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Molecular and symbiotic characterization of exopolysaccharide-deficient mutants of *Rhizobium tropici* strain CIAT899

Jocelyn L. Milner, Ricardo S. Araujo and Jo Handelsman*

Department of Plant Pathology, 1630 Linden Drive, University of Wisconsin-Madison, Madison, Wisconsin 53706, USA.

Summary

We studied the symbiotic behaviour of 20 independent Tn*5* mutants of *Rhizobium tropici* strain CIAT899 that were deficient in exopolysaccharide (EPS) production. The mutants produced non-mucoid colonies, were motile, grew in broth cultures at rates similar to those of the parent, and produced significantly less EPS than did CIAT899 in broth culture. A genomic library of strain CIAT899, constructed in pLA2917, was mobilized into all of the mutants, and cosmids that restored EPS production were identified. *Eco*RI restriction digests of the cosmids revealed nine unique inserts. Mutant complementation and hybridization analysis showed that the mutations affecting EPS production fell into six functional and physical linkage groups. On bean, the mutants were as efficient in nodulation and as effective in acetylene reduction as strain CIAT899, induced a severe interveinal chlorosis, and all but one were less competitive than CIAT899. On siratro, CIAT899 induced nodules that were ineffective in acetylene reduction, whereas the EPS-deficient mutants induced effective nodules. Microscopic examination of thin sections showed that nodules from both siratro and bean plants inoculated with either CIAT899 or an EPS-deficient mutant contained infected cells. These data indicate that EPS is not required for normal nodulation of bean by *R. tropici*, that it may contribute to competitiveness of *R. tropici* on bean, and that the loss of EPS production is accompanied by acquisition of the ability to reduce acetylene on siratro.

Introduction

Bacteria of the genus *Rhizobium* infect roots of leguminous plants and induce formation of nodules in which the bacteria reduce atmospheric nitrogen to ammonia. The process by which a successful symbiotic relationship is achieved requires a co-ordinated exchange of signals and responses between the plant and bacteria (Brewin, 1991; Reuber *et al.*, 1991b; Puhler *et al.*, 1991; Gray and Rolfe, 1990; Long, 1989).

Acidic exopolysaccharides (EPSs) are complex carbohydrates produced and secreted in large quantities by most *Rhizobium* species. EPS-deficient mutants of certain *Rhizobium* species are unable to induce the formation of nitrogen-fixing nodules on certain hosts. These symbioses include *Rhizobium meliloti* on alfalfa, *Rhizobium leguminosarum* bv. *viciae* on peas, *R. leguminosarum* bv. *trifolii* on clover, the broad-host-range *Rhizobium* sp. strain NGR234 on *Leucaena*, and *Rhizobium loti* on *Leucaena* (Leigh *et al.*, 1985; 1987; Muller *et al.*, 1988; Borthakur *et al.*, 1986; Diebold and Noel, 1989; Chakovorty *et al.*, 1982; Chen *et al.*, 1985; Djordjevic *et al.*, 1987; Hotter and Scott, 1991). In contrast, defects in EPS production do not interfere with the induction of nitrogen-fixing nodules by *Rhizobium fredii* on soybean, *R. loti* on *Lotus*, and *R. leguminosarum* bv. *phaseoli* on common bean (Kim *et al.*, 1989; Zdor and Pueppke, 1990; Hotter and Scott, 1991; Diebold and Noel, 1989). EPS also affects the nodulation competitiveness of *R. fredii* and *Bradyrhizobium japonicum* (Zdor and Pueppke, 1990; Bhagwat *et al.*, 1991).

Much is known at the molecular level about the genes involved in the biosynthesis and regulation of the two classes of EPS produced by *R. meliloti* (Finan *et al.*, 1985; Leigh *et al.*, 1985; Long *et al.*, 1988; Glazebrook and Walker, 1989; Muller *et al.*, 1988; Leigh *et al.*, 1987; Reuber *et al.*, 1991a). Studies using large numbers of mutants have shown that at least 14 loci participate in the biosynthesis of EPS-I and several more are involved in the biosynthesis of EPS-II, a chemically distinct molecule (reviewed by Reuber *et al.*, 1991b; Puhler *et al.*, 1991). Multiple genes have also been reported to be involved in the biosynthesis and regulation of EPS in *Rhizobium* sp. strain NGR234 and *R. leguminosarum* bv. *phaseoli* (Chen *et al.*, 1985; 1988; Zhan and Leigh, 1990; Gray *et al.*, 1990; Borthakur *et al.*, 1985; Borthakur and Johnston, 1987; Borthakur *et al.*, 1988; Diebold and Noel, 1989). Although EPS-deficient mutants of *Rhizobium tropici* strain CIAT899 have been described (O'Connell *et al.*, 1990;

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Kingsley and Bohlool, 1992) a molecular analysis of EPS production in *R. tropici* has not been reported.

Strains of *R. tropici* nodulate *Phaseolus vulgaris* (common bean), *Macroptilium atropurpureum* (siratro), and the tree legume, *Leucaena leucocephala* (Martinez-Romero *et al.*, 1991; Vargas *et al.*, 1990). We previously reported that six EPS-deficient, Tn*5* mutants of CIAT899 form nitrogen-fixing nodules and induce a severe interveinal chlorosis on bean (O'Connell *et al.*, 1990). In this study we isolated additional EPS-deficient mutants to define genetic regions involved in EPS production and we identified two additional symbiotic phenotypes of the EPS-deficient mutants on bean and siratro.

Results

Characterization of EPS-deficient mutants of CIAT899

We characterized 20 independent EPS-deficient mutants of CIAT899. Seventeen mutants produced non-mucoid colonies (Exo^-) and the three others produced colonies with a mucoid halo that was smaller than that produced by CIAT899 ($Exo^{+/-}$). Strain CT9111 was used as a representative Tn*5*-containing mucoid strain whose colony morphology was indistinguishable from that of CIAT899.

To determine whether the non-mucoid colony morphology was indeed due to the lack of EPS or was simply a result of slow growth, we tested the growth rates of several mutants in yeast extract/mannitol medium (YM) broth culture. The growth rates of the mutants (0.121 ± 0.006 to 0.167 ± 0.008 generations h^{-1}) were the same or faster than the growth rate of CIAT899 (0.126 ± 0.008 generations h^{-1}). CIAT899 and the mutants saturated the culture at approximately the same cell density. All of the mutants were motile in a plate swarm test. These data indicated that the mutations do not have detectable pleiotropic effects on growth.

To determine whether the mutants each contained a single Tn*5* insertion, we used pGS9 (which carries Tn*5*, see Table 1) to probe Southern blots of total genomic DNA of the mutants and CIAT899 digested with *Mlu*I or *Eco*RI, which do not cut within Tn*5*. pGS9 did not hybridize to CIAT899 genomic DNA. In most mutants, pGS9 hybridized to a single genomic *Mlu*I or *Eco*RI fragment, which indicated a single Tn*5* insertion. In five mutants (CT9005, CT9004, CT9102, CT9106, CT9107) pGS9 hybridized to four *Eco*RI fragments. Two bands (smaller than 5.4 kb) comigrated with two of three *Eco*RI fragments of pGS9. This suggested that the multiple bands were attributable to a single insertion of part or all of pGS9 in addition to the Tn*5*, rather than multiple Tn*5* insertions.

We evaluated the linkage between kanamycin resistance (Km^R) (Tn*5*) and the EPS-deficient phenotypes by a pJB3-mediated mobilization of the genome of each of the mutants into strain CT900 (Table 1), a spontaneous streptomycin-resistant (Str^R) mutant of CIAT899 that retained its mucoid colony morphology. For each mutant, over 1000 Str^R Km^R transconjugant colonies were scored for coinheritance of the EPS-deficient phenotype. For all but two mutants, all Str^R Km^R colonies were also non-mucoid. Of the transconjugants derived from mutants CT9108 and CT9110, 99% of the transconjugants coinherited Km^R and the Exo^- phenotype. These data indicated that the Tn*5* was likely to be causal to the EPS-deficient phenotype.

Table 1. Strains and plasmids.

Strain/Plasmid	Description	Origin/Source/ Reference
Strain		
CIAT899	*R. tropici* type strain, mucoid, Sp^R	P. Graham
CT900	Spontaneous Str^R mutant of CIAT899	This work
NGR234	Broad-host-range *Rhizobium* sp.	B. G. Rolfe
Rm1021	*R. meliloti*, Str^R derivative of SU47	F. Ausubel
Rm7020	Rm1021 *exoC020*::Tn*5*	Leigh *et al.* (1985)
Rm7031	Rm1021 *exoA031*::Tn*5*	Leigh *et al.* (1985)
Rm7055	Rm1021 *exoF055*::Tn*5*	Leigh *et al.* (1985)
Rm7094	Rm1021 *exoB094*::Tn*5*	Leigh *et al.* (1985)
Rm8274	Rm1021 *exoT274*::Tn*phoA*	Reuber *et al.* (1991a)
Rm8332	Rm1021 *exoQ332*::Tn*5*	Long *et al.* (1988)
Rm8431	Rm1021 *exoL431*::Tn*5*	Long *et al.* (1988)
Rm8457	Rm1021 *exoM457*::Tn*5*	Long *et al.* (1988)
DH5α	*E. coli*, *supE44*, *ΔlacU169*, (*Φ80lacZΔM15*), *hsdR17*, *recA1*, *endA1*, *gyrA96*, *thi-1*, *relA1*	Hanahan (1983)
Plasmid		
pRK2013	Km^R, helper plasmid	Figurski and Helinski (1979)
pGS9	p15A replicon, Tn*5*-carrying vector	Selvaraj and Iyer (1983)
pLA2917	Km^R Tc^R, broad-host-range cosmid vector	Allen and Hanson (1985)
pSUP104	Cm^R, Tc^R, IncQ vector	Long *et al.* (1988)
pD56	Rm1021-derived *exoB-F* complementing plasmid	Long *et al.* (1988)
pEX31	Rm1021-derived *exoB* subclone	Long *et al.* (1988)
pEX312	Rm1021-derived *exoAHF* complementing plasmid	Long *et al.* (1988)
pJB3	Conjugative plasmid, R68.45 derivative	Brewin *et al.* (1980)

EPS production

Visual evaluation of colony morphology was consistent with measurements of EPS accumulated by strains in

Table 2. Exopolysaccharide production by *R. tropici* strain CIAT899 and its derivatives.

Strain	Colony morphology[a]	Ethanol-precipitable Material[b]		Glucose Equivalents[c]	
		mg EPM ml^{-1}	mg EPM 10^{-9} cfu	mg hexose per mg EPM	mg hexose 10^{-9} cfu[d]
CIAT899	+	2.67 ± 0.38 ABCD	1.59 ± 0.26 ABC	0.587 ± 0.038 A	0.93
CT9111	+	2.19 ± 0.57 BCDE	1.43 ± 0.10 ABCD	0.419 ± 0.019 B	0.59
CT9105	+/−	1.30 ± 0.08 EFG	0.89 ± 0.20 CDEF	0.248 ± 0.014 CD	0.22
CT9109	+/−	1.38 ± 0.17 EFG	0.62 ± 0.03 DEF	0.384 ± 0.017 B	0.23
CT9101	−	0.52 ± 0.07 FG	0.30 ± 0.05 EF	0.255 ± 0.010 CD	0.07
CT9102	−	0.48 ± 0.15 FG	0.29 ± 0.01 EF	0.359 ± 0.016 BC	0.10
CT9103	−	0.73 ± 0.12 FG	0.35 ± 0.05 EF	0.411 ± 0.015 B	0.14
CT9104	−	0.60 ± 0.14 FG	0.36 ± 0.07 EF	0.355 ± 0.011 BC	0.13
CT9110	−	0.41 ± 0.05 FG	0.38 ± 0.07 EF	0.166 ± 0.006 D	0.06
CT9113	−	0.26 ± 0.11 G	0.17 ± 0.09 F	0.263 ± 0.009 CD	0.04
CT9002	−	0.68 ± 0.12 FG	0.30 ± 0.08 EF	0.260 ± 0.010 CD	0.08
CIAT899(pLA2917)	+	3.01 ± 0.09 ABC	1.46 ± 0.16 ABCD	0.629 ± 0.028 A	0.91
CT9101(pJM101)	+	3.62 ± 0.23 A	2.15 ± 0.18 A	0.610 ± 0.023 A	1.31
CT9103(pJM103)	+	3.33 ± 0.24 AB	1.78 ± 0.02 AB	0.622 ± 0.022 A	1.11
CT9002(pJM002)	+	1.74 ± 0.13 CDEF	1.18 ± 0.14 BCDE	0.553 ± 0.039 A	0.65

a. Colony morphology on BSM and YM media: +, mucoid colony morphology; +/−, reduced halo production; −, non-mucoid colony morphology.
b. The ethanol-precipitable material (EPM) in the culture supernatant was normalized to culture volume (mg EPM ml^{-1}) or the culture cell density (mg EPM 10^{-9} cfu, 1.2–2.5 × 10^9 cfu ml^{-1}). The mean ± the standard error of 2 to 6 determinations is reported. Values in the same column followed by the same letter are not significantly different at $P \leq 0.05$ according to the Tukey studentized range test.
c. The hexose content of the EPM, reported as glucose equivalents, was determined by the phenol–sulphuric acid assay as described in the *Experimental procedures*.
d. Calculated as the product of mg EPM 10^{-9} cfu and mg hexose per mg EPM.

supernatants of YM broth culture (Table 2). Most of the EPS-deficient mutants produced low but measurable amounts of EPS. These levels of EPS production were similar to those reported previously for CIAT899 and mutants CT9002, CT9003, CT9005, CT9009, and CT9010 (O'Connell *et al.*, 1990). Mutants CT9105 and CT9109 ($Exo^{+/-}$) produced significantly less EPS than CIAT899 but significantly more EPS than the Exo^- mutants.

We expressed EPS production as ethanol-precipitable material or glucose equivalents in the culture supernatant normalized to culture volume or cell number (Table 2). The proportion of the ethanol-precipitable material that was accounted for by glucose equivalents (mg hexose per mg EPM) was not consistent among the strains tested. This suggested that the mutants not only produced less EPS, but also may have produced altered forms of EPS. Alternatively, the proportion of ethanol-precipitable material not accounted for by glucose equivalents may have been higher in the culture supernatants of the mutants. Although we use the term EPS production, the measurements of EPS accumulation did not distinguish between the effects of the mutations on synthesis, degradation, export, or regulation of EPS production.

To determine if the mutants were also non-mucoid in the presence of seed exudate, we prepared growth medium from exudates of bean seeds. CIAT899 and its mutants grew poorly on bean-exudate medium. The addition of yeast extract improved their growth, but colonies of all the strains, including CIAT899, were non-mucoid. When mannitol was added, colonies of CIAT899 produced the characteristic mucoid halo but the mutants remained non-mucoid. This indicated that the composition of bean seed exudate did not enable the mutants to produce EPS.

Certain mutants deficient in EPS production are also altered in lipopolysaccharide (LPS) production (Diebold and Noel, 1989; Bhagwat *et al.*, 1991). We examined the LPS of the EPS-deficient mutants by SDS–PAGE (Diebold and Noel, 1989) and found that the patterns were indistinguishable among CIAT899 and all 20 mutants (data not shown).

Characterization of cosmids that restore EPS production

When the cosmid bank of strain CIAT899 was mobilized from DH5α into each EPS-deficient mutant, Exo^+ transconjugants, detected as mucoid colonies on selective medium, arose at a frequency of 0.1%. A single mucoid transconjugant derived from each mutant was isolated for further study. We will refer to this phenotypic restoration of EPS production by specific cosmids as complementation. We verified that the mucoid colony morphology corresponded to increased EPS production since representative cosmid-carrying mutants produced an amount of EPS similar to that produced by CIAT899 and CIAT899-

(pLA2917) (Table 2). Complemented mutants (Exo^+) that lost the cosmid in the absence of tetracycline (Tc) selection were Exo^- or $Exo^{+/-}$. Therefore, the cosmid was responsible for restoration of EPS production.

The EPS-restoring cosmids were isolated, transformed into DH5α, and mobilized into the mutant from which they were originally isolated to confirm their ability to restore EPS production. When they were mobilized into CIAT899, none of the cosmids altered its colony morphology. Among the 20 EPS-restoring cosmids, there were nine unique *Eco*RI restriction patterns. We probed digests of the nine cosmids with each of the nine cosmids to identify homologous insert DNA (Table 3). Each cosmid was mobilized into all of the mutants to identify additional mutants to which it could restore EPS production (Table 3). With the exception of pJM106, cosmids with homologous insert DNA complemented the same mutants.

We identified the genomic *Eco*RI fragment of CIAT899 corresponding to the Tn*5*-containing fragment of each mutant (Table 3). Southern blots of *Eco*RI digest of total genomic DNA from CIAT899 and each mutant were probed with the corresponding EPS-restoring cosmid(s). The Tn*5*-containing fragment was identified by a shift in mobility of 5.4 kb in the mutant as compared to its position in CIAT899. For mutants CT9004, CT9005, CT9102, CT9106, and CT9107, a single band replaced a band that was more than 5.4 kb smaller, which is consistent with our conclusion (discussed above) that the insertion in these mutants contained part or all of pGS9 in addition to Tn*5*. For all of the mutants, the Tn*5* probe also hybridized to the larger band. Four *Eco*RI restriction fragments were sites of more than one Tn*5* insertion (Table 3). These complementation and hybridization analyses demonstrated that the mutants defined six functionally and physically distinct regions that influence EPS production.

Cross-species complementation

Functionally interchangeable *exo* loci have been identified for *R. meliloti* strain RM1021 and *Rhizobium* sp. strain NGR234 (Zhan *et al.*, 1990; Gray *et al.*, 1991). Because the EPSs from Rm1021 and CIAT899 share common structural features (Gil-Serrano *et al.*, 1990; Aman *et al.*, 1981) we tested for functional homology between cloned *exo* genes from Rm1021 and CIAT899. None of the plasmids carrying *exo* genes of Rm1021 (Table 1) complemented *exo* mutants of CIAT899. However, when EPS-restoring cosmids derived from CIAT899 were mobilized into Rm1021-derived *exo* mutants (Table 1), pJM109 restored the Calcofluor-bright phenotype to Rm7020

Table 3. Molecular analysis of CIAT899 EPS-deficient mutants and EPS-restoring cosmids.

Mutant[a]	*Eco*RI fragment[b]	pJM101	pJM106	pJM102	pJM002	pJM010	pJM103	pJM008	pJM104	pJM109
CT9101	1.1	+								
CT9113	1.1	+								
CT9005	2.6	+								
CT9105	2.6	+	+/−							
CT9108	2.6	+	+/−							
CT9110	2.6	+								
CT9003	2.0	+								
CT9106	2.05		+							
CT9107	1.5		+							
CT9002	7.2		+/−	+	+	+				
CT9004	7.2		+/−	+	+	+				
CT9006	7.2		+/−	+	+	+				
CT9007	7.2		+/−	+	+	+				
CT9010	7.2		+/−	+	+	+				
CT9102	7.2			+	+	+				
CT9103	6.15						+	+		
CT9008	6.15						+	+		
CT9009	6.15						+	+		
CT9104	8.5								+	
CT9109	ND[c]									+
Estimated insert size[d]		22.3	23.6	18.4	25.8	29.8	26.1	30.2	24.0	25.8
Common *Eco*RI fragments[e]		2.0, 1.1		7.2, 2.5, 1.0			6.15			

a. Tn*5* mutants of CIAT899. Complementation by the cosmids listed across the top of the table is indicated: +, complete restoration of EPS production to the CIAT899-like colony morphology; +/−, partial restoration of EPS production; blank box, mutant colony morphology.
b. The estimated size (kb) of the genomic Tn*5*-containing *Eco*RI restriction fragment identified for each mutant.
c. ND, not determined.
d. The estimated size (kb) of insert DNA carried by each cosmid as estimated from restriction digests.
e. The estimated size (kb) of *Eco*RI restriction fragments that were common among the indicated cosmids as identified by hybridization analysis.

(*exoC*020::Tn*5*) and improved the poor growth of that strain. This result suggested that pJM109 carried a gene functionally interchangeable with *exoC* of Rm1021, a locus implicated in phosphoglucomutase activity (Uttaro *et al.*, 1990).

Symbiotic phenotype on bean

All of the mutants induced a similar number of nodules per plant at inoculum concentrations as low as 100 cfu ml^{-1}. Mean nodule numbers for beans inoculated with each of the mutants and CIAT899 were not significantly different at $P \leq 0.03$. A combined average of 25.6 ± 1.2 nodules/plant was induced on beans inoculated with CIAT899 and its EPS-deficient mutants. Beans inoculated with mutants or their cosmid-carrying derivatives reduced acetylene at rates not significantly different from CIAT899 (means of 3–7 plants, $P \leq 0.05$) and comparable to those previously reported for CIAT899 (O'Connell *et al.*, 1990).

O'Connell *et al.* (1990) described a severe interveinal chlorosis induced on beans inoculated with each of five of the Exo^- mutants of CIAT899 but not with Exo^- mutants derived from strains of *R. leguminosarum* bv. *phaseoli*. All of the Exo^- mutants described here also induced chlorosis. Some mutants (CT9104, CT9106, CT9003, CT9004) did not consistently induce chlorosis that was more severe than the chlorosis induced by CIAT899. For those mutants the results of two experiments are reported (Table 4). Beans inoculated with mutants containing EPS-restoring cosmids had significantly lower chlorosis ratings than those inoculated with the Exo^- mutants (Table 4). Thus, complementation of the *exo* mutations was sufficient to reduce chlorosis induction. These data strengthened the correlation between chlorosis induction and reduced EPS production by CIAT899 derivatives.

We examined the competitiveness of EPS-deficient mutants relative to CIAT899 (Table 5). Mixtures of CIAT899 and its mutants were applied to bean seeds at three ratios. The range of inoculum ratios was chosen so that CIAT899 or the mutant each occupied more than 50% of the nodules at a minimum of one ratio. The competitiveness index (CI) was calculated using a mathematical model, derived by Beattie *et al.* (1989), that relates the ratio of competing strains in a mixed inoculum to their ratio in the nodules. A CI of 0 is calculated when CIAT899 and the mutant occupy an equal proportion of the nodules at an inoculum ratio of 1:1 and is characteristic of a mutant that is equal in competitiveness to CIAT899. A CI greater than 0 indicates that the mutant is less competitive than CIAT899 and a CI less than 0 indicates that the mutant is more competitive than CIAT899.

Of 13 mutants examined, 12 were consistently reduced in competitiveness relative to CIAT899 (CI > 0, Table 5). Strain CT9109 ($Exo^{+/-}$) was similar in competitiveness to CIAT899 (CI = 0). Strain CT9111 (Exo^+) was slightly more competitive than CIAT899 (CI < 0). For the 12 mutants consistently reduced in competitiveness, a 10-fold excess of the mutant was generally required for the mutant to occupy a greater proportion of nodules than CIAT899. By comparison, certain Exo^- mutants of *R. leguminosarum* bv. *phaseoli* must out-number their parent by 50-fold in the inoculum to occupy the majority of the nodules (Araujo and Handelsman, 1990).

Table 4. Chlorosis ratings of beans inoculated with CIAT899, its EPS-deficient mutants, and their cosmid-carrying derivatives.

Strain	Cosmid	Chlorosis Rating[a]		
		without cosmid	with cosmid	*P* value[b]
CIAT899	pLA2917	1.2 ± 0.25	1.6 ± 0.21	0.25
		1.7 ± 0.21	2.1 ± 0.31	0.28
CT9101	pJM101	3.3 ± 0.33	1.6 ± 0.24	0.01
		5.0 ± 0.00	1.9 ± 0.28	0.00
CT9102	pJM102	3.6 ± 0.20	2.6 ± 0.37	0.04
CT9103	pJM103	3.3 ± 1.4	1.1 ± 0.14	0.00
CT9104	pJM104	1.8 ± 0.40	1.2 ± 0.20	0.20
		3.9 ± 0.42	1.9 ± 0.23	0.01
CT9106	pJM106	2.2 ± 1.8	1.0 ± 0.00	0.20
		4.1 ± 0.18	1.9 ± 0.30	0.00
CT9108	pJM101	3.6 ± 0.40	1.1 ± 0.14	0.00
CT9110	pJM101	4.0 ± 0.26	1.1 ± 0.14	0.00
CT9113	pJM101	3.3 ± 0.18	1.2 ± 0.20	0.00
CT9002	pJM002	2.7 ± 0.36	1.3 ± 0.18	0.01
CT9003	pJM101	2.3 ± 0.54	1.1 ± 0.14	0.09
		4.0 ± 0.29	1.8 ± 0.20	0.00
CT9004	pJM002	2.4 ± 0.60	1.6 ± 0.30	0.27
		4.2 ± 0.22	2.4 ± 0.34	0.00
CT9006	pJM002	3.1 ± 0.31	1.6 ± 0.24	0.00
CT9007	pJM102	4.0 ± 0.22	1.5 ± 0.22	0.00
CT9008	pJM008	3.8 ± 0.37	1.0 ± 0.00	0.00
CT9009	pJM103	4.0 ± 0.22	1.2 ± 0.20	0.00
CT9010	pJM010	3.2 ± 0.37	2.1 ± 0.26	0.05

a. Chlorosis was rated on a 1 (no chlorosis) to 5 (severe chlorosis) scale (O'Connell *et al.*, 1990). The mean of 3–10 plants ± the standard error is reported. No chlorosis was detected on uninoculated beans in the same experiments. The data presented are from two separate experiments.
b. *P* value for Student's *t*-test comparing the mutant and its cosmid-carrying derivative.

Symbiotic phenotype on siratro

Siratro plants inoculated with CIAT899, its derivatives, or *Rhizobium* sp. strain NGR234 grew equally well and could not be visually distinguished. However, siratro plants inoculated with mutants CT9101 and CT9104 reduced acetylene at significantly greater rates than plants inoculated with CIAT899 (Table 6) although the average number of nodules per plants was not significantly different at $P \leq 0.05$. This result was obtained in three experiments with siratro grown in test tubes and one experiment with siratro grown in Leonard jars. When the same bacterial cultures were used to inoculate bean they all induced equally effective nodulation on that host (Table

Table 5. Nodulation competitiveness of exopolysaccharide-deficient mutants relative to CIAT899 on bean.

Mutant[a]	EPS[b]	Number of experiments[c]	Competitiveness index (CIAT899: mutant)[d]
CT9111	+	2	−0.118 ± 0.058*
CT9101	−	3	0.525 ± 0.041*
CT9113	−	3	0.390 ± 0.045*
CT9105	+/−	3	0.162 ± 0.036*
CT9108	−	3	0.273 ± 0.038*
CT9110	−	3	0.240 ± 0.039*
CT9003	−	2	0.553 ± 0.068*
CT9106	−	1	0.476 ± 0.065*
CT9002	−	1	0.541 ± 0.127*
CT9102	−	1	0.660 ± 0.063*
CT9103	−	2	0.218 ± 0.059*
CT9008	−	1	0.148 ± 0.063*
CT9104	−	2	0.524 ± 0.053*
CT9109	+/−	3	−0.030 ± 0.034

a. Mutants are listed in the same order as in Table 3.
b. EPS, defined in Table 2.
c. The number of competition experiments performed with each mutant.
d. The competitiveness index was calculated according to Beattie *et al.* (1989) as described in the *Results*. In a single competition experiment, nodule occupancy was determined at three inoculum ratios (in the range 15:1 to 1:21, CIAT899:mutant) for each mutant mixed with CIAT899. The proportion of 8 nodules/plant, for 6 plants/inoculum ratio, occupied by each strain was determined as described in the *Experimental procedures*. Occupants of 144 nodules were determined in each experiment for each test strain. When more than one experiment was performed with a mutant, the competitiveness index was calculated using the data from all of the experiments taken together. *, the competitivenes index was significantly different from 0 at $P \leq 0.05$.

6). Strain CIAT899 was effective on beans grown at both 24°C (325 ± 28 nmol C_2H_2 reduced per hour per plant) and 28°C (518 ± 72 nmol C_2H_2 reduced per hour per plant); therefore the failure to reduce acetylene could not be attributed to the effect of the temperature at which the plants were grown. Acetylene reduction rates for siratro were consistently 100-fold lower than rates for bean. Siratro plants had an average of only two to five nodules per plant, independent of the inoculum strain, which partially accounted for that difference. Since siratro plants inoculated with *Rhizobium* sp. strain NGR234 produced a similar number of nodules and reduced acetylene at a rate similar to siratro inoculated with CT9101 and CT9104, the low acetylene reduction rates appear to be a property of siratro.

Nodules from siratro plants inoculated with CIAT899, its derivatives, or *Rhizobium* sp. strain NGR234 were similar in size, shape, and colour. Microscopic examination of thin sections of bean and siratro nodules (Fig. 1) from plants inoculated with CIAT899 or CT9101 showed that plant cells within the nodules were infected with bacteria. Therefore, the Fix$^-$ phenotype of CIAT899 on siratro may be attributed to a malfunction late in the establishment of the nitrogen-fixing nodule.

Discussion

To investigate the role of EPS in the symbiosis of *R. tropici* strain CIAT899 with its hosts, we generated a collection of 20 independent EPS-deficient mutants by screening Tn*5* mutants for non-mucoid colony morphology. We verified that the mutants grew at rates similar to that of CIAT899, carried single Tn*5* insertions, and produced significantly less EPS in broth culture than did CIAT899. All of the mutants produced normal LPS. EPS production was restored to each mutant by at least one of nine cosmids isolated from a gene library derived from CIAT899. At least six clusters of mutations altered EPS production in CIAT899. Four clusters were represented by multiple mutations and two clusters were represented by a single mutation each.

Nodulation competitiveness is the ability of one strain to occupy a greater proportion of nodules than the proportion at which it is present in a mixed inoculum (reviewed by Triplett, 1990). Bacterial cell surface changes alter the competitiveness of strains of *R. meliloti* (Handelsman *et al.*, 1984; Ugalde *et al.*, 1986). An Exo$^-$ mutant of *R. fredii* is increased in competitiveness (Zdor and Pueppke, 1990), and an Exo$^-$ Lps$^-$ mutant of *B. japonicum* is decreased in competitiveness and forms fewer nodules than its parent on soybean (Bhagwat *et al.*, 1991). On bean, we found that EPS-deficient mutants of *R. tropici* strain CIAT899 induced normal nitrogen-fixing nodules. We also found that 12 out of 13 EPS-deficient mutants (11 Exo$^-$ and one Exo$^{+/-}$) were less competitive than CIAT899, and one Exo$^{+/-}$ mutant was of equal competitiveness to CIAT899 (Table 5). We do not know if EPS has a direct or indirect effect on competitiveness. A severe reduction in EPS production appears to interfere with competitiveness because all of the Exo$^-$ mutants tested were reduced in competitiveness. However, because one mutant, CT9109 (Exo$^{+/-}$), was altered in EPS production, but was not altered in competitiveness, normal EPS

Table 6. Acetylene reduction by exopolysaccharide-deficient mutants on siratro and bean.

	Bean		Siratro	
Strain	C_2H_2 reduction rate[a]	*n*	C_2H_2 reduction rate	*n*
CIAT899	160 ± 28 A	17	0.6 ± 0.02 C	19
CT9101	169 ± 22 A	16	3.35 ± 0.47 A	23
CT9104	122 ± 25 A	8	1.80 ± 0.60 B	11

a. nmol per hour per plant. Means of *n* plants followed by the same letter, for a given plant species, did not differ according to the Tukey studentized range test at $P \leq 0.05$. The same inoculum was used for both plant species in this experiment. In a similar experiment, siratro inoculated with CIAT899 and CT9101 reduced acetylene at rates comparable to those reported in this table (0.03 ± 0.03 and 7.2 ± 2.7 nmol per hour per plant, respectively). Uninoculated control plants did not reduce C_2H_2 at detectable rates.

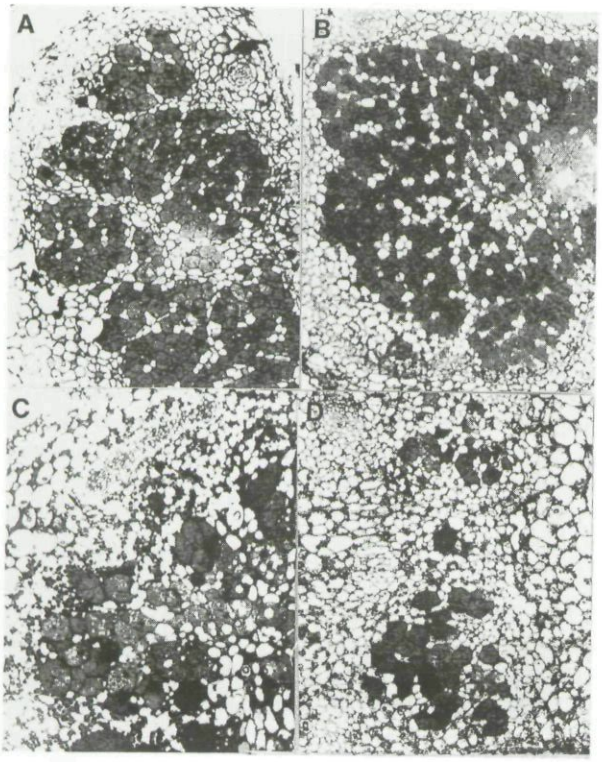

Fig. 1. Nodule cross-sections (2 μm thick) of a CIAT899-infected bean nodule (A), a CT9101-infected bean nodule (B), a CIAT899-infected siratro nodule (C), and a CT9101-infected siratro nodule (D). Magnification was 70×.

production is unlikely to be obligatory for normal competitiveness of CIAT899 on bean. In contrast, CT9105 ($Exo^{+/-}$), which produced a quantity of EPS similar to that produced by CT9109, was reduced in competitiveness. The molecular analysis of the mutants placed CT9105 in a group with six Exo^- strains but CT9109 was the only member of its group. We do not know whether CT9105 and CT9109 produce chemically indistinguishable EPSs. The nature of the defect in EPS production, as well as the quantity of EPSs produced, may also be relevant to the competitiveness phenotype. Our data indicate the importance of working with many mutants when studying relationships between complex, multigenic traits such as EPS production and competitiveness. Testing only one EPS-deficient mutant, such as CT9101, could have suggested a much simpler relationship between EPS production and nodulation competitiveness.

Cosmid loss in the rhizosphere and in nodules hampered our ability to determine whether the restoration of competitiveness would be concomitant with restoration of EPS production in the mutants by EPS-restoring cosmids (J. L. Milner and J. Handelsman, unpublished). However, we were able to observe that chlorosis, which was induced on bean plants by the mutants, was not induced by mutants restored to EPS production by specific cosmids. This result suggested that cosmids were sufficiently maintained so that the chlorosis phenotype of EPS-producing bacteria (mutants restored to EPS production by a specific cosmid) masked that of EPS-deficient bacteria (mutants) when the two were present in a mixed population.

Nitrogen-fixing nodules are induced on siratro by *Rhizobium* sp. strain NGR234 but not by some of its Exo^- mutants (Chen *et al.*, 1985; Djordjevic *et al.*, 1987). In contrast, we found that CIAT899 induced ineffective nodules on siratro, whereas its Exo^- mutants induced acetylene-reducing nodules (Table 6). Since the appearance of siratro nodules induced by CIAT899 or CT9101 and CT9104 was not distinguishable by macroscopic or microscopic inspection, the block is likely to be late in the development of the acetylene-reducing nodules. Reuber *et al.* (1991a) reported that an *exoF*::Tn*phoA* fusion in *R. meliloti* is expressed in the invasion zone, but not the bacteroid zone, of alfalfa nodules, which suggests that EPS production does not coincide with nitrogen fixation in alfalfa nodules. Additional evidence indicates that EPS production must be reduced to permit nitrogen fixation (Reuber *et al.*, 1991a). Mutations in *psi*, a negative regulator of EPS production, abolish the ability of *R. leguminosarum* bv. *phaseoli* to form nitrogen-fixing nodules on bean (Borthakur *et al.*, 1985) and *B. japonicum* bacteriods are thought not to produce EPS (Tully and Terry, 1985). Perhaps EPS production by rhizobia within the nodule prevents nitrogen fixation, and CIAT899 failed to fix nitrogen on siratro because it failed to repress EPS production within the siratro nodule.

Although it is not uncommon for wild-type rhizobia to fix nitrogen at low rates, we know of no other reports in which nitrogen fixation is enhanced in an EPS-deficient mutant of a strain that fixes poorly. Further investigation will be required to determine if this phenomenon is broadly exhibited by rhizobia or is specific to *R. tropici*, and to elucidate the role of EPS in the formation of a nitrogen-fixing nodule. This study highlights the multiplicity of functions of EPS, and the effects of mutations that affect EPS accumulation. It has now been demonstrated that in various host plants, EPS plays both positive and negative roles in competition, it affects root infection, chlorosis induction, and it has positive and negative effects on nitrogen fixation. It will be interesting to determine the molecular interactions that lead to such a wide range of roles for one group of macromolecules.

Experimental procedures

Strains, plasmids, and bacterial growth conditions

Strains and plasmids are listed in Table 1. Bacteria were grown in YM medium (Wacek and Brill, 1976), tryptone-yeast extract

medium (TY) (Beringer, 1974), and Luria–Bertani medium (Maniatis *et al.*, 1982). Antibiotics were added to solid medium at the following concentrations: spectinomycin (Sp), 200 μg ml^{-1}; Km, 25 μg ml^{-1} for *Escherichia coli* strains and 200 μg ml^{-1} for *Rhizobium*; Tc, 10 μg ml^{-1}; Str, 200 μg ml^{-1}. Calcofluor (Cellufluor) was added at 200 μg ml^{-1} (Long *et al.*, 1988).

Genetic and molecular techniques

Tn*5* mutagenesis of strain CIAT899 was conducted with the suicide plasmid vehicle, pGS9 (Selvaraj and Iyer, 1983). Tn*5*-containing transconjugants of CIAT899 were selected on Bergersen's mannitol-salts minimal medium (BSM) (Bergersen, 1961) with Sp and Km added. Colonies were screened visually for non-mucoid colony morphology. Putative EPS-deficient mutants arose at a frequency of 10^{-7}/recipient (10^{-4}/random Tn*5* mutant). One mutant from each of 20 independent mutageneses was streaked and colony-purified for further analysis (Table 3). Plasmids were mobilized into *Rhizobium* strains from *E. coli* by triparental matings with pRK2013 as a helper plasmid (Figurski and Helinski, 1979). Genomic DNA was isolated from *R. tropici* strains using CTAB to differentially precipitate EPS (Ausubel *et al.*, 1987). Plasmid DNA was isolated from *E. coli* by alkaline lysis and competent cells were prepared by the $CaCl_2$ method (Maniatis *et al.*, 1982). Restriction digests and agarose gel electrophoresis were performed according to standard methods. For Southern blot analysis, DNA was transferred to Magna nylon membrane (Micro Separations Inc.) according to the manufacturer's instructions. Digoxigenin labelling of probes, hybridization, and blot development were performed with a non-radioactive labelling and development kit (Boehringer-Mannheim Corp.).

To determine the linkage between the Tn*5* insertion and the Exo^- phenotype, pJB3 (Tc^R) was mobilized into all of the mutants (Sp^R Km^R) (Diebold and Noel, 1989; Brewin *et al.*, 1980). pJB3-containing mutants (Sp^R Km^R Tc^R) were mated with CT900, transconjugants were selected on BSM SpKmStr, and colonies were scored for mucoid or non-mucoid morphology. Spontaneous Str^R colonies of CIAT899 derivatives arose at a frequency of 10^{-6} and spontaneous Km^R colonies arose at less than 10^{-8}.

Construction of a gene library of strain CIAT899

Genomic DNA of CIAT899 was partially digested with *Sau*3A and the fragments were separated by agarose gel electrophoresis. Fragments sized between 20 and 25 kb were recovered from the gel by electro-elution and ligated into the unique *Bgl*II site of the cosmid vector pLA2917 (Allen and Hanson, 1985). The insert DNA interrupted the Km^R marker on the cosmid vector. Cosmids were packaged in lambda phage heads (DNA Packaging Kit, Boehringer-Mannheim Corp.) and transfected into *E. coli* strain DH5α resulting in 1263 Tc^R Km^S transformants. *Eco*RI restriction analysis of 25 random cosmids showed that the mean insert size was 24 kb.

The Km^R markers of Tn*5* and pLA2917 are from the same source (Allen and Hanson, 1985); therefore, it was important to verify that the cosmid did not insert into the genome by homologous recombination with the Tn*5*. Gels of *Hin*dIII digests of genomic DNA of the mutant were blotted and probed with pGS9, the Tn*5*-carrying vector. Tn*5* has two *Hin*dIII sites which generate a 3.4 kb internal fragment and two 1.2 kb flanking fragments. In each mutant, pGS9 hybridized to the three bands characteristic of Tn*5*. In cosmid-carrying mutants, pGS9 hybridized to two additional bands representing the interrupted Km^R marker of pLA2917 that flanks CIAT899 insert DNA.

Physiological characterization

Growth rates were determined by monitoring optical density at 600 nm of the culture during the logarithmic growth phase. Motility was assessed on soft TY agar (0.3% agar) by measuring the swarm diameter resulting from a point inoculum after 3 d of growth. Seed-exudate agar was prepared by germinating surface-disinfected beans in sterile water. After 4 d, equal parts of exudate, diluted to 1 ml per seed, were mixed with 3% water agar to prepare solid medium. The seed-exudate medium was supplemented with 0.1% yeast extract or 1% mannitol.

EPS production was quantified as described previously (O'Connell *et al.*, 1990). Ethanol-precipitable material (EPM) was recovered from the supernatants of three-day-old YM broth cultures. The proportion of ethanol-precipitable material represented by hexose was determined by the phenol–sulphuric acid assay for hexoses (Ashwell, 1966). Glucose was used as the hexose standard. Glucose equivalents were defined as the mass of EPM accounted for by the hexoses.

Lipopolysaccharide was examined by SDS–PAGE and silver staining as described previously (Diebold and Noel, 1989).

Symbiotic characterization

The common black bean cultivar, WBR22-34 (Bliss *et al.*, 1989), was used for all bean experiments. Planting, seed inoculation, and plant growth were carried out essentially as described previously (Araujo *et al.*, 1986; Beattie *et al.*, 1989; Beattie and Handelsman, 1989). Surface-disinfected seeds were planted individually in large test tubes that had been filled with a sand:vermiculite (1:1) mixture and then sterilized. Assays of the symbiotic phenotype on siratro were performed as described for bean except the plants were grown at 28°C rather than 24°C. In addition, 40–50 surface-disinfected siratro seeds were sown in a sterile mixture of Jiffy mix and vermiculite in modified Leonard jars (Vincent, 1970) and inoculated with 10 ml of a saturated three-day-old YM broth culture.

For chlorosis assays, bean seeds were inoculated with 1 ml of a saturated three-day-old YM broth culture. Bean plants were scored for interveinal chlorosis on a scale of 1 (no chlorosis) to 5 (severe chlorosis) 21–25 d after planting (O'Connell *et al.*, 1990).

Acetylene-reduction assays (Hardy *et al.*, 1968) were conducted on intact root systems in the growth tubes at 3 weeks after planting for bean and 4 to 6 weeks after planting for siratro. Roots were subsequently examined for nodules.

For competition experiments, YM broth cultures were grown for 2 d at 28°C and the culture density was adjusted to OD_{600} of 0.1 (approximately 10^8 cfu ml^{-1}). One millitre of these single inoculum suspensions was applied to seeds immediately after planting. Mixtures of the diluted cultures were prepared by combining the single inoculum suspensions at three ratios and 1 ml of the appropriate mixed inoculum was applied to each seed. Exact ratios of the mixed inocula were calculated following enumeration of the cell density of the single inoculum suspensions. The range

of inoculum ratios applied to seeds (CIAT899:mutant) was 15:1 to 1:21. At 21 d after planting, nodules were removed and nodule isolates were identified according to growth on YM agar containing appropriate antibiotics and by colony morphology (Beattie and Handelsman, 1989). The CI was calculated using a mathematical model that relates the ratio of the competing strains in the inoculum to their ratio in the nodules (Beattie *et al.*, 1989).

$$\log \frac{P_{CIAT899} + P_{CIAT899+mutant}}{P_{mutant} + P_{CIAT899+mutant}} =$$

$$CI_{CIAT899:mutant} + k\log \frac{I_{CIAT899}}{I_{mutant}},$$

where $P_{CIAT899}$ is the proportion of nodules occupied by only CIAT899, P_{mutant} is the proportion of nodules occupied by only the mutant, $P_{CIAT899+mutant}$ is the proportion of nodules occupied by both strains, $I_{CIAT899}/I_{mutant}$ is the ratio of CIAT899 to the mutant in the inoculum, k is a constant, and $CI_{CIAT899:mutant}$ is the competitiveness index.

Microscopy

Nodules were removed from bean plants at 3 weeks and from siratro plants at 4–6 weeks, fixed, embedded in Spurr's medium (Spurr, 1976), sectioned with a microtome, and stained as described by Vandenbosch *et al.* (1985) for examination by light microscopy. At least six bean nodules per treatment and 12 siratro nodules per treatment were examined microscopically. Representative cross-sections were photographed.

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