R. tropici* maintains only a single *nifH* gene copy on the symbiotic plasmid (17). *R. etli* and *R. tropici* show a broad host range but they nodulate different hosts (7, 17). Several new species among bean-nodulating strains have been proposed including *Rhizobium* sp. (*Phaseolus*) strain RCR 3618D with unknown geographical origin (42) and French isolates belonging to *Rhizobium* sp. (*Phaseolus*) strain R602sp and *Rhizobium* sp. (*Phaseolus*) strain H152 (14). The partial 16S rRNA gene sequence of strain R602sp was found to be identical to strain FL27 (14), a Mexican isolate from common bean which is poor in N_2-fixation (26). In general, strains of *Rhizobium* nodulating bean are of American origin as their host plant. For a long time it was believed that in Europe, *R. leguminosarum* bv. phaseoli was the only microsymbiont of common bean but recently *R. etli*, *R. tropici* and isolates belonging to different species were found (1, 14, 39).

Remarkably, strains recovered from common bean nodules from an Austrian soil showed high similarity to *Rhizobium* sp. strain R602sp isolated in France (39). The aim of this study was to characterize these bacteria phenotypically and to obtain more information on their phylogeny by sequence analysis of the 16S rRNA gene, determination of the copy number and heterogeneity of ribosomal genes, plasmid profiles and DNA-DNA hybridization. Based on this analysis we propose these isolates to belong to a new species, *Rhizobium pueblae*.

MATERIALS AND METHODS

Bacterial strains and phenotypic characterization. Four strains, CbS-1, CbS-3, CbS-17, CbS-18, were isolated from common bean grown in soil from fields around the Seibersdorf laboratory in Austria and characterized previously (39). *R.* sp. strain R602sp and reference strains were obtained either from G. Laguerre, France, or from the culture collection at the Seibersdorf laboratory. All rhizobial strains were maintained on yeast-extract-mannitol (YM) medium (44) and FL27, R602sp, CbS-1, CbS-3, CbS-17 and CbS-18 were tested for growth on LB medium (19) and on peptone-yeast extract (PY) medium (20). In order to test substrate utilization modified minimal B&D medium (46) was amended with the following carbon sources at a concentration of 1 g/liter: D-glucose, starch, maltose, urea, fructose, D-lactose, D-sorbit, D-xylose, D-mannose, arabinose, D-ribose, myo-inosite, melobiose, raffinose, D-trehalose, methanol and ethanol. The amino acids L-alanine, L-aspartic acid, L-asparagine, L-arginine, L-cysteine, L-glutamate, L-glutamine, L-histidine, L-leucine, L-methionine, L-phenylalanine, L-proline, L-serine, L-tryptophane, L-threonine, L-tyrosine and L-valine were each tested as sole carbon and nitrogen source at a concentration of 1 g/liter. Resistance to antibiotics was tested by plating the rhizobial strains on YM agar medium containing ampicillin (10 μg/ml), chloramphenicol (30 μg/ml), kanamycin (30 μg/ml), tetracycline (10 μg/ml), nalidixic acid (10 μg/ml), spectinomycin (100 μg/ml) or streptomycin (100 μg/ml).
The growth at different temperatures (20°C, 25°C, 30°C, 35°C, 37°C and 40°C), growth on YM containing 1.0, 1.5 and 2% NaCl and growth at different pH values (pH 4, 5, 6, 7, 8, 9 and 10) was determined in liquid culture.

Plasmid profiles, DNA isolation, and ribosomal DNA hybridization. Plasmid profiles of CbS-1, CbS-3, CbS-17, CbS-18, R602sp, FL27 and of CIAT899 were investigated as described by Hynes et al. (10). Total genomic DNA was prepared as described elsewhere (2) omitting the CsCl purification step. Genomic DNA was digested with *Hin*dIII and the resulting fragments were separated by electrophoresis using a 0.7% agarose gel, that was blotted onto a Hybond-N membrane (Amersham International, UK). A probe containing the 16S rRNA gene from strain R602sp was prepared by PCR using the primers rD1 and fD1 (45) as described previously (39). After isolating the resulting fragment from a 1% agarose gel utilizing the Geneclean II Kit (Bio 101, Inc., USA), it was labeled with α-[^{32}P]dATP by using the Multiprime DNA Labeling System (Amersham International, UK) according to the manufacturer's protocol. Hybridization was carried out at 65°C for 2 h in Rapid-Hyb

buffer (Amersham International, UK), washing and autoradiography was done as described elsewhere (37).

DNA-DNA hybridization. Dot blot hybridizations were performed as described previousy (13) using 25 µl samples containing 400 ng genomic DNA. In addition, 400 ng denaturated calf thymus DNA was transferred onto the membrane. The membranes were hybridized with 4 µg genomic DNA per dot previously digested with *Alu*I and labeled with α-[^{32}P] dATP by using the Multiprime DNA Labeling System. Hybridization was carried out under relaxed conditions at 55°C for 2 h in Rapid-Hyb buffer. The filters were washed under stringent conditions at 60°C in a solution containing 0.03 M NaCl, 0.003 M sodium citrate and 1% SDS. Membranes were cut and 9 x 9 mm pieces were counted with a liquid scintillation counter (Tri Carb 2200CA, Packard). The amount of radioactivity associated with calf thymus DNA was subtracted and the percentages of DNA relatedness were determined relative to the signal found in the homologous hybridization.

Analysis of the 16S rRNA ribosomal genes. The 16S rRNA genes of CbS-1, CbS-3, CbS-17, CbS-18 and of R602sp were amplified by using the primers FGPS6-63 with a *Bgl*II-site (14) and P1510Pst with a *Pst*I site (21) and a standard protocol (39). The amplified fragments were digested with *Bgl*II and *Pst*I and then cloned into pUC18Not that was previously cut with *BamH*I and *Pst*I. As the 16S rRNA gene of strain FL27 showed an internal *Pst*I restriction site, the gene was amplified by using the primers FGPS6-63 (14) and P1510HIII equipped with a *Hind*III-site (5'-GTG*AAGCTT*GGTTACCTTGTTACGACT-3'). The resulting fragment was digested with *Bgl*II and *Hind*III and then cloned into *Bgl*I-*Hind*III-cut pUC18Not using *Escherichia coli* strain DH5α as recipient. DNA sequence analysis of the cloned 16S rRNA fragments was done by using the dideoxy-chain termination method (33) using a LI-COR 4000L automated sequencer.
For the temperature gradient gel electrophoresis (TGGE) total genomic DNA was used as template to amplify a fragment of ca. 440 bp comprising the V6 - V8 variable regions of the 16S rRNA gene. The primers used, PCR and TGGE were previously described by Nübel et al. (22).

Sequence alignment and phylogenetic analysis. Gene banks were searched by using the FASTA tool (25) and alignments of selected 16S rDNA sequences were done with the ClustalW multiple-alignment programme (8). Short regions of uncertain alignment were excluded from further analyses. Calculation of evolutionary distances was done utilizing the Jukes and Cantor model (12). Phylogenetic trees based on the neighbour

joining method (32) were constructed with 100 bootstrap replicates using the TREECON for Windows software package (43). A Maximum Parsimony phylogenetic tree was created with the programme PAUP3.1 (41).

RESULTS

Phenotypic characterization. The strains CbS-1, CbS-3, CbS-17, CbS-18 and R602sp could not utilize the following compounds: starch, urea, methanol, ethanol, L-alanine, L-arginine, L-asparagine, L-cysteine, L-phenylalanine and L-tryptophane. The strains were not able to grow on LB medium or on YM supplemented with high (above 1%) NaCl concentrations, but they could be cultivated on PY medium. All strains were able to grow on D-glucose, maltose, fructose, D-lactose, D-sorbit, D-xylose, D-mannose, arabinose, D-ribose, myo-inosite, melibiose, raffinose, D-trehalose, L-aspartic acid, L-glutamate, L-glutamine, L-histidine, L-leucine, L-methionine, L-proline, L-serine, L-threonine, L-tyrosine and L-valine. The strains were able to grow on plates supplemented with ampicillin, chloramphenicol and nalidixic acid. The optimum pH range was from 6 to 8, while no growth occurred at pH 4, 9 and 10. All isolates were able to grow at a temperature up to 37°C but did not grow at 40°C.

Plasmid profiles. Plasmid analysis showed that the Austrian isolates CbS-1, CbS-3, CbS-17 and CbS-18 carried three plasmids identical in size while the French strain R602sp harboured two plasmids. Strain FL27 and *R. tropici* CIAT899 carried also three plasmids but had patterns different to each other and to the Austrian isolates. The largest plasmid of the Austrian isolates and of R602sp and FL27 appeared to have the same size (Fig. 1).

Ribosomal gene organization. Southern hybridization with the 16S rRNA gene of R602sp as a probe showed that isolates CbS-1, CbS-3, CbS-17, CbS-18, R602sp, FL27, *R. leguminosarum* bv. phaseoli strain H131 and *R. etli* strain CFN42 contained at least three copies of the 16S rRNA gene. Both *R. tropici* type CIAT899 and *R. tropici* type CFN299 carried one copy of the 16S rRNA gene. The Austrian isolates, R602sp and FL27 showed identical patterns with three hybridizing *Hin*dIII fragments of 4.6, 11.0 and 12.9 kb.

TGGE analysis of PCR-amplified segments of 16S rDNAs resulted in single band profiles for each tested strain (data not shown). This suggested identical sequences in those strains bearing more than one copy of the gene.

Figure 1. Plasmid profiles of CbS-1 (lane 1), CbS-3 (lane 2), CbS-17 (lane 3), CbS-18 (lane 4), R602sp (lane 5), FL27 (lane 6) and CIAT899 (lane 7).

DNA-DNA hybridization. Isolates CbS-1, CbS-3, CbS-17, CbS-18 and R602sp showed high levels of DNA relatedness ranging from 45 to 90%. Homologies between 26 and 65% were found among the European isolates and FL27 but the homology to *R. leguminosarum*, *R. tropici* and *R. etli* was very low (Table 1).

Analysis of the 16S rRNA gene sequence. The determined 16S rDNA sequences of strains CbS-1 and CbS-3 were identical and differed only in one nucleotide with those of strains CbS-18 and R602sp. Two substitutions at different positions were found in comaprison with the sequences of strains CbS-17 and FL27. FASTA analysis indicated several members of the alpha subclass of Proteobacteria as being the most related 16S rDNA sequences. All further comparisons were based on 1431 nucleotides comprising more than 93% of the 16S rRNA gene. *Rhizobium* sp. OK50 (24) and *R. leguminosarum* strain IAM12609 (24) had the highest homology values (Table 2). Phylogenetic dendrograms constructed by both distance and parsimony methods showed essentially the same topology and similar evolutionary distances, the latter method yielded only one most parsimonious tree. The phylogenetic tree (Fig. 2) showed a well-defined and compact *R. pueblae* cluster, here proposed to be a new species, including the proposed type strain R602sp, the Austrian isolates CbS-1, CbS-17 and CbS-18 and the Mexican isolate FL27. This cluster as well as other recognized rhizobial lineages were clearly separated and supported by high bootstrap values.

Table 1. Levels of DNA relatedness between CbS-1, CbS-3, CbS-17, CbS-29, R602sp and other common bean nodulating *Rhizobium* species.

	% DNA relatedness with									
Strain	CbS-1	CbS-3	CbS-17	CbS-18	R602sp	FL27	H131	CIAT899	CFN299	CFN42
CbS-1	100	76	75	69	90	60	7	7	19	11
CbS-3	83	100	80	87	83	65	7	5	13	12
CbS-17	90	83	100	88	82	62	6	5	9	14
CbS-18	45	68	86	100	87	52	2	1	7	7
R. sp. R602sp	55	82	79	74	100	61	5	3	8	14
R. sp. FL27	26	21	40	30	35	100	5	14	6	12
R. leg. bv. phaseoli H131	6	4	3	4	11	5	100	6	12	22
R. tropici type CIAT899	8	5	5	5	8	5	4	100	33	7
R. tropici type CFN299	7	5	5	4	5	5	3	14	100	11
R. etli CFN42	7	12	5	5	9	6	18	4	7	100

Table 2. Numbers of nucleotide differences and % 16S rDNA homologies in the aligned sequences of *Rhizobium pueblae* sp. nov. strains and most related species

	R602sp		CbS-1		CbS-3		CbS-17		CbS-18		FL27	
Species	ΔN[1]	%H[2]	ΔN	%H	ΔN	%H	ΔN	%H	ΔN	%H	ΔN	%H
R. sp. OK50	31	97.8	30	97.9	30	97.9	32	97.8	29	98.0	30	97.9
R. leg. strain IAM12609	31	97.8	30	97.9	30	97.9	32	97.8	29	98.0	30	97.9
R. etli	35	97.6	34	97.6	34	97.6	36	97.5	33	97.7	34	97.6
R. leg. bv. phaseoli	46	96.8	45	96.9	45	96.9	47	96.7	44	96.8	45	96.9
R. tropici	57	96.0	56	96.1	56	96.1	58	95.9	55	96.2	57	96.0
R. sp.3618D	58	95.9	57	96.0	57	96.0	59	95.9	56	96.1	57	96.0

1 number of nucleotide differences
2 % of 16S rDNA homology in 1431 nucleotides

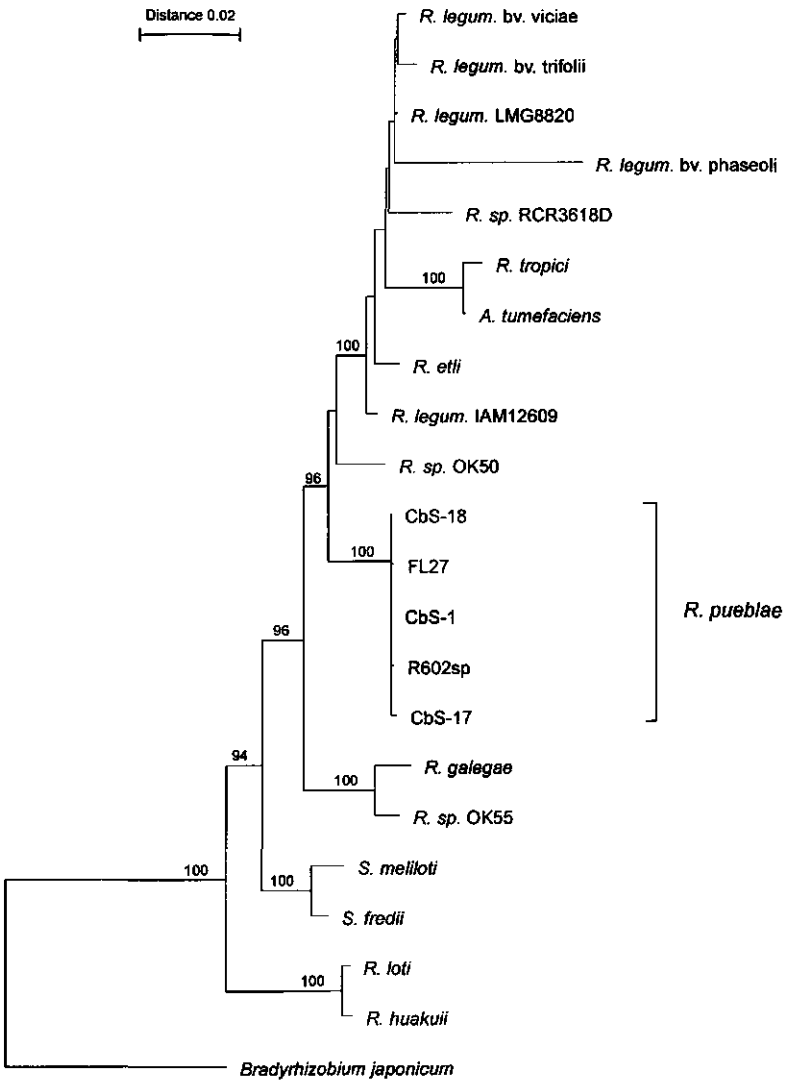

Figure 2. Neighbour-joining phylogenetic tree based on 1431 nucleotides of the 16S rDNA of different rhizobia and related genera. Percentage of 100 bootstrap replicates are shown at the left nodes when above 90%. The proposed *Rhizobium pueblae* type strain R602sp is included in the *R. pueblae* cluster, which is clearly separated from other rhizobial groups and supported by a 100% bootstrap value.

Accession numbers of the 16S rDNA sequences used are: *Rhizobium leguminosarum* (*R. legum.*) bv. viciae U29386, bv. trifolii U31074, bv. phaseoli U29388; for strains LMG8820 and IAM12609 are X67227 and D14513 respectively. *Rhizobium* sp. (*R. sp.*) strain RCR3618D is U29387, strain OK50 is D01271 and strain OK55 is D01266. U38469 for *R. tropici*, U38469 for *R. etli*, D11343 for *R. galegae*, D12791 for *R. loti* and D13431 for *R. haukuii*. *Sinorhizobium meliloti* (*S. meliloti*) is D14509, *S. fredii* is D14516, *Agrobacterium tumefaciens* (*A. tumefaciens*) is D14505 and *Bradyrhizobium japonicum* used as outgroup is D13429.

DISCUSSION

Recently, high relatedness among common bean nodulating strains isolated in Austria (CbS-1, CbS-3, CbS-17 and CbS-18) and the French strain *R.* sp. R602sp was demonstrated by using several approaches targeting symbiotic and chromosomal regions of the genome as well as the nodulation phenotype (39). Based on DNA-DNA hybridization and partial 16S rDNA sequences of 246 nucleotides, Laguerre et al. (14) suggested that strain R602sp belonged to a new species that also could contain the Mexican isolate FL27 as the latter showed identical partial sequences to R602sp. In the present study, sequence analysis of 93% of the 16S rRNA gene revealed high homology among the four Austrian isolates, R602sp and FL27 with a maximum of two nucleotide substitutions. One nucleotide difference in FL27 resulted in an internal *Pst*I restriction site that was absent in all European isolates. Comparison of the 16S rDNA sequences with those of other bacteria showed highest similarity to *R.* sp. OK50 isolated from *Pterocarpus klemmei* in Japan (24), to *R. leguminosarum* strain IAM12609 (24), and to *R. etli*. Phylogenetic dendrograms always positioned R602sp in the vicinity of the above mentioned species but was found to be a member of a lineage different from those of described *Rhizobium* species. This is in agreement with Eardly et al. (4) reporting high similarity between FL27 and *R. etli* by sequence analysis of a 260 bp segment of the 16S rRNA gene. Although ribosomal gene sequences play an important role in the description of new species, in many cases there is only a limited correlation between DNA relatedness and 16S rDNA homology. This was reported for *Rhizobium* (23) but also for other bacterial species such as *Aeromonas* and *Plesiomonas* (16). Recently, van Berkum et al. (42) suggested that although the level of 16S rDNA sequence similarity among bean rhizobia is high, the DNA relatedness data indicate different species. Stackebrandt and Goebel (40) demonstrated that the correlation between 16S rDNA homology and DNA-DNA reassociation is not necessarily linear indicating that distinct species can show high 16S rDNA sequence similarities. Assessment of DNA relatedness was proposed as an important criterion for the description of new species of root- and stem-nodulating bacteria (6). The *Rhizobium pueblae* sp. nov. strains tested shared very low DNA homology with the recognized common bean nodulating species, i.e. *R. leguminosarum*, *R. tropici* and *R. etli*. This is in agreement with Martínez-Romero (18), who found low DNA relatedness between *R. etli* and FL27. High DNA homology was found among the European isolates. The Mexican isolate FL27, however, shared lower DNA relatedness with the European strains but the values in combination with the 16S rDNA similarities are within the possible range suggested by Stackebrandt and Goebel (40). One explanation could be the presence of plasmids

in FL27 sharing little homology with those of the European isolates. These plasmids may carry up to 25% of the genetic information in *Rhizobium* (27) and plasmids are prone to losses or alterations. High-frequency plasmid-borne rearrangements including sequence amplification, deletion, cointegration and loss have been particularly observed in *R. etli* strain CFN42 plasmids (3, 31). In addition, exchange of plasmids among *Rhizobium* populations has been reported (29, 34). Interestingly, the Mexican, French and Austrian strains showed plasmid profiles that reflected their geographical origin. Data obtained by Martínez-Romero et al. (17) suggested that the RFLPs of rRNA in *Rhizobium* operons are species-specific, while riboprobing based on pulsed field gel electrophoresis fingerprints resulted in strain-specific patterns among *R. galegae* strains (9). The identical 16S rRNA organization in FL27, R602 and the Austrian isolates is an additional factor indicating the close relationship of these strains. Our study as well as results obtained by Huber and Selenska-Pobell (9) suggest that the presence of three rRNA operons is common among rhizobia. Previously, it was demonstrated that R602sp and the Austrian isolates contain one copy of the *nifH* gene (39). It is uncertain whether common bean is the true host of *Rhizobium pueblae* sp. nov. It has been shown that R602sp, CbS-1, CbS-3, CbS-17 and CbS-18 also nodulate *Leucaena*, *Gliricidia* and cowpea (39) and FL27 was isolated from bean grown in a *Leucaena* field (26). In addition, FL27 was demonstrated to be a poor N_2-fixer in common bean nodules (26). On the other hand, the Austrian isolates possess high competitive ability in nodulating bean in a soil where they adapted well (38, 39). Further studies will be needed to evaluate the agronomic value of *Rhizobium pueblae* sp. nov. inoculant strains.

Description of *Rhizobium pueblae* sp. nov. *Rhizobium pueblae* (pu' eb. lae. M. L. gen. n. *pueblae*, from Puebla, referring to the state Puebla in Mexico, where FL27 was isolated). These bacteria are aerobic, gram-negative, non-spore forming rods that are 0.5 to 0.8 by 0.9 to 1.3 μm. Colonies on YM are circular, convex, semitranslucent and reach about 3mm after 3 days. They grow on YM and on PY medium, but they are not able to grow on LB. The optimum pH range is from 6 to 8, and they are able to grow at higher temperatures up to 37°C. All strains tested are resistant to nalidixic acid, chloramphenicol and to ampicillin. They form nodules on *P. vulgaris, Leucaena leucocephala*, *Gliricidia* and *Vigna unguiculata*. *R. pueblae* sp. nov. possesses one copy of the *nifH* gene and at least three copies of the 16S rRNA gene. The 16S rRNA gene organization and its sequence is different to other species and the whole-DNA relatedness with other bean-nodulating rhizobia is low. Strain R602sp was chosen as the type strain as it is the best characterized strain of this species and it shows all characteristics described above.

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CHAPTER 7

Summary and concluding remarks

Summary and concluding remarks

The research described in this thesis was directed to the development of molecular identification and detection techniques for studying the ecology of *Rhizobium*, a nitrogen-fixing bacterium of agricultural importance. Competition of inoculant strains with indigenous microbes is a serious problem in agricultural practice and was therefore addressed in this work using the developed tools. Furthermore, various molecular techniques have been applied to analyse rhizobial populations nodulating common bean and a new species was characterized.

In this chapter the results obtained are summarized and potential future applications are discussed.

Development of gusA- and celB-minitransposons and their use in rhizobial competition studies

The use of marker genes in rhizobial competition studies is reviewed in Chapter 1. Specific attention is given to the *gusA* gene, encoding ß-glucuronidase (GUS). This gene is a highly suitable marker for studying plant-microbe interactions due to the absence of GUS activity in plants and in most bacteria that are of relevance in agriculture. In Chapter 2 the construction of several GUS transposons containing the marker gene in combination with different regulation systems to be used for ecological and genetic studies is described. The minitransposon mTn*5*SS*gusA*20 contains the *aph* promoter which was demonstrated to be expressed in a wide variety of Gram-negative bacteria (de Bruijn and Lupski, 1984) and its use in *Rhizobium* resulted in high-level constitutive expression. Transposon mTn*5*SS*gusA*21 is similar to mTn*5*SS*gusA*20, except that it contains a unique site for *Spe*I, a rare-cutting enzyme in bacteria with high G+C contents such as rhizobia (Sobral et al., 1991). The use of mTn*5*SS*gusA*21 therefore may be instrumental for the genetic mapping of insertions. The *tac* promoter was used to drive the expression of the *gusA* gene in the transposon mTn*5*SS*gusA*11 resulting in high GUS activity in the free-living state. The transposons with constitutive *gusA* expression are optimal for studying rhizosphere colonization and for studying nodule occupancy in young plants. In order to reduce any metabolic load due to GUS production, the mTn*5*SS*gusA*10 transposon with regulated *gusA* expression was constructed. It contains the *tac* promoter in combination with the $lacI^q$ repressor gene and *gusA* gene expression is repressed until an inducer, such as IPTG, is added. This regulation should avoid possible effects on

the ecological fitness. Two transposons carrying symbiotically activated *gusA* genes, mTn*5*SS*gusA*30 and mTn*5*SS*gusA*31, were made by using the *nifH* promoter of a *Rhizobium* and a *Bradyrhizobium* strain, respectively. The *nifH* gene encodes the Fe-component of nitrogenase and is only expressed in symbiotic or microaerobic conditions (Fischer, 1994). These constructs are recommended for longer-term nodule occupancy experiments. Furthermore, a promoter-less GUS transposon, mTn*5*SS*gusA*40, is described that should be of use for molecular genetic studies as well as for screening bacteria for their response to specific environmental conditions or signals. The developed transposons carry a gene conferring resistance to spectinomycin and streptomycin that proved to be an appropriate marker for many strains. Nevertheless, few strains with an endogenous resistance exist. Therefore, the development of additional transposons conferring an alternative resistance would be advantageous. This may be realized by inserting the developed GUS expression cassettes into minitransposons containing other antibiotic (de Lorenzo et al., 1990) or natural resistance markers (Herrero et al., 1990). Chapter 2 also addresses also the application of the different transposons in studies on root colonization and nodule occupancy, while various GUS assays are described in detail.

Potential effects on the fitness of a strain due to insertion of a GUS transposon were evaluated in Chapter 3. Only few data exist on the impact of foreign genes on the fitness of an organism (Doyle et al., 1995) and before using any marker system for ecological studies its ecological effects have to be studied rigorously. In the case of *Rhizobium*, it is essential that the nodulation behaviour and competitive ability are maintained. The competitive abilities, nodulation characteristics, and growth rates of five independent derivatives of *R. tropici* strain CIAT899 marked with the *gusA* gene on minitransposon mTn*5*SS*gusA*10 were determined relative to the parent strain. Insertion of mTn*5*SS*gusA*10 did not affect the nodulation or nitrogen fixation efficiency of the wild-type strain. Nevertheless, the competitiveness index of the different *gusA* derivatives relative to the parental strain CIAT899 varied between isolates. One isolate was less competitive than the wild-type strain in three independent experiments, while the other isolates proved to be either equally competitive or more competitive. The utilization of this methodology to assess competitivity resulted in highly significant calculations as all the nodules on each plant were analysed for nodule occupancy. The results showed that the insertion of mTn*5*SS*gusA*10 may have an impact on the ecological behaviour of a strain, but derivatives indistinguishable from the parent strain can be obtained. A primary selection of marked strains is recommended, which may be achieved by coinoculating the parent and the marked derivative in a one to one ratio and ensuring that the proportion of blue nodules does not differ significantly from the expected 50%.

Furthermore, in Chapter 3, the detection of dual nodule occupancy is discussed. The appearance of partially stained nodules in mixed inoculum treatments but not in single strain treatments led to the conclusion that these were due to mixed infections. This was confirmed by nodule isolation and plating experiments. Partially stained nodules were also observed by Krishnan and Pueppke (1992) who reported that nodules were occupied by either a *lacZ*-marked or a non-marked *R. fredii* strain and X-gal was used for detection. However, the *lacZ* marker system has several disadvantages due to high background activity in plant and rhizobia whereas the *gusA* marker gene can be used to readily detect dual nodule occupancy on plant.

A new marker gene system based on the *celB* gene is presented in Chapter 4. The *celB* gene has been isolated from the hyperthermophilic archaeon *Pyrococcus furiosus* and it encodes a thermostable and thermoactive ß-glucosidase with a high ß-galactosidase activity (Voorhorst et al., 1995). The latter enzyme activity can be used for the detection of rhizobia as endogenous background activity in plants as well as in bacteria can be easily eliminated by a heat treatment. Moreover, cheap histochemical substrates are available to determine ß-galactosidase activity. The *E. coli* ß-galactosidase gene, *lacZ*, has been used to monitor engineered soil bacteria (Drahos et al., 1986; Hartel et al., 1994) but was only found to be appropiate when used with Lac$^-$ bacteria. Transposons containing the *celB* gene were constructed in *E. coli*, based on the existing *gusA* transposons. The first transposon, mTn*5*SS*celB*10, contains the *tac* promoter which is regulated by the *lacI*^{q} gene product and should reduce any metabolic stress to the marked strain due to marker gene activity. Transposon mTn*5*SS*celB*31 carries *celB* expressed from a *Bradyrhizobium nifH* promoter and is active in nitrogen-fixing legume nodules. A third *celB* minitransposon, which contains the marker gene constitutively expressed is described elsewhere (Sessitsch et al., submitted). The *celB* marker gene system has several advantages in rhizobial competition studies over conventional techniques as the assay is simple to perform and the histochemical substrates are cheap. However, the greatest advantage is that *gusA* and *celB* marked strains can be localized simultaneously on a plant and a combined *gusA/celB* assay will enable studies of multi-strain rhizobial inocula competing with indigenous rhizobial populations. Although simultaneous detection of differently marked strains has been reported (Thompson et al., 1995; Bauchrowitz et al., 1996), the *celB* gene encoding the thermostable marker is better suited for double staining. In addition, a procedure has been described allowing detection of *gusA* and *celB* marked strains on plates (Sessitsch et al., submitted). In Chapter 4 the application of the *celB* marker gene is demonstrated for *Rhizobium*. However, because of the wide host range of the Tn*5* based transposons and the portable expression signals this marking system is suitable for use in a variety of Gram-negative bacteria.

The advantages of the different *gusA* and *celB* marker gene cassettes are discussed in Chapter 1, but also other reporter genes and their applications in studies on microbial ecology are presented. Moreover, the development of a GUS Gene Marking Kit is reported. This kit was made particularly for agronomists and microbiologists in developing countries who are not familiar with molecular techniques and who do not have the resources to establish this methodology in their laboratories. Meanwhile, a CelB Gene Marking Kit is also available that can be used either in combination with or instead of the GUS Gene Marking Kit (FAO/IAEA, 1992-1997).

Ecology of rhizobia nodulating common bean

In the fields around the Seibersdorf laboratory common bean has not been cultivated during the last decades but is well nodulated. In earlier studies, common bean rhizobia populations in this soil have been found to be very competitive in nodulation (see Chapter 1). They were shown to outcompete *R. tropici* strain CIAT899 when inoculating *Phaseolus vulgaris* at an inoculation level of 10^5 cells per seed. When increasing inoculation to 10^8 cells per seed, 65% of the nodules were still occupied by the native strains. In Chapter 5, rhizobial populations isolated from common bean nodules grown in the Seibersdorf soil were characterized. Molecular methods targeting the whole genome such as PCR with repetitive primers were used, and specific chromosomal loci such as the 16S rRNA gene or the 16S-23S rDNA intergenic spacer were analyzed. Plasmid profiles and Southern hybridization with a *nifH* probe gave information on symbiotic regions. In addition, the nodulation host range was determined. Two distinct groups were found, one of them was classified as *R. etli* according to the RFLP analysis of the 16S rRNA gene and because of the presence of three copies of the *nifH* gene. The members of the second group could not be assigned to any recognized common bean nodulating *Rhizobium* species, i.e. *R. leguminosarum* bv. phaseoli, *R. etli* and *R. tropici*, but showed high similarity to *Rhizobium* sp. (*Phaseolus*) strain R602sp isolated in France (Laguerre et al., 1993). Isolates of this group also formed nodules on cowpea, *Leucaena* and *Gliricidia*. For a long time, *R. leguminosarum* bv. phaseoli was believed to be the only microsymbiont in Europe, but recently *R. tropici* and two new species have been found in French soils (Laguerre et al., 1993; Amarger, 1994). These studies and the results obtained in Chapter 5 indicate that strains originating in Mesoamerica could establish well in European soils. However, diversity was not high among the Austrian isolates due to the long absence of the host plant in this soil.

The focus of Chapter 6 is on the taxonomy and phylogeny of the Austrian isolates showing high similarity to *Rhizobium* sp. R602sp. A Mexican common bean isolate, FL27, was included in this study since Laguerre et al. (1993) found the partial 16S rRNA gene sequence of R602sp to be identical to FL27. Sequence analysis of the 16S rRNA gene, determination of the copy number and heterogeneity of ribosomal genes, plasmid profiles and DNA-DNA hybridization resulted in valuable taxonomic information on these strains. Based on these results it was proposed that these strains belong to a new species that was named *R. pueblae* sp. nov., referring to the state Puebla, Mexico, where FL27 was isolated. The Mexican, French and Austrian isolates showed very similar 16S rDNA sequences with a maximum of two nucleotide substitutions. Comparison of the 16S rDNA sequences with those of other bacteria revealed highest similarity to *R. leguminosarum* strain IAM12609, *R.* sp. OK50 and to *R. etli*. Although phylogenetic dendrograms always positioned *R. pueblae* sp. nov. strains in the vicinity of the above-mentioned species, the new species was found to belong to a lineage different from those of described *Rhizobium* species. The whole DNA relatedness among the European isolates was very high but showed lower levels with FL27, probably due to the presence of different plasmids. The DNA homology to other bean-nodulating species was very low. *R. pueblae* sp. nov. strains possess at least three copies of the 16S rRNA gene and the ribosomal gene organization is different to other species. Despite the high competitive ability of some strains in the Seibersdorf soil, little is known on the agronomic value of this species.

Concluding remarks

To improve biological nitrogen fixation, adapted efficient nitrogen-fixing plant genotypes, effective rhizobial inoculants and appropiate agricultural management practices are needed. Consequently, plant breeders, microbiologists, soil scientists, agronomists as well as farmers have to cooperate in order to achieve this goal.

The selection of superior sources of natural plant genetic variability and plant breeding in the presence of rhizobia instead of applying nitrogen fertilizers may lead to the identification of high-fixing lines. Other desirable traits such as disease resistance or stress tolerance could be transferred by appropiate breeding methods or by genetic engineering. Soils, in which legumes are cultivated, vary greatly and can be opposed to various environmental stresses such as low pH or high temperature. Efforts have been undertaken to develop appropiate plant genotypes whereas for a long time the stress tolerance of inoculant strains has not been considered. Inoculant strains have been recommended based on good symbiotic performance in a particular environment

been recommended based on good symbiotic performance in a particular environment while the soil status or the agroecological zone of the final application has not been taken into account. The vast genetic pool of natural soils containing not yet identified strains and species can provide a variety of inoculant strains that may show better performance in the field. A strong correlation between the indigenous population size and the nodule occupancy of the inoculant strain has been established (Thies et al., 1991). However, the effect of the diversity of indigenous rhizobia on competition has not been determined. Probably, different strategies are needed in order to outcompete highly dominant field isolates or to achieve successful competition of an inoculant strain with a variety of different indigenous strains that are present in low numbers. In addition, inoculation practices have to be developed that are convenient for the farmer and that allow distribution of the introduced strain into the entire rooting zone.

It seems that the various aspects important for efficient nodulation and nitrogen fixation are presently not linked sufficiently. A more rational selection of efficient and competitive strains could be realized when a database existed containing data on soil properties, environmental conditions, rhizobial diversity and population size, as well as on the competitive ability and effectiveness of rhizobial strains in combination with particular plant genotypes. Furthermore, these data could be of use for the development of new strains or plants by genetic engineering.

In this thesis, the development of new methods to assess rhizobial competition is presented. These marker gene-based techniques are appropiate for the large-scale screening of inoculant strains but can be also used for genetic analysis of a variety of Gram-negative bacteria. Molecular methods have facilitated the analysis of strains nodulating common bean and resulted in the description of a new *Rhizobium* species that includes strains with possible beneficial properties.

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Samenvatting

Samenvatting en conclusies

Het in dit proefschrift beschreven onderzoek was gericht op de ontwikkeling van moleculaire technieken voor identificatie en detectie van de landbouwkundig belangrijke stikstofbindende bacterie *Rhizobium*. De ontwikkelde methodieken werden gebruikt bij het onderzoek naar de competitie van *Rhizobium*-stammen die als inoculum (entstof) worden gebruikt met in de grond aanwezige autochtone stammen. Hiernaast werden verschillende moleculaire technieken gebruikt voor het beschrijven van *Rhizobium* populaties in wortelknollen van de boon (*Phaseolus vulgaris*) en voor het karakteriseren van een nieuwe *Rhizobium* soort.

In dit hoofdstuk worden de verkregen resultaten samengevat en worden de toepassingsmogelijkheden besproken.

Ontwikkeling van gusA- en celB-minitransposons en het gebruik ervan in onderzoek naar competitie tussen Rhizobium-stammen

In hoofdstuk 1 wordt een literatuuroverzicht gegeven van het gebruik van merker genen in onderzoek naar competitie tussen *Rhizobium*-stammen. Speciale aandacht is besteed aan het *gusA* gen dat codeert voor ß-glucuronidase (GUS). Dit gen is een goede merker in onderzoek naar plant-micro-organisme interacties omdat GUS afwezig is in planten en in de meeste bacteriën die landbouwkundig relevant zijn. In hoofdstuk 2 wordt de constructie besproken van verschillende GUS transposons die het merker gen met verschillende regulatorsystemen bevatten ten behoeve van ecologisch en genetisch onderzoek. Het minitransposon mTn*5*SS*gusA*20 bevat de *aph* promoter waarvan bekend is dat deze tot expressie komt in veel Gram-negatieve bacteriën (de Bruijn en Lupski, 1984). Gebleken is dat de promoter in *Rhizobium* een hoge constitutieve expressie vertoonde. Transposon mTn*5*SS*gusA*21 is identiek aan mTn*5*SS*gusA*20, maar bevat een unieke knipplaats voor *Spe*I, een restrictie-enzym dat zelden knipt in DNA van bacteriën met hoog GC-gehalte, zoals rhizobia (Sobral et al., 1991). Transposon mTn*5*SS*gusA*21 kan daarom gebruikt worden voor genetische analyse van inserties. De *tac* promotor werd gebruikt om de expressie van het *gusA* gen in het transposon mTn*5*SS*gusA*11 aan te sturen, zodat een hoge GUS activiteit werd verkregen in de vrijlevende bacteriën. De transposons met een constitutieve *gusA* expressie zijn met name geschikt voor onderzoek naar de kolonisatie van de rhizosfeer en onderzoek naar de wortelknolvorming in jonge planten. Teneinde mogelijke effecten van GUS produktie op het celmetabolisme te voorkomen, werd het transposon mTn*5*SS*gusA*10 gemaakt waarbij de *gusA* gen-expressie gereguleerd is. Het bevat de *tac*

promoter in combinatie met het *lacI*^{q} repressor gen, waardoor *gusA* genexpressie onderdrukt blijft totdat een inducer, zoals IPTG, wordt toegevoegd. Door deze regulatie kunnen mogelijke effecten op de ecologische fitness voorkómen worden. Twee transposons, mTn*5*SS*gusA*30 en mTn*5*SS*gusA*31, die alleen in de symbiose actief zijn, werden gemaakt door de *nifH* promoter van resp. een *Rhizobium* en een *Bradyrhizobium* stam te gebruiken. Het *nifH* gen codeert voor de Fe-component van nitrogenase en komt alleen tot expressie onder micro-aërofiele condities en in de symbiose (Fischer, 1994). Deze constructies worden aanbevolen voor gebruik in langdurige nodulatie-experimenten. Verder werd een GUS transposon zonder promoter, mTn*5*SS*gusA*40, gemaakt voor moleculair-genetisch onderzoek en voor het screenen van bacteriën met hun reakties op specifieke milieuomstandigheden of signalen. De ontwikkelde transposons bevatten een spectinomycine en streptomycine resistentie-gen die goede merkers bleken voor veel stammen. Daar er toch enkele stammen met endogene resistentie bestaan, is het gebruik van transposons een extra voordeel. Dit kan worden gerealiseerd door de ontwikkelde GUS expressie casettes in te bouwen in minitransposons die andere antibioticum resistentie-genen herbergen (de Lorenzo et al., 1990) of natuurlijke resistentie merkers (Herrero et al., 1990) bevatten. In hoofdstuk 2 wordt ook de toepassing beschreven van de verschillende transposons in studies naar de kolonisatie van de wortels en wortelknolvorming, terwijl verschillende GUS bepalingsmethoden in detail worden beschreven.

In hoofdstuk 3 worden de mogelijke effecten van insertie van een GUS transposon op de fitness van een stam geëvalueerd. Er zijn slechts weinig gegevens gepubliceerd over het effect van vreemde genen op de fitness van een organisme (Doyle et al., 1995) en vóórdat een merker systeem in ecologisch onderzoek gebruikt wordt, moet het ecologisch effect ervan bepaald worden. In het geval van *Rhizobium* is het belangrijk dat de competitieve eigenschappen en het wortelknolvormend vermogen van de stam behouden blijven. Van vijf onafhankelijke isolaten van 24 *R. tropici* stam CIAT899, gemerkt met het *gusA* gen op het minitransposon mTn*5*SS*gusA*10, werden de competitieve eigenschappen, de nodulatie karakteristieken (= het vermogen wortelknollen de induceren), en de groeisnelheid vergeleken met die van de oorspronkelijke stam. Insertie van mTn*5*SS*gusA*10 veranderde niet het nodulatievermogen en de symbiontische stikstofbinding van de stam. Toch bleek het competitief vermogen van de verschillende *gusA* derivaten te variëren. Eén isolaat bleek minder competitief dan het wildtype in drie onafhankelijke experimenten, terwijl de andere isolaten even competitief of zelfs competitiever bleken dan het wildtype. Het gebruik van deze methode resulteerde in een zeer betrouwbare berekening van het competitief vermogen van een stam, omdat alle knollen van alle planten worden geanalyseerd. De resultaten toonden aan dat insertie van mTn*5*SS*gusA*10 een effect kan

hebben op het ecologisch gedrag van een stam, maar dat stammen kunnen worden verkregen, die op eenzelfde manier als de oorspronkelijke stam reageren. Een eerste selectie van gemerkte stammen is nodig door de gemerkte stam in gelijke hoeveelheden met de oorspronkelijke stam te mengen in het inoculum om te bevestigen dat het aantal blauwe knollen niet significant verschilt van de verwachtte 50 %. In hoofdstuk 3 wordt verder de detectie van meerdere stammen in één wortelknol besproken. Aangetoond werd dat gedeeltelijk gekleurde knollen alleen voorkwamen bij enten met een mengsel van stammen, en niet wanneer planten met één bacterie-stam werden geïnoculeerd, hetgeen wordt verklaard door dubbelinfecties. Dit werd bevestigd door isolaties van bacteriën uit de knollen. Gedeeltelijk gekleurde knollen werden eerder waargenomen door Krishnan en Pueppke (1992) bij inoculatie experimenten met een *lacZ*-gemerkte en een niet gemerkte *R. fredii* stam . Het gebruik van *lacZ* heeft echter een belangrijk nadeel vanwege de hoge achtergrond activiteit in het bacterie- en het plantenweefsel, in tegenstelling tot het gebruik van het *gusA* merker gen.

In hoofdstuk 4 wordt een nieuw merker systeem geïntroduceerd, gebaseerd op het *celB* gen. Dit merker gen werd geïsoleerd uit de hyperthermofiele Archaea *Pyrococcus furiosus* en codeert voor een thermostabiel ß-glucosidase met een hoge ß-galactosidase activiteit (Voorhorst et al., 1995). Laatstgenoemde enzymactiviteit kan worden gebruikt voor de detectie van rhizobia omdat endogene achtergrondactiviteit zowel in het plantenweefsel als ook in de bacteriën kan worden geëlimineerd door een warmtebehandeling. Bovendien zijn er goedkope histochemische substraten beschikbaar om ß-galactosidase activiteit te bepalen. Het *E. coli* ß-galactosidase gen, *lacZ*, is eerder gebruikt om genetisch veranderde bodembacteriën te monitoren in het milieu (Drahos et al., 1986; Hartel et al., 1994), maar dit bleek alleen geschikt bij gebruik van Lac$^-$ bacteriën. Transposons met het *celB* gen werden geconstrueerd in *E. coli*, gebaseerd op de aanwezige *gusA* transposons. Het eerste transposon, mTn*5*SS*celB*10 met de *tacI*^{q} gen-produkt is gebruikt om metabolische stress veroorzaakt door de aanwezigheid van de merker gen activiteit te onderdrukken. Het transposon mTn*5*SS*celB*31 met het *celB* met een *nifH* promoter uit een *Bradyrhizobium* stam, is alleen actief in de stikstofbindende wortelknol. Een derde *celB* minitransposon, die het merker gen constitutief bepaalt, is elders beschreven (Sessitsch et al., submitted). Het *celB* merker gensysteem heeft verschillende voordelen boven conventionele technieken in competitiestudies met rhizobia omdat de bepaling eenvoudig uit te voeren is en de histochemische substraten goedkoop zijn. Het grootste voordeel is echter dat *gusA* en *celB* gemerkte stammen gelijktijdig kunnen worden gelokaliseerd. Hierdoor is het mogelijk de competitie tussen multi-stam inocula met autochtone rhizobium populaties te bestuderen. Hoewel de simultane detectie van verschillend gemerkte stammen eerder is gerapporteerd (Thompson et al., 1995; Bauchrowitz et al., 1996), blijkt het *celB* gen

dat voor een thermostabiele merker codeert een betere kandidaat voor dubbel-merken. Een protocol is opgesteld voor detectie van *gusA* en *celB* gemerkte stammen op platen (Sessitsch et al., submitted). In hoofdstuk 4 wordt de toepassing van het gebruik van het *celB* gen als merker gen bij *Rhizobium* aangetoond. Gezien de brede waardplant range van het Tn5-transposon en de aanwezige expressie-signalen is dit merker systeem bruikbaar voor een aantal Gram-negatieve bacteriën.

De voordelen van de verschillende *gusA* en *celB* merker gen cassettes worden bediscussieerd in hoofdstuk 1, maar ook andere reporter genen en hun toepassing in de microbiële ecologie worden besproken. Bovendien wordt de ontwikkeling van een GUS Gene Marking Kit gerapporteerd. Deze kit werd gemaakt voor agronomen en microbiologen in ontwikkelingslanden die niet bekend zijn met moleculaire technieken en die geen mogelijkheid hebben deze methodologie in hun laboratorium op te zetten. Er is tevens een CelB Gene Marking Kit beschikbaar dat kan worden gebruikt in combinatie met of in plaats van het GUS Gene Marking Kit (FAO/IAEA, 1992-1997).

Ecologie van rhizobia die wortelknollen vormen bij Phaseolus vulgaris

Hoewel er gedurende de laatste tientallen jaren geen *Phaseolus*-bonen gekweekt waren in het veld rond het FAO/IAEA laboratorium in Seibersdorf, Oostenrijk, blijken *Phaseolus*-bonen in deze grond altijd goed genoduleerd. In eerdere studies werd aangetoond dat de autochtone *Rhizobium* populaties zeer competitief blijken (zie hoofdstuk 1). Wanneer bonen (*Phaseolus vulgaris*) worden geïnoculeerd met *Rhizobium tropici* stam CIAT899 (10^5 cellen per zaad) blijkt geen van de wortelknollen door deze stam gevormd te zijn. Zelfs bij verhoging van het inoculum tot 10^8 cellen per zaad blijkt nog 65% van de knollen door de autochtone stammen uit de grond gevormd te zijn. In hoofdstuk 5 worden de *Rhizobium* stammen gekarakteriseerd die werden geïsoleerd uit wortelknollen van planten gegroeid in grond uit Seibersdorf. Hiertoe werden moleculaire technieken gebruikt voor het gehele genoom als target, zoals PCR met repetitieve primers, en voor specifieke chromosomale loci, zoals het 16S rRNA gen of de spacer regio tussen het 16S en het 23S rDNA. Plasmiden profielen en Southern hybridizatie met een *nifH* probe gaven informatie over de symbiontische eigenschappen. Bovendien werd de waardplant specificiteit bepaald. Twee groepen werden onderscheiden, één groep werd geklassificeerd als *R. etli* op grond van RFLP analyse van het 16S rRNA gen en de aanwezigheid van drie kopieën van het *nifH* gen. De vertegenwoordigers van de tweede groep leken op geen van de op *Phaseolus* nodulerende *Rhizobium* soorten (*R. leguminosarum* bv. phaseoli, *R. etli* en *R. tropici*), maar vertoonden grote gelijkenis met een *Rhizobium* sp. (*Phaseolus*) stam R602sp die in

Frankrijk werd geïsoleerd (Laguerre et al., 1993). Isolaten van deze groep vormen eveneens wortelknollen bij *Vigna*, *Leucaena* en *Gliricidia* spp. Sinds lang werd aangenomen dat *R. leguminosarum* bv. phaseoli de enige microsymbiont van *Phaseolus* knolletjes in Europa was, maar recentelijk werden *R. tropici* en twee nieuwe soorten in Franse gronden gevonden (Laguerre et al., 1993; Amarger, 1994). Deze studies en de resultaten beschreven in hoofdstuk 5 wijzen er op dat stammen die uit Midden Amerika afkomstig zijn, zich goed in Europese grond kunnen vestigen. Echter de diversiteit van de isolaten die in Oostenrijk werden gevonden was gering, hetgeen veroorzaakt kan worden door de afwezigheid van de waardplant in deze gronden.

Hoofdstuk 6 is geconcentreerd op de taxonomie en fylogenie van de Oostenrijkse isolaten die grote gelijkenis vertonen met *Rhizobium* sp. R602sp. In deze studie werd tevens isolaat FL27, afkomstig uit Mexicaanse *Phaseolus* knollen, betrokken, omdat Laguerre et al. (1993) had gevonden dat de sequentie van een deel van het 16S rRNA gen van R602sp identiek bleek aan die van FL27. Sequentie analyse van het 16S rRNA gen, bepaling van het aantal kopieën en de heterogeniteit van de ribosomale genen, plasmiden profielen en DNA-DNA hybridizatie resulteerde in waardevolle taxonomische informatie over deze stammen. Op grond van deze resultaten is voorgesteld de stammen te groeperen in een nieuwe soort, *R. pueblae* sp. nov., refererend naar de staat Puebla, Mexico, waar FL27 was geïsoleerd. De Mexicaanse, Franse en Oostenrijkse isolaten vertonen grote overeenkomst in de 16S rDNA sequentie, met maximaal twee nucleotiden verschil. Vergelijking van de 16S rDNA sequenties met die van andere bacteriën vertoonde de grootste gelijkenis met *R. leguminosarum* stam IAM12609, *R.* sp. OK50 en met *R. etli*. Hoewel fylogenetische dendrogrammen de *R. pueblae* sp. nov. stammen altijd in de omgeving van de bovengenoemde soorten plaatsten, bleek de nieuwe soort te behoren tot een tak die verschillend is van de beschreven *Rhizobium* soorten. De totale DNA verwantschap tussen de Europese isolaten was erg groot maar vertoonde lagere waarden met FL27, hetgeen mogelijk wordt verklaard door de aanwezigheid van verschillende plasmiden. DNA homologie met andere *Phaseolus* nodulerende soorten was erg laag. *R. pueblae* sp. nov. stammen bezitten tenminste drie kopieën van het 16S rRNA gen en de ribosomale genorganisatie is verschillend van andere soorten. Ondanks de hoge competitieve eigenschap van sommige stammen in de grond in Seibersdorf, blijft de landbouwkundige kennis van deze soorten nog grotendeels onbekend.

Conclusies

Verbetering van de biologische stikstofbinding vereist aangepaste efficiënt stikstofbindende planten genotypen, effectieve rhizobia als entstof, en geschikte landbouwkundige management praktijken. Dit doel kan alleen bereikt worden wanneer plantenveredelaars, microbiologen, bodemkundigen, agronomen en boeren samenwerken.

De selectie van superieure bronnen van natuurlijke genetische variatie en plantenveredeling in aanwezigheid van rhizobia in plaats van gebruik van stikstofkunstmest, kan leiden tot het vinden van lijnen met een hoge stikstofbindings activiteit. Andere gewenste eigenschappen, zoals ziekteresistentie of stress tolerantie kunnen worden overgedragen via geschikte veredelings methoden of door genetic engineering. Gronden, waarin leguminosen worden gekweekt, variëren onderling sterk in pH en temperatuur. In het verleden is veel aandacht besteed aan het ontwikkelen van geschikte plantengenotypen, terwijl stresstolerantie van inoculum-stammen buiten beschouwing bleef. Inoculum stammen worden aanbevolen op grond van goede symbiontische eigenschappen in een bepaald milieu, terwijl de bodemgesteldheid of de agro-ecologische zone van de uiteindelijke toepassing niet meegerekend worden. Onderzoek heeft uitgewezen dat er een sterke correlatie bestaat tussen de grootte van de autochtone populaties en het wortelknolvormend vermogen van de inoculum stam (Thies et al., 1991). Echter het effect van de diversiteit van de autochtone rhizobia op de competitie is niet bepaald. Waarschijnlijk zijn er verschillende strategieën nodig om nodulatie door de sterk dominerende veldisolaten te onderdrukken. Bovendien dienen er inoculum praktijken ontwikkeld te worden, die gemakkelijk door de boer toegepast kunnen worden en die verspreiding van de geïntroduceerde stam over het gehele wortelstelsel mogelijk maken.

Het lijkt erop dat de verschillende aspecten die van belang zijn voor een efficiënte wortelknolvorming en stikstofbinding onvoldoende aan elkaar zijn gekoppeld. Een meer rationele selectie van efficiënte en competitieve stammen zou kunnen worden gerealiseerd wanneer er een databank komt met gegevens over bodemkarakteristieken, milieucondities, diversiteit van rhizobia en populatie grootte, alsmede de competitieve eigenschappen en effectiviteit van de stammen in combinatie met bepaalde planten genotypen. Een dergelijke databank zou ook bruikbaar zijn voor de ontwikkeling van nieuwe stammen of planten via genetic engineering.

In dit proefschrift wordt de ontwikkeling van nieuwe methodieken gepresenteerd om het competitief vermogen van rhizobia te bepalen. Deze op merker gen gebaseerde technieken zijn geschikt voor het grootschalig screenen van inoculum stammen maar kan ook worden gebruikt voor de genetische analyse van een aantal Gram-negatieve

bacteriën. Het gebruik van moleculaire methodieken hebben de analyse van *Rhizobium* stammen die noduleren op *Phaseolus* vereenvoudigd en heeft geleid tot de beschrijving van een nieuwe *Rhizobium* soort die stammen bevat met mogelijke gunstige eigenschappen.

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Curriculum vitae

The author of this thesis was born on the 1st of June 1964 in Graz, Austria. She studied Technical Chemistry at the University of Technology, Graz, and specialized in Biochemistry and Biotechnology. She obtained the M.Sc. in 1990 and her thesis was on "Evaluating the efficiency of a hydrobotanical sewage plant by chemical and microbiological parameters after seven years of operation". She joined 1990 the Soil Science Unit at the FAO/IAEA Agriculture and Biotechnology Laboratory as a technician and obtained in 1994 an Associate Professional Officer position. During this employment she was responsible for the development of molecular microbial identification techniques and their application in agriculture. An additional task was technology transfer. In 1992 she started a Ph.D. at the Department of Microbiology, Wageningen Agricultural University, The Netherlands, under the supervision of Prof. de Vos and Dr. Akkermans. The results of this Ph.D. study which was completed in 1997, are presented in this thesis.

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