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Rhizobium tropici teu genes involved in specific uptake of *Phaseolus vulgaris* bean-exudate compounds

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Abstract *Rhizobium tropici* nodulates and fixes nitrogen in bean. In the *R. tropici* strain CFN299 we identified and characterized *teu* genes (*tropici e*xudate *u*ptake) induced by bean root exudates, localized by insertion of a promoter-less Tn*5-gusA1* transposon. *teu* genes are present on a plasmid of around 185 kb that is conserved in all *R. tropici* strains. Proteins encoded by *teu* genes show similarity to ABC transporters, specifically to ribose transport proteins. No induction of the *teu* genes was obtained by treatment with root exudates from any of several other plants tested, with the exception of *Macroptilium atropurpureum*, which is also a host plant for *R. tropici*. It appears that the inducing compound is characteristic of bean and closely related legumes. It is present in root exudates, but not in seeds. This compound is removed, presumably by metabolism, from the exudates by the majority of bean-nodulating rhizobia (such as *R. etli*, *R. leguminosarum* bv. *phaseoli* and *R. giardinii*). The principal inducing compound has not been identified, but some induction was obtained using trigonelline. The CFN299 strain seems to have an additional uptake system, as no phenotype is observed in two different mutants. *R. tropici* strain CIAT899, on the other hand, must have only one uptake system, since a mutant bearing an insertion in the *teu* genes could not remove the compound from the exudates as efficiently as the wild type, and it showed diminished nodulation competitiveness.

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Introduction

The capacity to use a variety of carbon sources seems to be advantageous for soil bacteria. A number of sugars, as well as flavonoids, amino acids and vitamins are exuded into the rhizosphere by plants (Phillips et al. 1994; Rovira 1969). In some cases, the production of a specific carbon source by a plant may favor the growth of a particular bacterial population. For example, homoserine secreted by pea plants is assimilated by the pea symbiont *R. leguminosarum* bv. *viciae* (Van Egeraat 1975; Johnston et al. 1988; Hynes and O'Connell 1990). Mimosine, found in *Leucaena* plants, is a carbon and nitrogen source for around 40% of Rhizobium isolates from *Leucaena* (Soedarjo et al. 1994). *Agrobacterium* spp. transform plant cells to make them produce unusual amino acid derivatives, opines, that can be metabolized specifically by strains of *A. tumefaciens* (Scott et al. 1979). Similarly, rhizopines synthesized in nodules are catabolized by other rhizobia in the soil. Rhizopines are usable only by a limited number of *R. meliloti* and *R. leguminosarum* bv. *viceae* strains. It has been suggested that rhizopines could provide a new strategy for increasing the competitiveness and persistence of rhizobia in the soil, if the catabolic genes were transferred to the desired bacteria and the biosynthetic genes to the plant (Rossbach et al. 1995).

In *R. leguminosarum* and *R. etli*, the ability to utilize different carbon sources, such as adonitol, arabinose, catechol, glycerol, inositol, lactose, malate, rhamnose, sorbitol, dulcitol and melobiose, is encoded on different plasmids, and these genes could contribute to the symbiotic and saprophytic competence of rhizobia (Baldani et al. 1992; Brom et al. unpublished; Oresnik et al. unpublished). Sugar transporters belonging to the ABC superfamily are encoded on the symbiotic plasmid of NGR234 (Freiberg et al. 1997). Charles and Finan

(1991) have also shown that the *R. meliloti* megaplasmid pRmeSU47b carries genes involved in dulcitol, lactose, raffinose, melibiose, quinate and protocatechuate catabolism, so it appears that the presence of a wide variety of plasmid-encoded transport and catabolic genes may be a general feature of the fast-growing rhizobia.

The symbiotic capacity of *Rhizobium* is determined largely by *nod*, *nif* and *fix* genes, which are generally located on plasmids (Kondorosi et al. 1984). The expression of *nod* genes is inducible by plant-exuded flavonoids (reviewed by Phillips et al. 1994) or, in some cases, betaines (Phillips et al. 1992), and this constitutes one of the first steps in plant-*Rhizobium* signal exchange (Long 1989). *Rhizobium nod* genes are involved in the synthesis of Nod factors (lipochitooligosaccharides) that are triggers of the nodulation process (reviewed in Dénarié et al. 1992 and Schultze and Kondorosi 1996).

Other *Rhizobium* loci related to nodulation but not involved in Nod factor production have been described (Phillips et al. 1996; Freiberg et al. 1997) and recently reviewed (Pueppke 1996; Vlassak and Vanderleyden 1997). Some of these may act to ensure the survival of the bacteria in the rhizosphere or in other signaling processes between bacteria and plants that determine the colonization and infection of the root.

We have studied different *Rhizobium* populations that establish symbioses with *Phaseolus vulgaris* bean (Martínez et al. 1985; Martínez-Romero 1994; Piñero et al. 1988). *R. tropici*, one of the bean symbionts (Martínez-Romero et al. 1991), is capable of nodulating and fixing nitrogen in bean and in other legumes such as *Leucaena* and *Macroptilium* (Hernández-Lucas et al. 1995; Mavingui et al. 1997). *R. tropici* was originally isolated from acid tropical soils in South America, where it is successfully used as an inoculant for bean crops. Based on genetic and phenotypic characteristics, two types have been recognized among *R. tropici* strains. Other bean-nodulating species are *R. etli* (Segovia et al. 1993), *R. leguminosarum* bv. *phaseoli*, *R. gallicum* and *R. giardinii* (Amarger et al. 1997; Sessitsch et al. 1997).

With the aim of identifying and characterizing new *R. tropici* functions involved in the early interaction with the plant, we searched for genes that are inducible by bean exudates in a library of *R. tropici* CFN299 mutants obtained by the insertion of a promoter-less Tn*5-gusA1* transposon. We analyzed one of these mutants which is affected in the *teu* genes, which appear to be inducible by bean-exudated sugars.

Materials and methods

Bacterial strains, plasmids and media

Bacterial strains and plasmids are listed in Table 1. *Rhizobium* strains were grown in PY medium (per liter: 5 g peptone, 3 g yeast extract, and 1 g $CaCl_2 \cdot 2H_2O$). *E. coli* and *Agrobacterium* strains were grown in Luria-Bertani (LB) medium (Miller 1972). When required, antibiotics were used at the following concentrations for *Rhizobium tropici* CFN299: kanamycin, 25 mg/l; nalidixic acid, 10 mg/l; for CIAT899: kanamycin, 40 mg/l; nalidixic acid, 20 mg/l; tetracycline, 10 mg/l; for *E. coli*: ampicillin, 100 mg/l; tetracycline, 10 mg/l; gentamicin, 30 mg/l; for *A. tumefaciens*: rifampicin 100 mg/l.

Nodulation assays

Nodulation assays in *P. vulgaris* plants were performed as described (Martínez-Romero and Rosenblueth 1990) and followed up to 15 or 25 days after inoculation. Thin slices of nodules were stained and observed in the microscope as described (Martínez et al. 1987).

Genetic manipulations and hybridization

CFN299 derivatives carrying Tn*5-gusA1* were obtained from matings between strains pSB387 and CFN299. The transconjugants were selected for nalidixic acid and kanamycin resistance. Transfer frequencies were estimated with reference to the recipient cell number. Genomic DNA was isolated, digested with various restriction enzymes, electrophoresed in 1% agarose gels, blotted onto nylon membranes and hybridized with a random-primer labelled probe (Rediprime and Rapid-hyb buffer from Amersham Life Science, Little Chalfont, Bucks., England) under conditions of moderately high stringency (hybridization at 65° C, and washing at 55° C). Plasmid patterns were visualized by the Eckhardt (1978) technique, as modified by Hynes and McGregor (1990), blotted into nylon membranes and hybridized. To obtain the mutants carrying a *lacZ* cassette inserted in *teu* genes, pMR16-3 (an *Xba*I-*Xho*I subclone from pMR16) was inserted in the suicide plasmid pJQ200SK. An interposon containing the promoter-less structural gene for β-galactosidase and a kanamycin resistance gene (pKOK6) was inserted into one of the *teuB* internal *Bgl*II sites of pMR16-3 (Fig. 1). CFN299-191 and CIAT899-191 were obtained as double recombinants. The localization and orientation of the insert was checked by hybridization using as a probe an internal DNA fragment and the *lacZ* cassette.

DNA manipulations and sequencing

A clone (pcosMR11) carrying the entire *teu* operon was obtained by hybridizing a 2.6-kb *EcoR*I-*Hind*III fragment (pMR19) to a cosmid library of total DNA from CFN299 in pSUP205 (Laeremans et al. 1996). Two *EcoRV* fragments from this cosmid were cloned into the Bluescript II SK(−) vector (Stratagene, La Jolla, Calif.) and transformed into *E. coli* DH5α, yielding pMR16 and pMR34 (Fig. 1). For sequencing, smaller subclones were obtained by recloning with a variety of enzymes into the same vector. Double-stranded DNA was isolated with the Wizard Miniprep DNA purification system (Promega, Madison, Wis.). Universal oligonucleotide primers and some synthesized oligonucleotides were used with an automatic 373 A DNA Sequencing System (Applied Biosystems, Foster City, Calif.). DNA was sequenced at least twice on both strands.

Sequence analysis

Identification of ORFs and motifs was performed with the GCG package (Gelassemble, Map and Motifs programs). Sequence similarities were searched with the BLAST network service from GCG (in the databases of the National Center for Biotechnology Information, Bethesda, Md.), using the peptide sequence databases of nr (non-redundant GenBank CDS translations + PDB + Swiss Prot + PIR). To predict intracellular location and transmembrane regions of predicted proteins, the programs Psort (Nakai and Kanehisa 1991) and TMpred were used (Hofmann and Stoffel 1993). Hydropathy profiles were performed also with TMpred and the Gene Works package (Kyte and Doolittle 1982), with a window

Table 1 Strains and plasmids used in this study

Strain	Relevant characteristics	Reference/source
Rhizobium tropici		
CFN299	Type A, wild type	CFN[a]
CFN299-19	CFN299 *teuC1*::Tn*5-gusA1*, Km^r	This study
CFN299-191	CFN299 *teuB*::*lacZ*, Km^r	This study
CFN299 pb^-	CFN299 cured of pb	This study
BR864	Type B, wild type	CFN[a]
CIAT899	Type B, wild type	Graham et al. (1982)
CIAT899-191	CIAT899 *teuB*::*lacZ*, Km^r	This study
Rhizobium etli		
CFN42	Wild type	CFN[a]
CFNX184	CFN42 cured of pc	Brom et al. (1992)
CFNX89	CFN42 cured of pd	Brom et al. (1992)
BRA-5	Wild type	CFN[a]
F8	Wild type	CFN[a]
Rhizobium leguminosarum bv. *phaseoli*		
4292	8002, Rif^r	Lamb et al. (1982)
8401	8002, Str^r cured of pSym	Lamb et al. (1982)
Rhizobium leguminosarum bv. *viciae*		
VF39SM	VF39, Sm^r	Priefer (1989)
Rhizobium leguminosarum bv. *trifolii*		
ANU 843	Wild type	B. G. Rolfe[b]
W14-2	Wild type	Baldani et al. (1992)
Rhizobium giardinii bv. *phaseoli*		
Ro84	Wild type	Amarger et al. (1997)
bv. *giardinii*		
H152	Wild type	Amarger et al. (1997)
Rhizobium gallicum bv. *gallicum*		
FL27	Wild type	Piñero et al. (1988) Sessitsch et al. (1997)
Rhizobium spp		
Cli80	Wild type	CFN[a]
CFN234	Wild type	CFN[a]
CFN265	Wild type	CFN[a]
Rhizobium meliloti		
2011	Wild type	Casse et al. (1979)
Rm41	Wild type	Bánfalvi et al. (1981)
Rhizobium sp.		
NGR234	Wild type	Trinick (1980)
Agrobacterium spp		
KAg3	Wild type	Sawada and Ieki (1992)
ChAg-4	Wild type	Sawada and Ieki (1992)
Agrobacterium tumefaciens		
GMI9023	C-58 cured of its native plasmids	Rosenberg and Huguet (1984)
Apa	GMI9023 harboring pRtrCFN299a	This study
Apb	GMI9023 harboring pRtrCFN299b::Tn*5-mob*, Km^r	CFN[a]
Apc	GMI9023 harboring pRtrCFN299c::Tn*5-mob*, Km^r	CFN[a]
Apa19	GMI9023 harboring pRtrCFN299-19a, Km^r	This study
Apa191	GMI9023 harboring pRtrCFN299-191a, Km^r	This study
Escherichia coli		
DH5α	*recA*$^-$, Nal^r	Bethesda Research Laboratories
DH5α (pcosMR11)	DH5α harboring pcosMR11	This study
Plasmids		
pUC18	Cloning vector Ap^r	Norrander et al. (1983)
pJQ200SK	*B. subtilis sacB* containing suicide vector, Gm^r	Quandt and Hynes (1993)
pRK2013	Helper plasmid for triparental mating, Km^r	Figurski and Helinski (1979)
pSB387	pRK606Ω::Tn*5-gusA1*, Km^r Tc^r	Sharma and Signer (1990)

Table 1 (contd.)

Strain	Relevant characteristics	Reference/source
pMR19	2.6-kb *EcoR*I-*Hin*dIII insert derived from *EcoR*I fragment cloned from CFN299-19 in pUC18, Ap^r (*Hin*dIII site is from Tn*5-gusA1*)	This study
pcosMR11	40-kb cosmid from a library in pSUP205 of total DNA from CFN299 containing the wild type *teu* locus, Tc^r	This study
pMR16	4.0-kb *EcoR*V fragment of pcosMR11 in pBluescript II Sk^-, Cb^r	This study
pMR16-3	2.65-kb *Xba*I-*Xho*I fragment of pMR16 in pBluescript II SK^-, Cb^r (*Xba*I site is from the vector)	This study
pMR34	5.2-kb *EcoR*V fragment of pcosMR11 in pBluescript II SK^-, Cb^r	This study
pKOK6	*lacZ*-Km^r cassette in pKOK4, Km^r, Cb^r	Kokotek and Lotz (1989)

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of 20 amino acids. Identification of promoter regions was done using Promoter Prediction by Neural Network (Reese et al. 1996).

Induction of gene expression by plant exudates

Induction studies were done with seed and root exudates from bean (*P. vulgaris* cv. Negro Xamapa, N-8-116, Peruano, Bronco, L-3-1-1-1, Bat 47 and wild type), *Clitoria ternatea*, *Leucaena leucocephala*, *Macroptilium atropurpureum*, soybean (*Glycine max* cv. Peking), alfalfa (*Medicago sativa* cv. Valenciana), pea (*Pisum sativum* cv. Trapper), and maize (*Zea mays* cv. Piedra blanca). All seeds were sterilized for 1 min in ethanol and 15 min in 1.25% (w/v) sodium hypochlorite, and rinsed five times with sterile water. To obtain seed exudates, the seeds were imbibed in water for 6 h in darkness at 28° C and drained. The amount of water used was four times the volume of the seeds for the smaller seeds (wild-type bean, *L. leucocephala*, *M. atropurpureum*, soybean and alfalfa) and two seeds per ml of water for the large ones (bean, *C. ternatea*, pea and maize). Root exudates were obtained from plants grown for 5 days in Fahraeus (1957) solution without nitrogen. Three milliliters per plant (or 1.5 ml in the case of alfalfa) of exudate were obtained. Exudates were tested for the presence of bacteria or fungi by plating on PY medium. The exudates were kept at −20° C, if not used immediately.

Bean nodule extracts from plants nodulated by CFN299 were obtained 15 days after inoculation. The nodules were sterilized for 2 min in 1.25% (w/v) sodium hypochlorite and rinsed five times with sterile water. Ten nodules of average size were crushed per ml of water and filtered through Millipore 0.45 μm filters.

β-Glucuronidase (GUS) activity was detected with X-Gluc or with MUG as described (Gallagher 1992; Jefferson 1987). For the first screening, in order to identify genes inducible by bean exudates, all mutants were grown overnight in minimal medium (MM) (0.05% K_2HPO_4, 0.95% KH_2PO_4, 0.02% $MgSO_4 \cdot 7H_2O$, 0.02% $CaCl_2$, 0.001% ferric citrate, 0.2% glutamic acid, 10 mM mannitol,

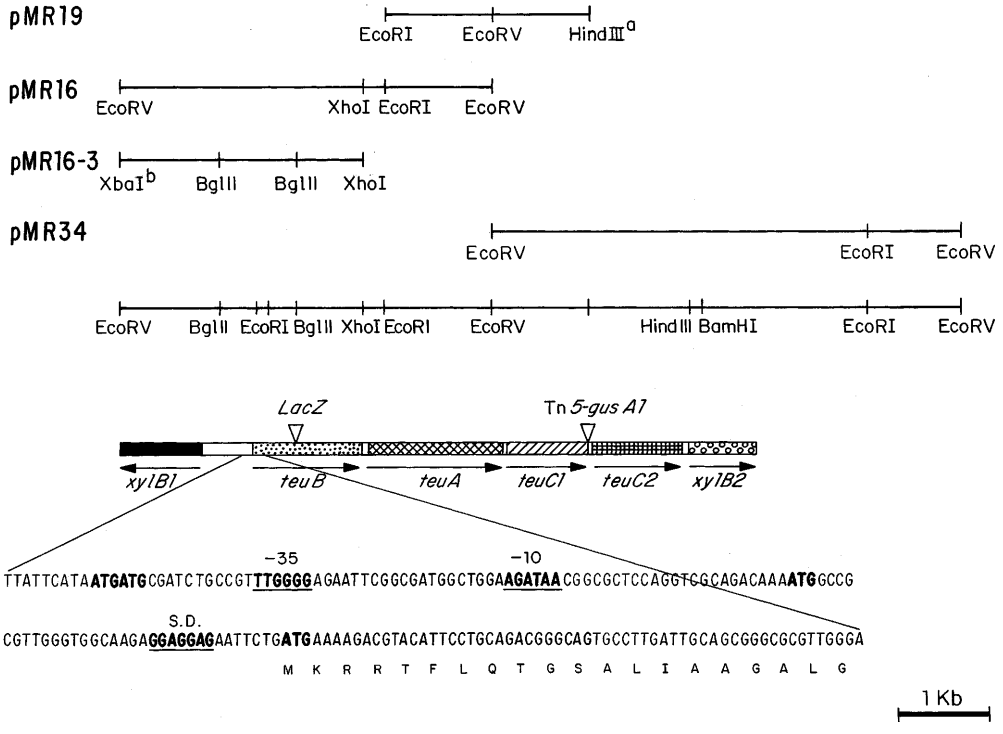

Fig. 1 Diagram of the organization of the *teu* operon and adjacent genes. Some restriction sites are indicated and fragments contained in clones pMR19, pMR16, pMR16-3, and pMR34 are shown. *Hin*dIII[a] is from Tn*5-gusA*; *Xba*I[b] is from the pSK(−) vector. The *vertical arrowheads* indicate insertion sites in the independent mutants obtained. The upstream regulatory sequence is shown at the bottom, including different possible ATG initiation sites, −35 and −10 motifs and a putative Shine-Dalgarno sequence. The *arrows* show the orientation of transcription

and Fahraeus trace minerals) and diluted 1:1 with bean-root exudates. GUS activity was detected with X-Gluc using 50 µl of each culture.

Other assays were performed with CFN299-19 cultures (inoculated at a concentration of 0.2 OD600 units) and grown for 3, 5 and 20 h in MM plus exudates or nodule extracts, using MUG as a substrate. GUS activity in the nodules was observed by incubating sterilized nodules with X-Gluc or with MUG in the presence of unfiltered bean-nodule extracts.

Induction of gene expression by different compounds

Induction of CFN299-19 (measured using MUG) was tested in microplates (Biolog, Haywood, Calif.) which contained 95 different carbon sources. Compounds which gave detectable fluorescence were further tested at 1, 10, 20 and 50 mM, along with other available compounds, performing the induction assays after 3 h of growth (initial OD of 0.2) in liquid MM without mannitol. The compounds tested were the following: α-D-glucose, maltose, D-fructose, α-D-lactose, ribose, sucrose, L-arabinose, melibiose, D-raffinose, L-rhamnose, stachyose, xylose, uridine, inosine, glucosamine hydrochloride, N-acetyl-D-glucosamine, D-galactose, galactosamine hydrochloride, N-acetyl-D-galactosamine, N-N-diacetylchitobiose, N-N-N-triacetylchitotriose, trigonelline, carnitine, stachydrine, polygalacturonic acid, nicotinic acid, D-galacturonic acid, D-mannitol, adonitol, erythritol, myo-inositol, xylitol, dulcitol, D-sorbitol, trehalose, and 2-amino ethanol. The following flavonoids were also tested for induction activity: naringenin, genistein, apigenin, quercetin and luteolin. The induction assays with exudates and carbon compounds were repeated at least five times, and a single representative experiment is reported.

HPLC analysis

The polar fraction of bean root exudates was separated on an HPLC system equipped first with a reverse-phase C-18 column following the conditions used by Hungria et al. (1991). Compounds were also separated on the basis of molecular weight on a Waters Ultrahydrogel 120 column (Millipore, Milford, Mass.), eluting with water at 1.125 ml/min and monitoring at 195 nm.

Results

Selection of Tn*5-gusA1* mutants

Out of 2000 Tn*5-gusA1 R. tropici* mutants, 270 were selected because they had insertions in plasmids pa, pb or pc. These mutants were identified by their ability to transfer kanamycin resistance to *A. tumefaciens* recipient strains in plate matings. When these mutants were assayed for the expression of β-glucuronidase, we found eight that expressed GUS activity in the presence of bean root exudates.

Single Tn*5-gusA1* insertions were present in each mutant. Insertions were either located on the Sym plasmid (pc) (in three mutants) or on a smaller plasmid (pa) (in five mutants). *E. coli* clones containing pUC18 with *Eco*RI DNA fragments derived from the CFN299 mutants were selected by their resistance to kanamycin. The sequence analysis, starting from a primer within Tn*5*, showed that an insertion on the Sym plasmid was in the *nodB* gene of *R. tropici* (not shown). Other sequences, with the exception of pMR19 (derived from CFN299-19) were not clearly related to known genes. Due to the instability of the Tn*5-gusA1* inserts in the majority of the cases, we were unable to recover the original mutants from which the mutations had been cloned. By hybridizing with Tn*5* as a probe, we found a very high frequency of rearrangements of the Tn*5-gusA1* during subculture of the mutant strains (not shown). We also became aware that the version of Tn*5-gusA1* that we were using has been observed to be unstable in other laboratories. Nonetheless, we decided to continue the work with CFN299-19 since it proved to be very stable, even after 4 years of repeated subculturing. The 2.6-kb *Eco*RI-*Hin*dIII fragment in pUC18, pMR19 (Fig. 1), was used as a probe to select a cosmid clone (pcosMR11) carrying the wild-type locus from a library of CFN299. The location of the cloned fragment is on pRTrCFN299a, a 185-kb plasmid that is conserved in the two *R. tropici* types (Fig. 2). This plasmid is autotransferable to *A. tumefaciens* at very high frequencies (around 1×10^{-1}).

DNA sequence analysis

A 6936-bp DNA region flanking the site of insertion of Tn*5-gusA1* in the original mutant CFN299-19 was completely sequenced. The sequence has been deposited in GenBank under accession number AF036920. The transposon insertion in CFN299-19 is in a genetic region whose deduced protein products show similarity to ABC transporters, specially with sugar transport proteins from a family which includes Ara (arabinose), Mgl (galactose), and Rbs (ribose) proteins (Table 2). The best homology was with Rbs proteins from *E. coli*.

The predicted products of the ORFs found are the following. The first, TeuB (362 amino acids, encoded from nucleotides 1556–2641) has homology, albeit low (60% at the C-terminal end), to RbsB, the periplasmic protein precursor (Table 2). The second, TeuA (508 amino acids, encoded from nucleotides 2700–4223),

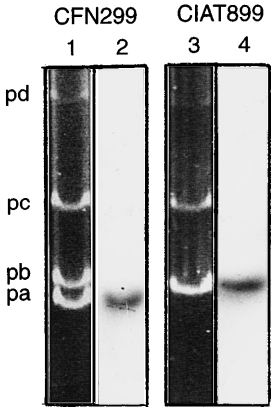

Fig. 2 Ethidium bromide-stained plasmid profiles (odd-numbered lanes) and Southern blots of each plasmid profile hybridized to pMR19 containing an internal fragment of *teuA* and *teuC1* (even-numbered lanes). The *R. tropici* strain CFN299 has four plasmids, pd of 1500 kb, pc of 450 kb (pSym), pb of 220 kb and pa of 185 kb

Table 2 Comparisons of Teu and Xyl proteins with their closest relatives

Protein	Best score homology[a]	Identity[b]	Similarity[b]	Probability[c]	Motifs	Amino acids[d]
XylB1	XylB P39849 Aryl-alcohol dehydrogenase *P. putida*	163/299 (54%)	211/299 (70%)	2.2×10^{-114}		
TeuB	RbsB P02925 D-ribose binding periplasmic protein precursor *E. coli*	49/230 (21%)	102/230 (44%)	6.0×10^{-18}		
TeuA	RbsA P04983 Ribose transport ATP-binding protein *E. coli*	188/450 (42%)	283/450 (63%)	1.4×10^{-113}	ATP/GTP - A binding site ABC transporter	GENGAGKS (37–44) LSGGNQQKVLLCRWL(399–413)
TeuC1	RbsC P04984 Ribose transport system permease protein *E.coli*	92/237 (39%)	143/237 (60%)	2.7×10^{-50}	Hydrophobic membrane proteins; periplasmic permeases of the AraH family	LYLFASGGNDFNARMNGVPVERTVI KAYILSALFACAAGM (185–225)
TeuC2	RbsC P04984 Ribose transport system permease protein *E.coli*	72/244 (30%)	126/244 (52%)	1.1×10^{-28}	Hydrophobic membrane proteins; periplasmic permeases of the AraH family	RYFRAVGNNPRAAEITGIPLARTIFF SHTLAGTLTGIAAL (204–244)
XylB2	XylB P39849 Aryl-alcohol dehydrogenase *P. putida*	98/217 (45%)	140/217 (65%)	4.3×10^{-64}		

[a] Protein, data base accession number and source organism
[b] Number of amino acids with identity or similarity/total number of amino acids compared
[c] Probability that the scores have occurred by chance
[d] Amino acids contained in the motif and localization in the protein

shows the highest similarity to sugar transport ATP-binding proteins. TeuA should have two ATP-binding motifs, but with the program used only one was detected (Table 2). By direct inspection we found a sequence that may constitute the other ATP binding site, VFIFDE (163–168 amino acids) (Higgins 1992). The third and fourth proteins, TeuC1 and TeuC2 (313 and 332 amino acids, encoded from nucleotides 4236–5174 and 5183–6178, respectively), had homology to the permease protein (RbsC). The TeuC1 and TeuC2 have the conserved motif found in hydrophobic membrane proteins of the periplasmic permease type, being most similar to the AraH family (Table 2) (Saurin et al. 1994). TeuC1 and TeuC2 show only 25% amino acid identity (51% similarity).

The deduced proteins are predicted to have the same cellular location as the corresponding Rbs proteins (Bell et al. 1986). TeuB is, according to our analysis, a periplasmic protein with a cleavable N-terminal signal sequence which may interact with the compound that is transported. TeuA is in the cytoplasm and is bound to the inner membrane via one transmembrane segment. TeuC1 and TeuC2 are highly hydrophobic (as is RbsC), having eight and nine transmembrane segments in the inner membrane, respectively.

We found other two ORFs, one on each side of the four described above. Both of these had homology to *xylB* from *Pseudomonas putida*, but the one that is close to *teuB* is transcribed in the opposite direction relative to the other genes (Fig. 1 and Table 2). They were called *xylB1* and *xylB2* because of their high similarity to *xylB*. They were partially sequenced and compared, showing 61% homology over 609 nucleotides.

The intercistronic spaces between *xylB1*, *teuB*, *teuA*, *teuC1*, *teuC2*, and *xylB2* are 661, 58, 12, 8 and 50 bp long, respectively. Potential Shine-Dalgarno sequences (ribosome binding sites) were found preceding each of the genes. No transcriptional terminators were found in the 50 bp between *teuC2* and *xylB2*.

The probable translation start codon for the *teu* operon is at nucleotide 1556, since a Shine-Dalgarno sequence was found seven bases upstream. Other methionines were found in this region, but none of them is near a Shine-Dalgarno sequence (Fig. 1). A probable promoter region (−10 −35) was found 54 bases upstream of this ATG.

Under the hybridization conditions tested, no homology was found with *R. etli* strain CFN42 using as a probe the *Eco*RI-*Hin*dII insert from pMR19 (Fig. 1). This probe includes a large part of *teuA* and *teuC1*, which are the most conserved genes in the sugar transport operons.

Induction of *teu* gene expression by plant exudates

Reproducible GUS activity in CFN299-19 was detected on incubation with Negro Xamapa bean-root exudates. At least 20 independent assays were performed. The highest GUS activities (517-465 nM MU/min/10^6 cells) were obtained with CFN299-19 cultures at the beginning of the exponential phase (at 0.2–0.6 OD600) and lower inducibility (159 nM MU/min/10^6 cells) was observed with cultures in late exponential or stationary phase (at 1.8 OD600). Root-exudates obtained from a bean cultivar with a high capacity to fix nitrogen (N-8-116) or from light-seed beans, as well as from wild *P. vulgaris*, had an inducing capacity similar to that of Negro Xamapa. Induction was observed also with *Macroptilium atropurpureum* root exudate. There was also a slight induction with alfalfa root exudates (Fig. 3). No induction was observed with seed exudates. GUS activity was not observed in bacteroids treated with X-Gluc or MUG, or with extracts from bean nodules. To determine whether an inhibitor of gene expression was present in bean-seed exudates or in nodule extracts, they were mixed 1:1 with bean-root exudates. No inhibition was observed, compared to the control (water + bean-root exudates) (Fig. 3). No induction was observed with seed or root exudates from the other plants tested.

Utilization of the inducing compound

To test if the inducing compound was utilized by various *Rhizobium* species, including *R. tropici*, we incubated the bean-root exudate-medium with various strains (inoculated at an OD600 value of about 0.1) for 24 h. The exudate-containing media were filtered to remove the bacteria and tested for their inducing activity with CFN299-19. All the inducing compound was removed by *R. tropici* type A strain CFN299, type B strains CIAT899 and BR864, and by other bean-nodulating bacteria such as *R. etli* strains CFN42, Bra5 and F8; *R. leguminosarum* bv. *phaseoli* 4292 and *R. giardinii*

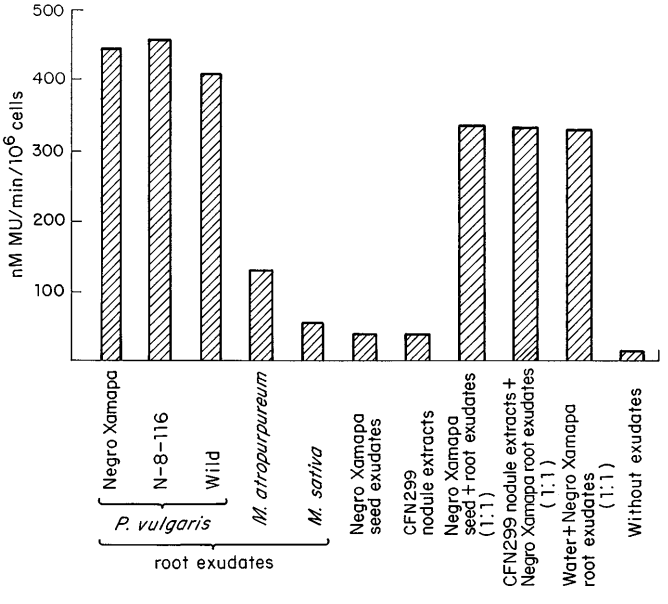

Fig. 3 β-Glucuronidase specific activities measured for CFN299-19 after incubation for 3 h in the presence of plant exudates or nodule extracts

strains H152 and Ro84 (Fig. 4). *A. tumefaciens* cells bearing the *R. tropici* plasmids pRtrCFN299a (Apa) or pRtrCFN299c (Apc) were also proficient at metabolising the inducer. The ability to use the inducer was dependent on the presence of pSym in the *R. leguminosarum* bv. *phaseoli* and in *R. etli* (Fig. 5). *E. coli* strain DH5α, *A. tumefaciens* strain GMI9023 and the *Agrobacterium* spp. strains KAg-3 and Chag-4 (closely related to *R. tropici*), *Rhizobium* spp. related to *R. etli* (Cli 80, CFN234, CFN 265), *R. gallicum* bv. *gallicum* strain FL27, *R. meliloti* strains 2011 and Rme41, *R. leguminosarum* bv. *viciae* strains 3841 and VF39SM, bv. *trifolii* strains ANU843 and W14-2, and *Rhizobium* sp. strain NGR234 were unable to use the compound and the exudate retained its inducing capacity (Fig. 4). *E. coli* strain DH5α acquires the capacity to use the inducing compound when it harbours the CFN299 cosmid with the *teu* genes (pcosMR11) (Fig. 5).

Induction of *teu* gene expresssion by various compounds

On the basis of the sequence similarity of the *teu* genes to sugar transport genes we examined whether the inducing activity in the exudate was attributable to a commercially available sugar. We tested galactose, ribose, and arabinose as first choices based on the gene sequence homologies and we also used trisaccharides and tetrasaccharides, such as raffinose and stachyose, that are common in bean plants. Out of 114 compounds that may be usable as carbon sources in bacteria, we found a group of sugars (inosine, uridine, galactosamine, glucosamine, N-acetyl galactosamine, N-acetyl glucosamine, α-cyclodextrin) and a few alcohols (2-aminoethanol and xylitol), that had a low to medium level of inducing capacity (around 30% of the bean-root exudate) but only when tested after bacteria had been incubated in their presence for 20 h. Only basal levels of induction were obtained with mannitol, which was used as a control, and with many other of the non-inducing sugars (Fig. 6). We tested whether combinations of the different sugars could enhance their effects but we found that this was not the case, and we never attained the levels of induction observed with bean-root exudates. Induction with the exudate always occurred faster than with any of the commercial compounds tested (Fig. 6). Trigonelline induction was clearly dependent on its concentration. Optimum induction was observed with 10^{-3} M trigonelline. CFN299 can grow well in 10^{-6} to 10^{-3} M trigonelline as the only carbon source, but cannot grow at 10^{-2} M (Fig. 7). With trigonelline we obtained 30% of the GUS activity as compared to the bean-root exudate when tested in 3-h induction reactions, but with a longer induction period, we found a level of activity similar to that seen with the bean-root exudate. This was partly because the β-glucuronidase activity detected with bean-root exudates declines after 3 h of induction (Fig. 6), perhaps implying that the inducing compound

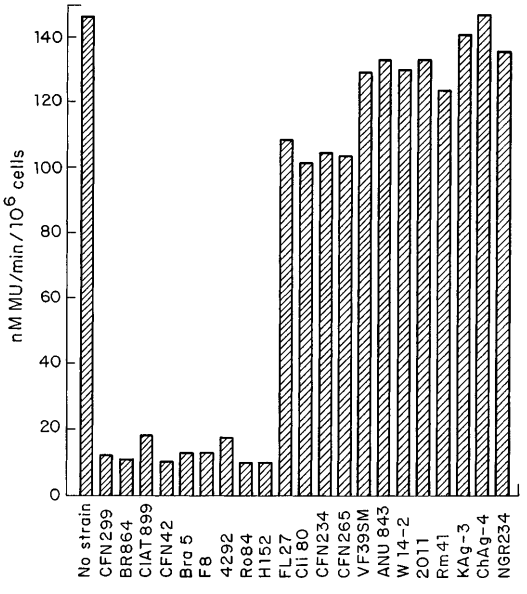

Fig. 4 β-Glucuronidase specific activities obtained from CFN299-19 induced by bean exudates (No strain) and by bean exudates pre-incubated with the *Rhizobium* and *Agrobacterium* strains indicated below each bar. *R. tropici*: strains CFN299, BR864, CIAT899; *R. etli*: strains CFN42, Bra5, F8; *R. leguminosarum* bv. *phaseoli*: strain 4292; *R. giardinii*: strains Ro84 and H152; *R. gallicum* bv. *gallicum*: strain FL27; *Rhizobium* spp.: strains Cli80, CFN234, CFN265; *R. leguminosarum* bv. *viciae*: strain VF39SM; *R. leguminosarum* bv. *trifolii*: strains ANU 843, W14-2; *R. meliloti*: strains 2011, Rm41; *Agrobacterium* spp.: strains KAg-3, ChAg-4; *Rhizobium* sp.: strain NGR234

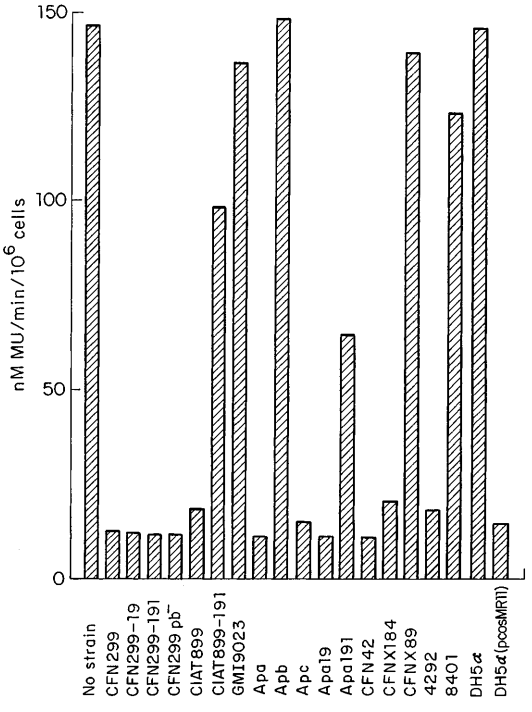

Fig. 5 β-Glucuronidase specific activities obtained as in Fig. 4. In this assay, mutant strains, transconjugants and plasmid-cured strains (see text) were tested for their capacity to remove the inducer from the root exudate

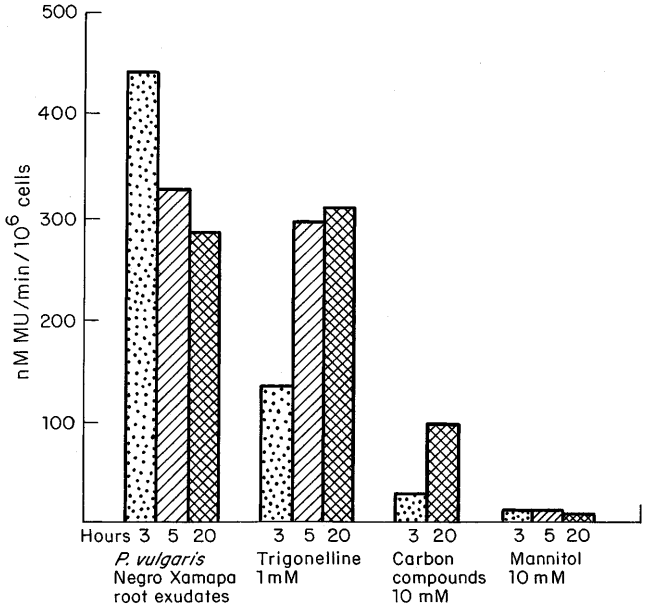

Fig. 6 β-glucuronidase specific activities from CFN299-19 incubated for different periods of time (3, 5, and 20 h) with plant root exudates and various defined compounds. The bars labelled Carbon compounds show the average level of β-glucuronidase expression obtained with inosine, uridine, galactosamine, glucosamine, N-acetyl galactosamine, N-acetyl glucosamine, α-cyclodextrin, 2-aminoethanol and xylitol

is catabolized during the first 3 h. No induction was observed with any of the flavonoids tested.

To establish whether trigonelline was the main compound determining the inducing activity of the exudate, a *R. etli* CFNX184 (CFN42 cured of pc) strain that is incapable of growth on trigonelline as sole carbon source was tested for its capacity to remove the inducer from the exudate (Fig. 5). After the strain had been growing on the exudate for 24 h very little inducer activity was recovered from the exudate. Similar observations were made with CIAT899, which cannot catabolize trigonelline (Fig. 5). *R. meliloti* and *R. leguminosarum* bv. *viciae* VF39SM, although able to catabolize trigonelline, were incapable of using the exudate-inducing compound.

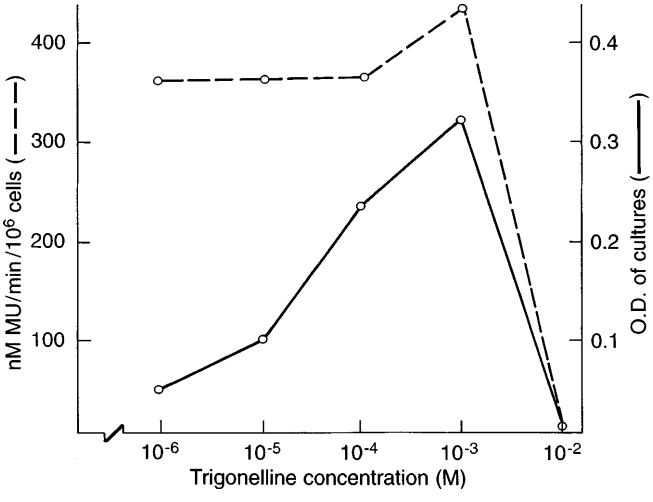

Fig. 7 β-glucuronidase specific activities (*continuous line*) of CFN 299-19 cultures grown in the presence of different trigonelline concentrations. The OD600 value of the cultures is indicated by the *dashed line*

HPLC analysis

We recovered the inducing compound in the polar fraction of the root exudate. This fraction was chromatographed either on a C18 column, for separating flavonoids, or on an Ultrahydrogel 120 column that separates compounds according to molecular weight. In the C-18 chromatograph a fraction that eluted before trigonelline and another that coeluted with trigonelline had inducing activity. In the Ultrahydrogel chromatograph it emerged that the fraction that had inducing activity had a molecular weight between 150 and 350 (data not shown).

Phenotypic analyses of the mutants

To analyze the role of the *teu* genes in symbiosis, we obtained independent mutants, both in the *R. tropici* type reference strain CIAT899 (Type B) and in CFN299, by site-directed insertion of a *lacZ* cassette into the *teuB* gene. The original mutant CFN299-19, as well as the new construction CFN299-191, was able to utilize the bean-inducing compound like the wild type strain CFN299 (Fig. 5). No deficiency in symbiosis was observed with the CFN299 mutants. On the other hand, CIAT899-191 was less efficient in removing the inducing activity from exudate when grown for 24 h in the exudate medium (Fig. 5). When CIAT899-191 was inoculated by itself, nodule number, persistence, and senescence were as in the wild type and microscopic analysis revealed that the nodules were normally infected, but CIAT899-191 showed reduced competitiveness for nodule formation. It formed significantly less than half of the nodules when inoculated in a 1:1 ratio with the wild type. Different inoculum ratios showed that the mutant was significantly less competitive than the wild type (Table 3). Different inoculum sizes were tested, varying between 1000 and 100 000 total bacteria per plant, without affecting the proportion of wild type and mutant cells recovered from nodules.

A. tumefaciens strain GMI9023, which is unable to metabolize the bean compound, acquires this capacity from plasmid pa (from wild type CFN299). It is worth noting that the mutant plasmid from CFN299-191 is incapable of complementing this function, whereas the plasmid from CFN299-19 confers this capacity. This may be related to the different positions of the two mutations in the *teu* operon (Fig. 5).

All three mutants (CIAT899-191, CFN299-19, CFN299-191) and the wild types were able to grow on all of the compounds that were used in the induction assays, indicating that they were not auxotrophs. This

Table 3 Relative nodule occupancy by the *R. tropici* wild type strain CIAT899 and the mutant CIAT899-191

Inoculum ratio[a]	Nodule occupancy		Statistical significance[c]
	% CIAT899	% CIAT899–191	
1:1[b]	71.6	28.4	**
1:1	60.3	39.7	*
1:2	57.1	42.9	**
1:20	15.2	84.8	**

[a] Ten nodules from 10 plants were analyzed for each inoculum ratio. Mixed nodules were not considered. The [b] inoculum ratio 1:1 was tested in two independent experiments
[c] Probably significant differences ($P < 0.05$) and highly significant differences ($P < 0.01$) from the expected values deduced from the inoculum ratio using proportion contrast analyses are indicated by *single* and *paired asterisks*, respectively

would indicate that the inducing compound transported by the *teu* genes does not correspond to any of the commercially available ones we have tried until now.

Discussion

The *A. tumefaciens* strain GMI9023 bearing the *R. tropici* CFN299 symbiotic plasmid (pRtrCFN299c) is able to form nitrogen-fixing nodules on bean, albeit at a reduced efficiency both for nodulation and nitrogen fixation. If, in addition to the Sym plasmid, a 200-kb plasmid (pRtrCFN299b) is transferred to *A. tumefaciens* recipient strains, then nodulation rates of up to 50% of the wild-type rate and nitrogen fixation at 25% of the wild-type rate are obtained with the transconjugants (Martínez et al. 1987). It therefore seemed likely to us that the *R. tropici* plasmids contained some of the genetic information involved in symbiosis, and for this reason we decided to focus our efforts on plasmid-borne genes that are inducible with exudates.

The locus at which the Tn*5-gusA1* was inserted was originally identified as being inducible by bean-root exudates, and sequencing revealed it to contain genes related to sugar transport genes. The uptake of sugars into the cell requires an assemblage of transport proteins. In bacterial sugar transport systems, the genes encoding the proteins that are directly involved in the binding of the sugar (e.g. *rbsB*) are the most divergent ones (Ames 1986; Higgins 1992). This accounts for the different sugar specificities. This is also the case with the *teu* genes: *rbsB* and *teuB* show only low similarity, restricted to the C-terminal end. In addition, if the start codon is the one proposed, the encoded TeuB protein would be 66 amino acids larger than RbsB. In contrast, the ATP-binding proteins for sugar transport systems (TeuA in this case) show the highest degree of similarity and they are the most conserved of the ABC transporters (Higgins 1992). TeuC1, TeuC2, and TeuA have similar numbers of amino acids to the respective Rbs proteins. Since we found no homology to RbsD, we suppose that one of the RbsC homologs may substitute for RbsD in the inner membrane.

The bean inducing compound could be unrelated to sugars and might resemble opines, betaines or even an alcohol, as discussed later. The nicotinic-methylated betaine trigonelline occurs widely in plants (Tramontano et al. 1986). Seeds and roots of alfalfa release the betaines trigonelline and stachydrine, which act as inducers of transcription of some *R. meliloti nod* genes (Phillips et al. 1992). Betaine biosynthetic pathways include amino acids as precursors. Genes for the catabolism of trigonelline are located on the symbiotic plasmid of *R. meliloti* and have been cloned and sequenced (Boivin et al. 1991). They bear no significant homology to the *R. tropici teu* genes. Plant secondary metabolites catabolized selectively by microorganisms have been called nutritional mediators (Tepfer et al. 1988). These, in addition to being energy and carbon sources, may act as additional plant-signal molecules. Strains such as VF39SM and *R. meliloti* 2011 do catabolize trigonelline yet do not consume the inducer. CIAT899 and the c plasmid-cured CFN42 (CFNX184) do not use trigonelline but consume the inducing activity. Thus we conclude that, in addition to trigonelline, there is another compound in the bean exudates that acts as the primary inducer. In addition, *xylB2* could be part of the *teu* operon. In *P. putida* this gene belongs to *xylCAB* operon whose proteins convert aromatic compounds to benzoic acids. XylB is a benzyl alcohol dehydrogenase that oxidizes toluol to tolualdehyde (Shaw and Harayama 1990). On this basis, the presence of *xylB2* could be an indication that the inducing compound is related to an alcohol (perhaps an aryl alcohol).

The majority of bean-nodulating rhizobia are able to utilize this compound, with the exception of the *Rhizobium* spp. (Cli80, CFN234, and CFN265) and *R. gallicum* bv. *gallicum* (FL27), which are related to *R. etli*. These have been reported to form few nodules on bean as compared to the other species and they do not harbor a "*phaseoli*" type plasmid (Martínez et al. 1985; Martínez et al. 1987).

The fact that *R. etli* strain CFN42 has no clear structural homologs of the *R. tropici* genes, in spite of its ability to take up and remove the inducer from the exudate, may be explained if these two species have independent and converging uptake systems. These might have been selected by the host plant. We are interested in cloning and sequencing genes from *R. etli* strain CFN42 that are involved in this uptake in order to answer this question. We speculate that CFN299 has an additional system for uptake and thus no phenotype is observed in

the CFN299 mutants. The other system of CFN299 is perhaps homologous to that of *R. etli*, and is encoded on the pSym, as in *R. etli* and *R. leguminosarum* bv. *phaseoli*. CIAT899 must, then, have only one of these uptake systems. The remaining nodulation of CIAT899 is explained because this strain, like many other rhizobia, is capable of using carbon sources present in the exudate, such as sucrose and glucose.

Periplasmic sugar-binding proteins play a role in chemotaxis in *E. coli*, *Salmonella*, *Azospirillum* and *Agrobacterium*, and certainly could do so in rhizobia. It remains to be evaluated if *teu* mutants are affected in chemotaxis.

Only after chemical characterization of the bean exudate inducer and the identification of the compound that is transported by the wild types but not by the mutants, will we be able clearly and definitively to determine the function of the *teu* genes. It is worth pursuing the definition of the chemical structure of this bean-exudate compound and we are working on this problem. Preliminary evidence indicates that the compound is present at low concentration in the exudate (in agreement with the fact that the bacteria seem to remove it completely within 3 h), that it might be equivalent in weight to a mono- or a disaccharide and is stable at 95° C.

Macroptilium and *Phaseolus* belong to the Phaseolinae subtribe of the subfamily Papilionoideae (Bruneau et al. 1990). *Macroptilium* is the closest to *Phaseolus* among all the plant root exudates tested and it has *teu* gene-inducing capacity. The compound in *Macroptilium* could be identical or similar to that of *Phaseolus*. The inducing compound seems to be restricted to this group of related legumes and it may form part of specificity mechanisms that may be further manipulated to improve inoculant competitiveness in the field. It is remarkable that all bean-nodulating rhizobia tested had the capacity to metabolize the bean exudate compound. This characteristic, although not indispensable for symbiosis, may be advantageous for rhizosphere survival and efficiency as a bean symbiont.

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References

Amarger N, Macheret V, Laguerre G (1997) *Rhizobium gallicum* sp. nov. and *Rhizobium giardinii* sp. nov. from *Phaseolus vulgaris* nodules. Int J Syst Bacteriol 47:996–1006

Ames GF-L (1986) Bacterial periplasmic transport systems: structure, mechanism and evolution. Annu Rev Biochem 55:397–425

Baldani JI, Weaver RW, Hynes MF, Eardly BD (1992) Utilization of carbon substrates, electrophoretic enzyme patterns, and symbiotic performance of plasmid-cured clover rhizobia. Appl Environ Microbiol 58:2308–2314

Bánfalvi Z, Sakanyan V, Koncz C, Kiss A, Dusha I, Kondorosi A (1981) Location of nodulation and nitrogen fixation genes on a high molecular weight plasmid of *R. meliloti*. Mol Gen Genet 184:318–325

Bell AW, Buckel SD, Groarke JM, Hope JN, Kingsley DH, Hermodson MA (1986) The nucleotide sequences of the *rbsD*, *rbsA* and *rbsC* genes of *Escherichia coli* k12. J Biol Chem 261:7652–7658

Boivin C, Barran LR, Malpica CA, Rosenberg C (1991) Genetic analysis of a region of the *Rhizobium meliloti* pSym plasmid specifying catabolism of trigonelline, a secondary metabolite present in legumes. J Bacteriol 173:2809–2817

Brom S, Garcia de los Santos A, Stepkowsky T, Flores M, Dávila G, Romero D, Palacios R (1992) Different plasmids of *Rhizobium leguminosarum* bv. phaseoli are required for optimal symbiotic performance. J Bacteriol 174:5183–5189

Bruneau A, Doyle JJ, Palmer JD (1990) A chloroplast DNA structural mutation as a subtribal character in the Phaseoleae (Leguminosae). Syst Bot 15:78–86

Casse F, Boucher C, Julliot JS, Michel M, Dénarié J (1979) Identification and characterization of large plasmids in *Rhizobium meliloti* using agarose gel electrophoresis. J Bacteriol 113:229–242

Charles TC, Finan TM (1991) Analysis of a 1600-kilobase *Rhizobium meliloti* megaplasmid using defined deletions generated in vivo. Genetics 127:5–20

Dénarié J, Debellé F, Rosenberg C (1992) Signaling and host range variation in nodulation. Annu Rev Microbiol 46:497–531

Eckhardt T (1978) A rapid method for the identification of plasmid deoxyribonucleic acid in bacteria. Plasmid 1:584–588

Fahraeus G (1957) The infection of clover root hair by nodule bacteria studied by a single glass slide technique. J Gen Microbiol 16:374–381

Figurski DH, Helinski DR (1979) Replication of an origin-containing derivative of RK2 dependent on a plasmid function provided in trans. Proc Natl Acad Sci USA 76:1648–1652

Freiberg C, Fellay R, Bairoch A, Broughton WJ, Rosenthal A, Perret X (1997) Molecular basis of symbiosis between *Rhizobium* and legumes. Nature 387:394–401

Gallagher SR (1992) Quantitation of GUS activity by fluorometry. In Gallagher SR (ed) GUS Protocols. Using the GUS gene as a reporter of gene expression. Academic Press, San Diego, pp 47–59

Graham PH, Viteri SE, Mackie F, Vargas AAT, Palacios A (1982) Variation in acid soil tolerance among strains of *Rhizobium phaseoli*. Field Crops Res 5:121–128

Hernández-Lucas I, Segovia L, Martínez-Romero E, Pueppke SG (1995) Phylogenetic relationships and host range of *Rhizobium* spp. that nodulate *Phaseolus vulgaris* L. Appl Environ Microbiol 61:2775–2779

Higgins CF (1992) ABC transporters: from microorganisms to man. Annu Rev Cell Biol 8:67–113

Hofmann K, Stoffel W (1993) TMbase – a database of membrane-spanning protein segments. Biol Chem Hoppe-Seyler 347:166

Hungria M, Joseph CM, Phillips DA (1991) *Rhizobium nod* gene inducers exuded naturally from roots of common bean (*Phaseolus vulgaris* L.). Plant Physiol 97:751–758

Hynes MF, McGregor NF (1990) Two plasmids other than the nodulation plasmid are necessary for formation of nitrogen-fixing nodules by *Rhizobium leguminosarum*. Mol Microbiol 4:567–574

Hynes MF, O'Connell MP (1990) Host plant effect on competition among strains of *Rhizobium leguminosarum*. Can J Microbiol 36:864–869

Jefferson RA (1987) Assaying chimeric genes in plants: the GUS gene fusion system. Plant Mol Biol Reporter 5:387–405

Johnston AWB, Burn JE, Economou A, Davis EO, Hawkins FKL, Bibb MJ (1988) Genetic factors affecting host range in *Rhizobium leguminosarum*. In: Palacios R, Verma DP (eds) Molecular genetics of plant-microbe interactions. APS Press, St. Paul, Minn., pp 378–384

Kyte J, Doolittle RF (1982) A simple method for displaying the hydropathic character of a protein. J Mol Biol 157:105–132

Kokotek W, Lotz W (1989) Construction of a *lacZ*-kanamycin resistance cassette, useful for site-directed mutagenesis and as promoter probe. Gene 84:467–471

Kondorosi A, Kiss GB, Dusha I (1984) Plasmids governing symbiotic nitrogen fixation. In: Sobba-Rao NS (ed) Current developments in biological nitrogen fixation. Edward Arnold, London, pp 135–171

Laeremans T, Caluwaerts I, Verreth C, Rogel MA, Vanderleyden J, Martinez-Romero E (1996) Isolation and characterization of the *Rhizobium tropici* nod factor sulfation genes. Mol Plant-Microbe Interact 9:492–500

Lamb JW, Hombrecher G, Johnston AWB (1982) Plasmid-determined nodulation and nitrogen-fixation abilities in *Rhizobium phaseoli*. Mol Gen Genet 186:449–452

Long SR (1989) *Rhizobium*-legume nodulation: life together in the underground. Cell 56:203–214

Martínez E, Pardo MA, Palacios R, Cevallos MA (1985) Reiteration of nitrogen fixation gene sequences and specificity of *Rhizobium* in nodulation and nitrogen fixation in *Phaseolus vulgaris*. J Gen Microbiol 131:1779–1786

Martínez E, Palacios R, Sánchez F (1987) Nitrogen-fixing nodules induced by *Agrobacterium tumefaciens* harboring *Rhizobium phaseoli* plasmids. J Bacteriol 169:2828–2834

Martínez-Romero E (1994) Recent developments in *Rhizobium* taxonomy. Plant Soil 161:11–20

Martínez-Romero E, Rosenblueth M (1990) Increased bean (*Phaseolus vulgaris* L.) nodulation competitiveness of genetically modified Rhizobium strains. Appl Environ Microbiol 56:2384–2388

Martínez-Romero E, Segovia L, Martins Mercante F, Franco AA, Graham P, Pardo MA (1991) *Rhizobium tropici*, a novel species nodulating *Phaseolus vulgaris* L. beans and *Leucaena* sp. trees. Int J Syst Bacteriol 41:417–426

Mavingui P, Flores M, Romero D, Martínez-Romero E, Palacios R (1997) Generation of *Rhizobium* strains with improved symbiotic properties by random DNA amplification (RDA). Nature Biotechnol 15:564–569

Miller JH (1972) Experiments in bacterial genetics. Cold Spring Harbor Laboratory Press, Cold Spring Harbor, New York

Nakai K, Kanehisa M (1991) Expert system for predicting protein localization sites in Gram-negative bacteria. Proteins 11:95–110

Norrander J, Kempe T, Messing J (1983) Construction of improved M13 vectors using oligonucleotide-directed mutagenesis. Gene 26:101–106

Pardo MA, Lagunez J, Miranda J, Martínez E (1994) Nodulating ability of *Rhizobium tropici* is conditioned by a plasmid-encoded citrate synthase. Mol Microbiol 11:315–321

Phillips DA, Joseph CM, Maxwell CA (1992) Trigonelline and stachydrine released from alfalfa seeds activate NodD2 protein in *Rhizobium meliloti*. Plant Physiol 99:1526–1531

Phillips DA, Dakora FD, Sande E, Joseph CM, Zon J (1994) Synthesis, release, and transmission of alfalfa signals to rhizobial symbionts. Plant Soil 161:69–80

Phillips DA, Streit WR, Volpin H, Palumbo JD, Joseph CM, Sande ES, de Bruijn FJ, Kado CI (1996) Plant regulation of bacterial root colonization. In: Stacey G, Mullin B, Gresshoff PM (eds) Biology of plant-microbe interactions. International Society for Plant-Microbe Interactions, St. Paul, Minn., pp 481–486

Piñero D, Martínez E, Selander RK (1988) Genetic diversity and relationships among isolates of *Rhizobium leguminosoarum* biovar *phaseoli*. Appl Environ Microbiol 54:2825–2832

Priefer UB (1989) Genes involved in lipopolysaccharide production and symbiosis are clustered on the chromosome of *Rhizobium leguminosarum* biovar *viciae* VF39. J Bacteriol 171:6161–6168

Pueppke SG (1996) The genetic and biochemical basis for nodulation of legumes by rhizobia. Crit Rev Biotechnol 16:1–51

Quandt J, Hynes MF (1993) Versatile suicide vectors which allow direct selection for gene replacement in Gram-negative bacteria. Gene 127:15–21

Reese MG, Harris NL, Eeckman FH (1996) Large-scale sequencing: specific neural networks for promoter and splice site recognition. In: Hunter L, Klein T (eds) Proceedings of the Pacific Symposium on Biocomputing. Kona, Hawaii

Rosenberg C, Huguet T (1984) The pATC58 plasmid of *Agrobacterium tumefaciens* is not essential for tumor induction. Mol Gen Genet 195:533–536

Rossbach S, Rasul G, Schneider M, Eardly B, de Bruijn FJ (1995) Structural and functional conservation of the rhizopine catabolism (*moc*) locus is limited to selected *Rhizobium meliloti* strains and unrelated to their geographical origin. Mol Plant-Microbe Interact 8:549–559

Rovira AD (1969) Plant root exudates. Bot Rev 35:35–57

Saurin W, Köster W, Dassa E (1994) Bacterial binding protein-dependent permeases: characterization of distinctive signatures for functionally related integral cytoplasmic membrane proteins. Mol Microbiol 12:993–1004

Sawada H, Ieki H (1992) Phenotypic characteristics of the genus *Agrobacterium*. Ann Phytopath Soc Japan 58:37–45

Schultze M, Kondorosi A (1996) The role of Nod signal structures in the detemination of host specificity in the *Rhizobium*-legume symbiosis. World J Microbiol Biotechnol 12:137–149

Scott IM, Firmin JL, Butcher DN, Searle LM, Sogeke AK, Eagles J, March JF, Self R, Fenwick GR (1979) Analysis of a range of crown gall and normal plant tissues for Ti plasmid-determined compounds. Mol Gen Genet 176:57–65

Segovia L, Young JPW, Martínez-Romero E (1993) Reclassification of American *Rhizobium leguminosarum* biovar *phaseoli* type I strains as *Rhizobium etli* sp. nov. Int J Syst Bacteriol 43:374–377

Sessitch A, Ramírez-Saad H, Hardarson G, Akkermans AD, de Vos WM (1997) Classification of austrian rhizobia and the mexican isolate FL27 obtained from *Phaseolus vulgaris* L. as *Rhizobium gallicum*

Sharma SB, Signer ER (1990) Temporal and spatial regulation of the symbiotic genes of *Rhizobium meliloti* in planta, revealed by transposon Tn*5*-*gusA*. Genes Dev 4:344–356

Shaw JP, Harayama S (1990) Purification and characterization of TOL plasmid-encoded benzyl alcohol dehydrogenase and benzaldehyde dehydrogenase of *Pseudomonas putida*. Eur J Biochem 191:705–714

Soedarjo M, Hemscheidt TK, Borthakur D (1994) Mimosine, a toxin present in leguminous trees (*Leucaena* spp.), induces a mimosine-degrading enzyme activity in some *Rhizobium* strains. Appl Environ Microbiol 60:4268–4272

Tepfer D, Goldmann A, Fleury V, Maille M, Message B, Pamboukdjian N, Boivin C, Dénarié J, Rosenberg C, Lallemund JY, Descoins C, Charpin I, Amarger N (1988) Calystegins, nutritional mediators in plant-microbe interactions. In: Palacios R, Verma DP (eds) Molecular genetics of plant-microbe interactions. APS Press, St. Paul, Minn., pp 139–144

Tramontano WA, McGinley PA, Ciancaglini EF, Evans LS (1986) A survey of trigonelline concentrations in dry seeds of the Dicotyledoneae. Environ Exp Bot 26:197–205

Trinick MJ (1980) Relationships among fast-growing rhizobia of *Lablab purpureus*, *Leucaena leucocephala*, *Mimosa* spp., *Acacia farnesiana* and *Sesbania grandiflora*, and their affinities with other rhizobial groups. J Appl Bacteriol 49:39–53

Van Egeraat AWSM (1975) The possible role of homoserine in the development of *Rhizobium leguminosarum* in the rhizosphere of pea seedlings. Plant Soil 42:381–386

Vlassak KM, Vanderleyden J (1997) Factors influencing nodule occupancy by inoculant rhizobia. Crit Rev Plant Sci 16:163–229