Characterization of Two Plasmid-borne *lps* β Loci of *Rhizobium etli* Required for Lipopolysaccharide Synthesis and for Optimal Interaction with Plants

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Received 13 March 1997. Accepted 11 June 1997.

In *Rhizobium etli* CFN42, both the symbiotic plasmid (pd) and plasmid b (pb) are required for effective bean nodulation. This is due to the presence on pb of a region (*lps* β) involved in lipopolysaccharide (LPS) biosynthesis. We report here the genetic array and functional features of this plasmid-borne region. The sequence analysis of a 3,595-bp fragment revealed the presence of a transcriptional unit integrated by two open reading frames (*lps* β 1 and *lps* β 2) essential for LPS biosynthesis and symbiosis. The *lps* β 1 encodes a putative 193 amino acid polypeptide that shows strong homology with glucosyl-1P and galactosyl-1P transferases. The deduced amino acid sequence of the protein encoded by *lps* β 2 was very similar to that of proteins involved in surface polysaccharide biosynthesis, such as *Pseudomonas aeruginosa* WpbM, *Bordetella pertussis* BpIL, and *Yersinia enterocolitica* TrsG. DNA sequences homologous to *lps* β 1 and *lps* β 2 of *R. etli* CFN42 were consistently found in functionally equivalent plasmids of *R. etli*, *R. leguminosarum* bv. *viciae*, and *R. leguminosarum* bv. *trifolii* strains, but not in *R. meliloti*, *R. loti*, *R. tropici*, *R. fredii*, *Bradyrhizobium*, *Azorhizobium*, and *Agrobacterium tumefaciens*. Even though *Rhizobium* and *Agrobacterium* do not share *lps* β sequences, their presence is required for crown-gall tumor induction by *R. etli* transconjugants carrying the Ti plasmid.

Additional keywords: nitrogen fixation.

Soil bacteria of the genus *Rhizobium* are able to induce the formation of nitrogen-fixing nodules on roots of leguminous plants. This symbiotic interaction requires the expression of many bacterial and plant genes.

The genetic information in *Rhizobium* is distributed among the chromosome and numerous large plasmids. The molecular characterization of the *Rhizobium* genome has been focused mainly on the symbiotic plasmid (pSym), where most of the genes required for symbiotic nitrogen fixation are located. Many of these genes have been isolated and significant advances have been made in the study of their structure, regulation, and functional role (Schultze et al. 1994).

Plasmids other than the pSym may also influence the symbiotic process, by encoding functions such as biosynthesis of surface polysaccharides, nodulation competitiveness, and nitrogen fixation (see García-de los Santos et al. [1996] for a review). However, very few sequences from these plasmids have been isolated and characterized at the molecular level. The best-studied sequences from a nonsymbiotic plasmid are those located on the *exo* megaplasmid of *R. meliloti*, which are involved in exopolysaccharide (EPS) biosynthesis (Leigh and Walker 1994) and C₄-dicarboxylate transport (Watson 1990), and the *nfe* genes, which are located on a self-transmissible plasmid of *R. meliloti* GR4 and are involved in nodule efficiency and competitiveness (Soto et al. 1994). *R. etli* CFN42 (formerly *Rhizobium phaseoli* [Segovia et al. 1993]) contains six plasmids (pa to pf), ranging in size from 180 to 600 kb (Quinto et al. 1982). Analysis of derivatives cured of each individual plasmid has shown that in addition to the symbiotic plasmid pd, a second plasmid, pb, is also indispensable for nodule formation, due to the presence on this plasmid of sequences required for lipopolysaccharide (LPS) biosynthesis (Brom et al. 1992).

LPS is the major structural component of gram-negative bacteria outer membrane, and plays an important role in bacteria-host interactions. Although its precise function in the symbiotic process is still unclear, it has been suggested that it participates in evasion of plant defense mechanisms, or in bacteroid release into the nodules (Noel 1992).

Mutants of *R. etli* CFN42 defective in LPS biosynthesis have been isolated through Tn5 insertion. Such mutants are less glossy than the wild type, clump in liquid culture, are nonmotile on swarm plates, lack O-antigen in the LPS, and induce the formation of incompletely developed (Ndv⁻ Fix⁻) nodules (Cava et al. 1989). Three distinct genetic *lps* regions have been identified. Two separate regions, named α and γ , have been associated with the chromosome, and a third one, named β , has been located on pb (Brom et al. 1992; Noel 1992). The *lps* α is the best characterized of these three LPS regions. It comprises nine complementation groups spanning over 18 kb of chromosomal DNA (Cava et al. 1990). Partial chemical characterization of the LPSs synthesized by strain CFN42 and derivatives with mutations in the α region (Carlson et al. 1987, 1989) led to the assignment of probable biosynthetic functions for particular genetic loci in this region (Carlson et al. 1995). However, definitive confirmation of the biochemical function of individual gene products is still lack-

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Nucleotide and/or amino acid sequence data are to be found at GenBank as accession number U56723.

ing. A common *lps* locus located on the chromosome of *R. leguminosarum* bvs. *viciae* and *phaseoli* has been recently described. Its 2-kb region contains two genes, apparently involved in the sequential addition of galactose and 3-deoxy-D-manno-2-octulosonic acid (Kdo) to the core tetrasaccharide. The name δ -*lps* has been suggested for this locus since these genes do not correspond to any of the previously identified regions (Allaway et al. 1996).

In contrast to the well-characterized *lps* α , little information is available on the plasmid-borne *lps* β region. Within this region, contained in the 7.5-kb *EcoRI* fragment of pDEL27 (Brink et al. 1990; Cava et al. 1989), only one Tn5 insertion affecting LPS synthesis has been located (Cava et al. 1989). pDEL27 complements an LPS⁻ Ndv⁻ Fix⁻ plasmid-less derivative of *R. leguminosarum* bv. *viciae* VF39 (Hynes and MacGregor 1990) and a *R. leguminosarum* bv. *trifolii* ANU843 Tn5 mutant (Brink et al. 1990) for the flocculation phenotype characteristic of LPS mutants and for effective nodulation. These data suggest that homologous sequences could be located on plasmids of these strains. Support for this proposal would be gained by visualizing the structural homology among them and by showing that *R. leguminosarum* bvs. *viciae* and *trifolii* LPS sequences also complement the *R. etli*, as well as their own LPS⁻ derivatives.

In contrast to *R. etli* CFN42 in which, in addition to the pSym, the presence of the *lps* β region of pb is essential for nodulation, the *A. tumefaciens* GMI9023 strain harboring only the pSym from *R. etli* CFN42 induces nodule formation on bean plants (Brom et al. 1988). Such data suggest a functional equivalence among surface polysaccharides from both *A. tumefaciens* and *R. etli*.

To initiate the analysis of *R. etli* plasmids other than the pSym, we chose the *lps* β region of pb due to its relevance in symbiosis. In this paper, we report its genetic analysis, as well as the DNA sequence of two open reading frames (ORFs) designated *lps* β 1 and *lps* β 2 that are indispensable for LPS biosynthesis and symbiosis. Also, we determined the distribution of these *lps* β ORFs among *Rhizobiaceae* by structural hybridization and functional complementation, and examined their involvement in crown-gall tumor induction.

RESULTS

Genetic analysis and nucleotide sequence of the *lps* β region.

A 7.5-kb *EcoRI* fragment carried by plasmid pDEL27 was previously identified as the *lps* β region of *R. etli* CFN42, through complementation of insertional LPS mutants (Brink et al. 1990; Cava et al. 1989). The restriction map of this fragment is shown in Figure 1A. Four subclones were constructed and analyzed for complementation of the Tn5 mutant CE168 and the pb-cured derivative CFNX230. Among the clones tested, pAGS10 was able to complement both mutants for flocculation, nodulation, and nitrogen fixation, while pAGS11 and pAGS13 were unable to complement either. Clone pAGS15 complemented only the insertional mutant CE168. The fact that the plasmid-cured mutant CFNX230 was not complemented by pAGS15 suggests that, in addition to the gene interrupted by Tn5 in strain CE168, at least another gene is required for LPS synthesis. To confirm this possibility, the *EcoRI*-*Bam*HI fragment (3,595 bp) from pAGS10 was se-

quenced as described in Materials and Methods, with the clones shown in Figure 1B.

The DNA sequence of this fragment together with the deduced amino acid sequences are shown in Figure 2. Analysis of the DNA sequence shows the presence of one incomplete ORF, ORF1, and two complete ORFs, *lps* β 1 and *lps* β 2 (Fig. 2). The deduced amino acid sequence of each coding region was compared with the protein data bases contained in the NCBI (see Material and Methods). Sequences similar to polypeptides encoded in ORF1, *lps* β 1, and *lps* β 2 are shown in Table 1. The incomplete ORF1 (520 bp) constitutes the 3' region of a hypothetical gene. The product encoded by this partial ORF (173 amino acids) is 60% identical to the carboxy termini of *R. leguminosarum* RepC (Turner and Young 1995) and *A. rhizogenes* RepC (Nishiguchi et al. 1987) proteins involved in plasmid replication.

Downstream of ORF1, we located *lps* β 1, which is 582 bp long and potentially encodes a 193 amino acid protein. ORF1 and *lps* β 1 are separated by a noncoding 258-bp track, which

Fig. 1. Physical map and complementation analysis of CFN42 *lps* β . **A**, Restriction map of the 7.5-kb *EcoRI* fragment of cosmid pDEL27. Subclones introduced by conjugation into CE168 and CFNX230 LPS⁻ ndv⁻ strains are shown below restriction map. In pAGS13, a deletion of the 715-bp *Xho*I-*Xho*I fragment is indicated by a dotted line. Transconjugants were checked for complementation of flocculation, nodulation, and nitrogen fixation phenotypes. **B**, Subclones used for DNA sequencing of the 3,595-bp *Eco*RI-*Bam*HI fragment. **C**, Deduced ORF1, *Lps* β 1, and *Lps* β 2 are indicated below the pAGS10 subclone. Location of the Tn5 insertion in strain CE168 is indicated by arrowhead. Abbreviations: E, *Eco*RI; P, *Pst*I; S, *Sal*I; X, *Xho*I; B, *Bam*HI; H, *Hind*III. Symbols: +, complementing subclone; -, non-complementing subclone.

contains a potential σ^{70} promoter, and a putative ribosome binding site (Fig. 2).

The polypeptide encoded by *lps* β 1 shows high-level sequence similarity with a number of proteins (Table 1). Among them are Cap5M and Cap8M, whose precise function is unknown, although they are involved in the synthesis of capsular polysaccharide (CPS) in *Staphylococcus aureus* (Sau and Lee 1996). A significant homology over the entire length of the protein was also found with *Campylobacter hyoilei* RfbP, *R. leguminosarum* Pss2, *R. meliloti* ExoY, *Salmonella enterica* RfbP, *Vibrio cholerae* Orf7, and *Anabaena* RfbP. All of them

are potential galactosyltransferases involved in the synthesis of surface polysaccharides. The Lps β 1 protein also showed a significant similarity with the C-terminal part of *S. typhimurium* RfbP and *Xanthomonas campestris* GumD, the best-studied sugar transferases. RfbP is a galactosyltransferase, which catalyzes the transfer of UDP-galactose to the C55 lipid carrier. GumD catalyzes the transfer of UDP-glucose to an isoprenyl phosphate carrier. Both enzymes participate in the first step of polysaccharide synthesis.

The alignment of the amino acid sequences of Lps β 1 with Pss2 (Borthakur et al. 1988), ExoY (Müller et al. 1993), GumD

Fig. 2. Nucleotide sequence of the 3,595-bp *EcoRI*-*BamHI* fragment from *Rhizobium etli* CFN42 *lps* β region. Derived amino acid sequences of ORF1, *lps* β 1, and *lps* β 2 are given in the standard one-letter code. Putative ATG start codons are marked in bold. Potential σ^{70} promoters are overlined; potential ribosome-binding sites are underlined. Restriction enzyme sites corresponding to those shown on physical map in A are in bold. Putative NAD-binding site in *lps* β 2 is boxed. The Tn5 insertion in *lps* β 2 is indicated (▼) and the nucleotides among which the Tn5 was inserted are underlined. Stop codons are in bold and indicated by an asterisk.

(Coplin and Cook 1990), and *S. typhimurium* RfbP (Jiang et al. 1991) is shown in Figure 3. In addition to the conserved residues among all these proteins, their potential transmembrane regions are also highly conserved. The *lps*β2 gene contains 2,052 bp and presumably encodes a protein of 683 amino acids (Fig. 2). The *lps*β1 and *lps*β2 genes are separated by a noncoding region of 123 bp in which no σ^{70} promoter sequence was detected by sequence analysis. This *lps*β2 is preceded by a putative Shine-Dalgarno ribosome-binding site.

Analysis of the deduced amino acid sequence of *lps*β2 indicates that this polypeptide shows significant similarity with proteins required for polysaccharide synthesis (Table 1). The most striking similarity was found with the products of *Pseudomonas aeruginosa* wpbM, *Bordetella pertussis* bplL, and *Yersinia enterocolitica* trsG over their entire lengths. More limited homology was found among the Lpsβ2 and other smaller proteins (Table 1): *Vibrio cholerae* ORF11, *Neisseria meningitidis* GalE, *N. meningitidis* RfbB, *R. meliloti* ExoB, and *N. gonorrhoeae* RfbB. The homology of these proteins with the Lpsβ2 extends from the middle of the protein to the C-terminal end (Fig. 4). GalE and ExoB enzymes catalyze the epimerization of UDP-glucose to UDP-galactose, while RfbB are enzymes involved in synthesizing 6-deoxy and dideoxy sugars.

The alignment of the amino acid sequence from WpbM (Burrows et al. 1996), BplL (Allen and Maskell 1996), TrsG (Skurnik et al. 1995), ExoB (Buendía et al. 1991), GalE (Jennings et al. 1993), and *N. meningitidis* RfbB (Jennings et al. 1993) with Lpsβ2 is shown in Figure 4. As can be seen, the degree of homology among all these proteins increases toward the C-terminal end: *P. aeruginosa* (61.2% identity), *B. pertussis* (58.9% identity), *Y. enterocolitica* (56% identity).

All these proteins have potential transmembrane domains analogous to those observed in Lpsβ2 (Fig. 4).

As in other epimerases (Wyk and Reeves 1989) and dehydratases (Macpherson et al. 1994), a putative NAD-binding domain was also located in all seven proteins between amino acids 305 and 335. These sequences align very well with the consen-

sus fingerprint proposed by Wierenga et al. (1986), especially in the three glycine residues and the acid residue at the end of the fingerprint, which are strictly required for the βαβ three-dimensional structure of NAD-dependent proteins. (Fig. 4).

The precise site of the Tn5 insertion of strain CE168 was mapped by sequencing two polymerase chain reaction (PCR) products (see Materials and Methods) and found to interrupt the *lps*β2 near to its C-terminal end (Fig. 2).

Localization of sequences homologous to *R. etli* CFN42 *lps*β in rhizobia.

The 7.5-kb *Eco*RI fragment from pDEL27 was subcloned in pSUP202 and used as probe in high stringency hybridizations with blots of *Eco*RI-digested total genomic DNA of different strains (Table 2). Homology was detected in all *R. etli* (9 strains), *R. leguminosarum* bv. *viciae* (3 strains), and *R. leguminosarum* bv. *trifolii* (6 strains) tested. Homology was not detected in *R. meliloti* (5 strains), *R. tropici* (8 strains), *Rhizobium* sp. NGR 234, *R. fredii* USDA 191, *R. loti* NZP 2037, *A. caulinodans* ORS 571, *B. japonicum* (3 strains), and *A. tumefaciens* (4 strains).

Plasmid profiles from the *R. etli* and *R. leguminosarum* strains were also hybridized with an internal probe of *lps*β1 (see Materials and Methods) and with pAGS4 containing an internal fragment of *lps*β2. In all cases, homologous sequences to both ORFs were present in the same plasmid. However, the size of the hybridizing plasmid varied among strains. Some examples are shown in Figure 5.

Functional equivalence among *R. etli* and *R. leguminosarum* plasmid-borne LPS sequences.

The *lps*β-plasmid cured derivatives of two *R. etli* strains (CFN42 and TAL182) and one *R. leguminosarum* bv. *trifolii* (ANU 843) strain were used as receptors to construct transconjugants containing *R. etli* (pCFN42b, pTAL182a, pVikingIb), *R. leguminosarum* bv. *viciae* (pVF39c), or *R. leguminosarum* bv. *trifolii* (pANU843b) *lps*β-carrying plasmids.

Table 1. Identity and similarity of ORF1, Lpsβ1, and Lpsβ2 with homologous proteins^a

ORFs (length) ^b	Similar polypeptides	Length ^b	Degree of identity/similarity	Putative functions	Data base accession number
ORF1 (173)	<i>Rhizobium leguminosarum</i> RepC	405	60.8/74.1	Plasmid replication	X89447
	<i>Agrobacterium rhizogenes</i> RepC	405	60.5/73.4	Plasmid replication	X04833
Lpsβ1 (193)	<i>Staphylococcus aureus</i> Cap5M	185	43.4/66.8	Undetermined	U81973
	<i>Staphylococcus aureus</i> Cap8M	185	42.9/66.8	Undetermined	U73374
	<i>Campylobacter hyoilei</i> RfbP	200	39.0/63.1	Galactosyltransferase	X91081
	<i>R. leguminosarum</i> Pss2	200	38.6/61.3	Undetermined	P10498
	<i>R. meliloti</i> ExoY	226	37.9/60.9	Galactosyltransferase	Q02731
	<i>Salmonella enterica</i> RfbP	125	37.8/60.5	Galactosyltransferase	X60666
	<i>Vibrio cholerae</i> Orf7	186	36.9/55.9	Galactosyltransferase	U47057
	<i>Anabaena</i> sp. RfbP	252	33.1/56.8	Galactosyltransferase	U75690
	<i>Salmonella typhimurium</i> RfbP	476	38.9/62.1	Galactosyltransferase	S15314
	<i>Xanthomonas campestris</i> GumD	484	38.4/64.7	Glucosyltransferase	U22511
	<i>Pseudomonas aeruginosa</i> WbpM	665	51.0/69.0	Epimerase or dehydratase	U50396
Lpsβ2 (683)	<i>Bordetella pertussis</i> BplL	624	45.5/64.8	dTDP-glucose 4,6-dehydratase	X90711
	<i>Yersinia enterocolitica</i> TrsG	636	45.1/66.2	dTDP-glucose 4,6-dehydratase	Z47767
	<i>S. aureus</i> CapD	599	38.0/63.6	Undetermined	U10927
	<i>V. cholerae</i> ORF11	378	54.1/69.8	Undetermined	U47057
	<i>Neisseria meningitidis</i> GalE	339	22.7/48.0	UDP-glucose 4-epimerase	L09188
	<i>N. meningitidis</i> RfbB	360	22.0/46.0	dTDP-glucose 4,6-dehydratase	L09188
	<i>R. meliloti</i> ExoB	328	21.6/45.0	UDP-glucose 4-epimerase	P26503
	<i>N. gonorrhoeae</i> RfbB	346	21.0/46.5	dTDP-glucose 4,6-dehydratase	P37761

^a Values are in percentage (see Materials and Methods).

^b Amino acids.

The free-living and symbiotic phenotype of these transconjugants was determined (Table 3). It can be seen that all the plasmids cross-complement the plasmid-less derivatives for flocculation and motility as well as for effective nodulation. Despite repeated attempts, no transconjugants could be obtained with a plasmid-cured derivative of *R. leguminosarum* bv. *viciae* VF39 as receptor.

*lps*β is required for tumor induction by rhizobia harboring Ti plasmids.

Rhizobia carrying *Agrobacterium* Ti plasmid are capable of inducing tumor formation (Hooykas et al. 1977). We took ad-

vantage of this fact to determine if the surface polysaccharide alteration of a *R. etli* CFN42 *lps*β mutant affects the tumor formation process. The Ti plasmid of *A. tumefaciens* strain NT1 was introduced into the wild-type *R. etli* strain CFN42, its pb⁻ derivative (CFNX183), and finally the pb⁻ transconjugant complemented with the *lps*β-carrying cosmid pDEL27 (CFNX188). Interestingly, plasmid a of CFN42 was always lost upon introduction of the Ti plasmid, suggesting that these plasmids are incompatible.

The tumor-inducing capacity of the different derivatives was examined on *Kalanchoe* plants (Table 4). In plants inoculated with *A. tumefaciens* NT1, CFNX227 (CFN42/pTi), or

StRfbP		...mdnidn	kynpqlckif	LaIsDLiffn	lalwfsllgcV	YfiFdqvqrF	ipqdqldrv	56
XcGumD	mlladlssa	tyttssprll	skysaaadlv	LrVfDLtmv	asgliayriV	FgtWvpaapY	rvaiaattlly	69
Consensus	-----	-----	-----	L---DL---	-----V	-----	-----	
				*	**	*		
StRfbP	ithfilsvvc	vgwFWiRlrh	ytyrkpfwye	lkeifrtiVi	faIfdlalia	ftkWqfsRYV	wvFcwtfAli	126
XcGumD	svicfalflp	yrswRgRgll	selvvlggaf	ggvfalfavh	alIvqvgeqv	srGWvglWVF	ggLvslvAar	139
Consensus	-----	-----R---	-----	-----V-	--I-----	---W-----V	-----A--	
		*		*	*	*	*	
StRfbP	lvpffraltk	hllnklgiWk	kktiilgsgq	nargaySalq	seeMmgfdvi	affdtdAsda	einmLPvikD	196
XcGumD	tllrgflnhl	rtqgvdvqRv	vvvglrhvpm	kishylSrnp	wvgMnmvgyf	rtpydlAvae	qrqgLpclgD	209
Consensus	-----	-----	-----	-----S---	---M-----	---LP---	---D	
				*	*	*	**	*
StRfbP	tEiiwDlnrt	gdVhyIlaye	ytelekthfw	Lrelskhhcr	SVTivVPSFrg	LpLyNtdmsF	IfShEVmlLR	266
XcGumD	pDeliEylkn	nqVeqVwisl	plgerdhikq	Llqrldrypi	nVklVPdLfd	FgLlNqsaec	IgSvpVinLR	279
RmExoY			mk	SaTrsaS...	spF	fipeEtgavR	22	
Consensus	-----	--V-----	-----	L-----	SVT-VPS---	--L-N-----F	I-S-EV---LR	
		*	*				*	
StRfbP	iqnnlAKrSs	rFlkrtFDIV	csimiliIaS	PLMiYlWkV	t.rdgGPaiY	ghqRVGRhGk	lFpcYKFRSM	335
XcGumD	qgGvdrcnyF	VvakALqDki	LAVIALMgLv	PLMLaIAvgV	KMSPPGPVFF	rqRRhGlgGr	eFymFKFRSM	349
RmExoY	piGmAkrS.	FDVL	aAsVALLIFS	PLFLlImaLV	KFSdgGsVFY	ghRRIGHNGq	sFKcLKFRtM	85
RlPss2		mdL VLKRAF.DIF	sslsALLVLA	PFLFLVALLI	KLdSPGPVLF	kqtRwGkNck	aiKvYKFRSM	62
ReLpsB1	...meevlgm	gLKrAv.DfL	LAlIASvILl	vpilvVAFsV	rLtSPGPIly	wsKRIGRfnq	iFlmpKFRSM	66
Consensus	--G--AK-S-	VLK-A-FDIL	LA-IALILIS	PLML--A-LV	KLSSPGPV-Y	--RRIGRNG-	-FK-YKFRSM	
		*		*		*	***	*
StRfbP	vmnsqEVLkE	LLandPiAra	EWekdfKLKN	DPR.ITaVGr	FiRktSLDEL	PQLFNVLkGD	MSlVGPRPI.	403
XcGumD	RVhdDhgtT.		IqQatKN	DtR.ITRfGs	FLRrsSLDEL	PQiFNVLgGs	MSlVGPRP..	402
RmExoY	mekgDEVLeE	FFrinPdAye	EWratrKLqN	DPR.VTtVGa	vLRKlSLDEL	PQLNtiirGE	MSVVGPRPV.	153
RlPss2	RtDlCDVsg.		VaQtvKN	DPR.ITRIGA	iLRrtnvDEL	PQLNVLIgh	MSVVGPRc..	115
LpsB1	RVDtptVaT.		hlLeN	perflTpIGS	FLRKsSLDEL	PQLWcILaGk	MSFVGpaly	120
Consensus	RVD-DEVlTE	-----P-A--	EW---QKLKN	DPR-ITR-G-	FLRK-SLDEL	PQL-NVL-G-	MSVVGPRP--	
			*	*	*	*	***	*
StRfbP	...vsDELE	rYcDdVdYYL	ma...kPGMT	GLwQVsGrnd	VdYdt	RVyFDswYVv	NwTLWmDial	461
XcGumD	HAA...qhnt	hYEKLInhYM	QRHyvkPGiT	GwAQVnGfRg	ETPelrtmkk	RIqYDLdYIr	RWSLWLDiRI	469
RmExoY	...veDELE	LYDsaavFYl	rs...rPGLT	GLwQIsGrnd	VsYaT	RVAFDttqYVq	NWSLFAdLVI	211
RlPss2	HAigmraggm	LYEELVpeYh	QRHaMrPGMT	GLAQmrGlrG	pTdrpakara	RIASDLrYVg	NFSivMDMRI	185
LpsB1	nqydliElrt	vYgv.....	..dkLlPGLT	GwAQInG.RD	ElP...ipe	kVvFDveYle	rRSLGfDMRI	177
Consensus	HA----DEL-	LYE-LV--YL	QRH---PG-T	GLAQ--G-RD	ETP--V-Y-T	RVAFDL-YV-	NWSLW-DMRI	
		*	*	*	*	*	*	*
StRfbP	IFkTAKrVLR	RdGAY.	476					
ExoY	IvltAVrVLg	srGsY.	484					
XcGumD	VFkTipaVcl	qKtAY.	226					
RlPss2	IFgTvVs=lt	RgkgF.	200					
LpsB1	IFlTAcKvVR	RKGikh	193					
Consensus	IF-TAV-VLR	RKGAY-						
	*							

Fig. 3. Alignment of the deduced amino acid sequence of *Rhizobium etli* Lpsβ1 with galactosyl or glucosyl transferases from *Salmonella typhimurium* RfbP, *Xanthomonas campestris* GumD, *R. meliloti* ExoY, *R. leguminosarum* Pss2. Amino acid residues identical in all five proteins are indicated by asterisks. Highly conserved hydrophobic regions among all these proteins are boxed.

CFNX229 (CFNX188/pTi), the presence of tumors was evident 3 weeks after inoculation, whereas, in plants infected with the *lps* β ⁻ derivative (CFNX228), only after 6 weeks did tumors start to emerge. The pTi carried by strain CFNX228 was introduced by conjugation into *A. tumefaciens* GMI9023. The resulting strain (GMI9023-1) formed visible tumors 3 weeks after inoculation, indicating that the altered phenotype of CFNX228 was solely due to the absence of the *lps* β -carrying plasmid (Table 4).

DISCUSSION

In this paper, we have described the identification of two ORFs (*lps* β 1 and *lps* β 2) located in the *lps* β region of pb of

R. etli CFN42. The genetic analysis of this region indicates that both ORFs are indispensable for LPS biosynthesis and nodule infection. pAGS15 has a deletion covering 399 bp from the 5' end of *lps* β 1. The presence of an incomplete *lps* β 1 impaired the ability of pAGS15 to complement the CFNX230 pb-cured derivative, which lacks both *lps* β 1 and *lps* β 2, but did not affect its capacity to complement the *lps* β 2::Tn5 mutant (CE168). CFNX230 could only be complemented by pAGS10 containing the entire *lps* β 1 and *lps* β 2 genes. The presence of a putative σ^{70} promoter upstream of *lps* β 1 (Fig. 2) and the absence of a transcription termination structure in the spacing sequence between *lps* β 1 and *lps* β 2 support our hypothesis that *lps* β 1 and *lps* β 2 form a tran-

LpsB2	mpenttptetp rrrfvlvMq AlvapLLaMP RaaKRoLAIL vDsgLcvLti WLAYcRLne Wtv...LVGV dWLoVfysl cMAInPiFvW GMYRAIFRYa	96
YeTrsG	 mPIVFLsLP RPyKRTimil LDtILlialAY WaAFvRL...DvdSPPTsI cswvalAait pptLlAYikL GLYRVVLRVY	77
PaWbpM	 MLd nLRlKLGLP RYKRMLcva aDvLVWLSL WLAFVRLet eDmISPPsGH aWLFIApLV AipLE...IrF GMYRAVMRYL	81
BpIL	 Mtlpy AArLrLVdLP RPFKMLaiv LDaVilLgAF hLdWLRPef FeltqdyVfL sLIacaggia ALA...aF GYVIVLRYM	80
Consensus	----- ALR--LL-LP RP-KRMLA-L LD--L--LA- WLAFW-RL-- -D--SPPTG- -NL--A--A-- A-ALP--I-F GMYRA-LRY-	
LpsB2	nmaaPIAVLK aTatv...va FmtiFtaLsv pcVPRTVgIL qpLILLIaIG LSRLqirYwL gdtY..... qrilhQntL akVliYAGn AGRLAGALt	187
YeTrsG	 SakiysiVLv dVLISSqllv LgsYPL...g vvlPRTVsVM PFISIVLILG GSRLffRmLL...nYgvrgq IP..... .VviYAGA sGRQLlpAlm	161
PaWbpM	 gndalIAIKa avTISaLVls LlvYVYrscp AvVPSslyfn VMWLSMILIG GLRLamRqYf mgdWysavqs VPflnrQDgU prVaLYAGA AanQLvaALR	181
BpIL	 SerylaAILG qVWVSVMVt agmtFLqL... AtISRoVLVL YaaLaVgLI GvRLiaRkLI fpadh..... hmaDpr tpVLIYAGG AGsQLanALR	169
Consensus	S---LTA-LK ---VIS--V-- L--YPL-L-- A-VPRTV-VL Y--LSLVLIG GSRL--R--L ---Y----- P----QD-L -VLIYAGA AGRLA-ALR	
LpsB2	nsaElvVgY IDDDpRLksg ImgGlpIYdP sdLpVtLaL GVhnVLLALP SASRqrRnEI lEhirkarVn VRLtPdLtaL aqGRVaVSDI RELIEEDLLG	287
YeTrsG	 qaaEYfPIAF vDdpkLkka VInGvtVYPS EkLeyLlgrY GikKvLLAMP SvsgsqRRaV vnkLhNsece VLSlPGMSdL VEGRaQISsL kkvVsIEELLG	261
PaWbpM	 lGramRPVAF IDDDkqianr VtaGRVYta khirgMIDet GageVLLaIP SATarRREI lEsLEPPpLh VRSmPGmDL tsGRVkvDdL qGVIdaLLG	281
BpIL	 tGphyRPVam IDDDkkrkIr VnGLRVYPP BqLPkLIDRH nIrqLLIAMP SAppqIRsI vBaaEPYRLr IRlvPSMrEL IDptngVr.L RDVVEDLLG	268
Consensus	-G-E-RPVAF -DD-RLH-- VI-GLRVYPP E-LP-LIDR- GI--VLAMP SASR--RREI -E-LEP-RL- VRS-PGM-DL --GRV-VSDL REV-TIEDLLG	
LpsB2	REAVAPRGL LDKamrKV VMTGAGSGIG gELCRQILrN aPSSeLILIdq nEPaLYNIHa ELLKLAeVqY hEgl...qivP ILcSVRDqDR MEhImeswRp	385
YeTrsG	 RDPVpdiRkL LdknItgKV VMTGAGSGIG SELCRQIive kPSLILFDI SEFSYsIten E...MAaICk knkietEFva ILGSVgsekR LqvImnfhV	358
PaWbpM	 RDSVAPRkEL LERCIrqqV VMTGAGSGIG SELCRQImc sPSvLILFh SEYNLYsIHQ E...LerriK rEsIsvnlIP ILGSVRnpER LvdVMrtwkV	378
BpIL	 RDPVAPIDL LgrCVtdrV VMTGAGSGIG SELCRQILal rPrkLVLFBI aEPaLYaIeQ DLrq....r igEEniElaG VLGSVRDeah claqLqEhVp	363
RmExoB	mgnni LVVgaGyIG ShtCQLlaak GyqpvV.YD...NLsNgHe EPVkw..... .gvLE... .kGdIRDRqR LDvLArhVp	69
NmGalE	mkKI LVtGgcGfIG ShtvvsLLks Ghqvwi.LD...NLNssi niLprlktit ggEipPy... .qGdIRDREI LrrIFABhRI	75
NmRfbB	mqntanKtI LVtGgaGfIG SavvRhiIn tqdsVnDl...Klyag. nLestLDIad npryaFE... .qvdrCDrae LDvVFAqhRp	80
Consensus	RDVAPR-EL L--CI--KTY VMTGAGSGIG SELCRQIL-N GPS-LILIDF SEYNLYNIHQ ELLKLA-I-K -EE--PE-VP ILGSVRDRER LD--MAE-RV	
LpsB2	qTLYHAAAYK HVP1VEHtav EG1kNNVnGT LitArAA... ..nkYg VsNFVLISTD KA..... ..VR PTNmMGASKR LAEMVLQALa	460
YeTrsG	 nTVYHAAAYK HVP1VENvI EGvNNIEGT LycAKAA... ..IksG VekFVLISTD KA..... ..VR PTNmMGATKR MAELVLQALs	433
PaWbpM	 nTVYHAAAYK HVP1VEHNIa EGV1NNVIGT LhavqAA... ..VQvG VqNFVLISTD KA..... ..VR PTNmMGATKR LAEMVLQALs	453
BpIL	 qTLYHAAAYK HVP1VEHNVs EG1rtNaGT LmAbtAA... ..IQAG VdFVLISTD KA..... ..VR PTNmMGASKR LAELVLQAHa	438
RmExoB	 raLIHfAAMI eVgesvkdpa afydnNVIGT LtLlAA... ..laAG IdaFVFSStC atYG.dPGkV PMdEs.hkga PiNpyGrtKW icEgALkdgy	158
NmGalE	 dsViHfAgIK aVgesvaeqm kyynNVsGs LvlAEem... ..arAG VfsiVFSsa tvYG.dPGkV PytEDmPlgd tTspYgSKS MvErLLtdiq	165
NmRfbB	 daVmHIAAes HVdsisgag EfiqtNIVGT FhLEAArey wggpmQhe aFrFhIISTD evYGdLhGtd dLftEttyPa PsspyASKA ssDhlvrAw.	179
Consensus	-TVYHAAAYK HVP-VEHNV- EG1-NNVIGT L-LAEAA--- -IQAG VNFVLISTD KAYG-LPG-V P--E---VR PTN-MGASKR LAE-VLQAL-	
LpsB2	 aElEtAD rL..... .rThPSMVR FGNVLSSGS VVPLFRqQik EGGPVTLTHP kItIRYFMTIS EAsQLVIQAG AMaGGGDVfL	536
YeTrsG	tEQ..... .nKtKfCMVR FGNVLSSGS VVPLFkQIA BGGPITLTHK dIIRYFMTIp EAAQLVIQAG AMGqGGDVfV	505
PaWbpM	NESapl LFGdrkdvhk vnKtrFtMVR FGNVLSSGS VVPLFRqQik rGGPVTVINP sItIRYFMTIp EAAQLVIQAG sMGqGGDVfV	539
BpIL	giQ..... .dKTrFaMVR FGNVLSSGS VVPLFRqQil EGGPITLTHP eItIRYFMTIp EAAQLVIQAG AMGqGSVfV	510
RmExoB	 lykylRSVI. LRYFNaagAD FeGIGBwhe petha.ipla idaalGzreg fkvFgtDydt rdG....Tcv RdyihvDla DhavraVdyl leGgesvaLn	262
NmGalE	 kadprMSVII LRYFNgigah eSGLIGBpn gipnnlLpyi cqvaagKlpq lavFgdDypt pG....Tgm RdyihvDla FghvaamQak ggvaGvhlFn	251
NmRfbB	LRTYglptiv tncsnnygpy hfpekLiplm ilNaLdgp. lpvlg..... .dG....mqi RdwLPvedha rA...lyQvv teGvVGETYn	255
Consensus	-----SVI- LRYFNEQ-AD --G-IGE--- -KT-F-MVR FGNVLSSGS VVPLFRQID- EGGP-TLTHP RI-RYFMTI- EAAQLVIQAG AMG-GGDVf-	
LpsB2	 LDMGEPVrIA DLArkWvLS GlTVrGvDnP eGDIElaVtG LRPGEKLYEE LLIGDmpetT eHPRIMkArE dFLWaELsk kInsLnasLD rNDMvaarAt	636
TrsG	 LDMGDPVKIi DLAKrMinLS GLsIkseEnl dGDIAIEISG LRPGEKLYEE LLIGDsVq... hHPRIMkArE imLewDdLnI llnkietacn DfnyecIRsL	603
WbpM	 LDMGpPVKII ELAEKMIhLS GLsVrsersP hGDIAIEfSG LRPGEKLYEE LLIGDnVnpT dHPmIMRAnE ehLwEaFkv vLEqLLaavE kDdsVrRqL	630
BpIL	 LDMGEPVlIr ELAErMvrlY GlTVvnsDpG dGDIEIRITG LRPGEKLYEE LLIGBdsReT lHPRIMkArE ystLPYETLmg qlrnlDsrslq mcsprGaal	619
RmExoB	 LgtGgttVvL ELIDai..... .ekVakrpfm iG.....y. aerrE..... .GDettlv anndkaRqvl GWePayDLaa itEsennWb... .sRfngGg	328
NmGalE	 LgsGraysVl Eliraf..... .e..aasgn ip.....yqi qorra..... .GDlacsF adPshktqet GwetkrsLq mMEDswrWv... .sRnpdOy	337
NmRfbB	 igghnekani EvvtictlL eelVpekpag varyBdlITf vqdrp..... .GhDvRYa vdaakiRrdL GWPLlEtPes glRktvGyLl DnktWpqnVL	347
Consensus	LDMGEPVrI- ELAE-M--LS GL-V---D-P -GDIEIEITG LRPGEKLYEE LLIGD-VR-T -HPRIMRA-E GWLPWEDLA- -LE-L--W-- DND-RR-RGL	
LpsB2	LaElVSGYss TGEVSDaFt gaetnPAhed Vnalasggn qlrttga	683
YeTrsG	 LLEaptGFqP TdgicDvVWq kthsEnAKnv Ivh.....	636
PaWbpM	 LREtVSGYAP dGEIvDwYr qrrrEP.....	665
BpIL	 LGqiVreYAs vtya.....	624
RmExoB		
NmGalE		339
NmRfbB	 nGsyrlerlg Tgk.....	360
Consensus	LGE-VSGYA- TGEI-D--- -EPA--- -	

Fig. 4. Comparison of the deduced amino acid sequence of *Rhizobium etli* LpsB2 with *Pseudomonas aeruginosa* WpbM, *Bordetella pertussis* BpIL, *Yersinia enterocolitica* TrsG, *R. meliloti* ExoB, *Neisseria meningitidis* GalE, and *N. gonorrhoeae* RfbB. Identical or conserved residues are in uppercase. Consensus shows residues that are conserved in at least 50% of the sequences; residues conserved among LpsB2, WpbM, BpIL, and TrsG sequences are in bold. Hydrophobic regions strongly conserved in the N termini of LpsB2, WpbM, BpIL, and TrsG sequences are underlined. The putative NAD-binding domain is in a box; residues identical to the consensus NAD-binding domain (Wierenga et al. 1986) are indicated by an asterisk; amino acids strictly required for the β o fold common to NAD-dependent proteins are in bold in all seven sequences.

scriptional unit. If this is true, the *lps*β2 of pAGS15, which lacks the promoter region, is probably transcribed from the *Escherichia coli lacZ* promoter, as has been reported for other *Rhizobium* genes cloned in plasmids carrying the *lacZ* promoter (Müller et al. 1993). *lps*β1 and *lps*β2 seem to be the only genes present in this region of pb involved in LPS biosynthesis, as the sequence upstream of *lps*β1 was found to be homologous to a gene involved in replication (*repC*), and the sequence downstream of *lps*β2 could be deleted without altering LPS biosynthesis.

The core oligosaccharides of *R. etli* and *R. leguminosarum* LPS have been shown to have a similar composition, constituted by two Kdo, one galactose, one mannose, and three galacturonic acid residues (Carlson et al. 1995). pDEL27 completely restores a *R. leguminosarum* bv. *trifolii* *lps*β mutant whose chemical composition displayed a drastic reduction in galactose and galacturonic acid as well as absence of the O-antigen (Brink et al. 1990). These chemical composition data, plus the striking similarity of Lpsβ1 with galactosyltransferases, suggest that Lpsβ1 may be responsible for the transfer of galactose or galacturonic acid to the core oligosaccharide. The activity of galactosyltransferase has recently been ascribed to the product of *lpcA*, a chromosomal gene located in the δ-*lps* region of *R. leguminosarum* bv. *phaseoli* RU301 (Allaway et al. 1996). The role of LpcA as a galactosyltransferase is supported by the complementation of a *R. leguminosarum* bv. *viciae* VF39 chromosomal mutant in which the galactose residue from the core tetrasaccharide is missing. Lpsβ1 is not able to substitute for LpcA, as a *R. leguminosarum* bv. *phaseoli* *lpcA* mutant is not complemented by a cosmid corresponding to *R. etli* CFN42 *lps*β region (Allaway et al. 1996). Also, the amino acid sequences of LpcA and Lpsβ1 show a low level of similarity (21% identity, 47% similarity). To explain these data, we propose that Lpsβ1 and LpcA are different sugar transferases, one of them being responsible for the transfer of galactose and the other for the transfer of galacturonic acid.

The biosynthetic pathways of bacterial polysaccharides have been shown to be linked (Breedveld et al. 1993; Diebold and Noel 1989). Similar to LPS, the EPS of *R. etli* and *R. leguminosarum* contain galactose (Diebold and Noel 1989; Canter-Cremers et al. 1990). Additionally, galactose has also been found in *R. leguminosarum* CPS (Zevenhuizen and Van Neerven 1983); these data hint that the putative galactosyltransferase encoded by *lps*β1 is a common link in the synthesis of LPS, EPS, and CPS.

*lps*β2 codes for a putative polypeptide of 683 amino acids, which showed strong homology with WpbM from *P. aeruginosa*, BpIL from *B. pertussis*, and TrsG protein from *Y. enterocolitica* serotype O:3. Although the N-terminal part of Lpsβ2, WpbM, BpIL, and TrsG showed the lowest level of homology among them (31% identity and 56% similarity, average), three potential transmembrane domains were found to be highly conserved in this fragment in all four proteins (Fig. 4), indicating the possibility that the N-terminal portions of these proteins have a similar function: anchoring the protein to the inner membrane.

Similar to that reported for WpbM and BpIL protein sequences, the region extending from amino acid 310 to the C-terminal end of *lps*β2 showed limited homology (21% identity, 46% similarity) to prokaryotic epimerases and dehydrata-

ses, including the ExoB protein from *R. meliloti*, which catalyzes the epimerization of UDP-glucose to UDP-galactose. Previous reports have shown that *R. meliloti* and *Rhizobium* sp. (*Acacia*) *exoB* mutants have multiple carbohydrate defects, including LPS biosynthesis (Leigh and Lee 1988; López-Lara et al. 1995). We obtained derivatives of mutant CE168 harboring the plasmid pMP2603 that contains the *exoB* gene of *R. leguminosarum* (Canter-Cremers et al. 1990); however, the transconjugants were not complemented by this plasmid (data not shown). Furthermore, Southern blot hybridization with the pMP2603 as probe showed sequences homologous to *exoB* located on the chromosome of *R. etli* CFN42 (data not shown). The dTDP-glucose 4,6-dehydratases are enzymes involved in the synthesis of 6-deoxy and dideoxy sugars (Wang and Gabriel 1969). These kinds of sugars have been localized in the O-polysaccharide of *R. etli* CFN42. Since *R. etli* mutant CE168 lacks the O-antigen, Lpsβ2 could be involved in the synthesis of the 6-deoxy or dideoxy sugar that links the O-antigen to the core oligosaccharide.

In the present work, we have demonstrated that all *R. etli* and *R. leguminosarum* bvs. *viciae* and *trifolii* strains tested share a common *lps* region, constituted by genes *lps*β1 and *lps*β2, which are structurally homologous to those of *R. etli* CFN42. Furthermore, this region was always localized on a plasmid, allowing us to conclude that the presence of an *lps*β plasmid is a widespread feature among, and confined to (see below), *R. etli* and *R. leguminosarum* strains.

Here, we have further shown that the native *lps*β plasmids from strains of either *R. leguminosarum* bv. *trifolii*, *R. leguminosarum* bv. *viciae*, or *R. etli* fully restore the phenotype of *R. etli* and *R. leguminosarum* bv. *trifolii* plasmid-cured derivatives.

In an extensive screening, in which a variety of *Rhizobium*, *Bradyrhizobium*, and *Azorhizobium* strains were probed with the *R. etli* *lps*β region, no homologous sequences were found under our hybridization conditions. These data could be explained by a complete absence of, or a high divergence for, these loci in these genera.

Sequences homologous to *R. etli* CFN42 *lps*β were not found in the different *Agrobacterium* strains analyzed, even though, as has been previously reported, *A. tumefaciens* GMI9023 harboring only the pSym from *R. etli* CFN42 is capable of eliciting nodule formation on *Phaseolus vulgaris* roots. Despite the fact that these species do not share *lps*β sequences, we have demonstrated that the surface polysaccharides of *Rhizobium* and *Agrobacterium* are functionally equivalent for symbiosis and crown-gall tumor induction.

The conservation of key sequences for bacterial surface polysaccharide biosynthesis in plasmids of *R. etli* and *R. leguminosarum* is an unexpected feature, as these organisms belong to taxonomically diverse lineages (Martínez-Romero 1994) with different geographical origin and host range. We have recently performed hybridization experiments with random clones containing sequences from pCFN42b as probes. Our results show that *R. leguminosarum* plasmids pANU843b and pVF39c share other sequences besides the *lps*β genes (A. García-de los Santos, *unpublished*). Furthermore, we have found that pCFN42b and pANU843b belong to the same incompatibility group (A. García-de los Santos, *unpublished*). These results suggest a broader relationship among these plasmids, not confined to the *lps*β region.

MATERIALS AND METHODS

Bacterial strains, plasmids, and growth conditions.

The different strains and plasmids used in this work are listed in Table 2. *Rhizobium*, *Bradyrhizobium*, and *Azorhizobium* strains were grown on PY (Noel et al. 1984) at 30°C. *E. coli* and *Agrobacterium* strains were grown on LB (Miller 1972) at 37 and 30°C, respectively. Antibiotics were added at the following concentrations: nalidixic acid, 20 µg ml⁻¹; rifampicin, 100 and 50 µg ml⁻¹ for *Agrobacterium* and *Rhizobium*, respectively; neomycin, 60 µg ml⁻¹; kanamycin, 30 µg ml⁻¹; spectinomycin, 75 µg ml⁻¹; tetracycline, 10 and 7.5 µg ml⁻¹ for *E. coli* and *Rhizobium*, respectively; carbenicillin, 100 µg ml⁻¹; chloramphenicol 20 µg ml⁻¹. Sucrose was added at a final concentration of 12.5% (wt/vol).

Genetic manipulation and bacterial matings.

Rhizobium derivatives carrying Tn5-*sac-mob* insertions in

plasmids were obtained as previously described (Hynes et al. 1989). The Tn5-*sac-mob*-labeled derivatives were used to generate plasmid-cured derivatives through selection of sucrose-resistant derivatives as described (Brom et al. 1992), and also as donors to construct transconjugants harboring them. Transconjugants were obtained by conjugation, in triparental crosses with pRK2013 (Figurski and Helinski 1979) as a helper and selecting for suitable markers on PY plates. Transformation of *E. coli* HB101 strains (Boyer and Roulland-Dussoix 1969) was done as described (Sambrook et al. 1989). The strains constructed in this work are listed in Table 2.

Filter blot hybridization and plasmid profiles.

Genomic DNA was isolated, digested with *EcoRI*, electrophoresed in 1% agarose gels, blotted onto nitrocellulose membranes, and hybridized under stringent conditions as described elsewhere (Flores et al. 1987). Plasmid patterns were

Table 2. Strains and plasmids used in this study

Strain	Homology to <i>lps</i> β	Relevant characteristics	Reference or source ^a
<i>Rhizobium etli</i>			
CFN42	(+) ^b	Wild type	Quinto et al. 1982
CE168	(+)	CFN42 <i>lps</i> β::Tn5, Lps ⁻ , Ndv ⁻	Cava et al. 1989
CFNX183	(-)	CFN42 cured of pCFN42b	Brom et al. 1992
CFNX188	(+)	CFNX183 harboring pDEL27	Brom et al. 1992
CFNX190	(+)	CFNX183 harboring <i>R. etli</i> CFN42 plasmid pCFN42b::Tn5- <i>mob</i>	Brom et al. 1992
CFNX230	(-)	CFNX183 Rif ^r	This study
CFNX227		CFN42 harboring <i>A. tumefaciens</i> NT-1 plasmid pTiC58Tra ^c ::Tn517-52, Km ^r	This study
CFNX228		CFNX183 harboring <i>A. tumefaciens</i> NT-1 plasmid pTiC58Tra ^c ::Tn517-52, Km ^r	This study
CFNX229		CFNX228 complemented with pDEL27, Tc ^r	This study
CFNX231	(+)	CFNX230 complemented with <i>R. etli</i> plasmid pTAL182a::Tn5- <i>mob-sac</i>	This study
CFNX232	(+)	CFNX230 complemented with <i>R. etli</i> plasmid pViking Ib::Tn5- <i>mob-sac</i>	This study
CFNX233	(+)	CFNX230 complemented with <i>R. leguminosarum</i> plasmid pANU843b::Tn5- <i>mob-sac</i>	This study
CFNX234	(+)	CFNX230 complemented with <i>R. leguminosarum</i> plasmid pRleVF39c::Tn5- <i>mob-Sp</i>	This study
CFNX235	(+)	CFNX230 complemented with pAGS10	This study
Brasil 5	(+)	Wild type	Piñero et al. 1988
CFN285	(+)	Wild type	Martínez et al. 1985
Nitragin 8251	(+)	Wild type	Piñero et al. 1988
TAL 182	(+)	Wild type	Piñero et al. 1988
TAL 182-1		pTAL 182a::Tn5- <i>mob-sac</i>	This study
TAL 182-2	(-)	TAL 182 cured of plasmid pTAL182a	This study
TAL 182-3	(+)	TAL 182-2 complemented with <i>R. etli</i> plasmid pCFN42b::Tn5- <i>mob</i>	This study
TAL 182-4	(+)	TAL 182-2 complemented with <i>R. leguminosarum</i> plasmid pVF39c::Tn5- <i>mob-Sp</i>	This study
Viking I	(+)	Wild type	Piñero et al. 1988
Viking I-1		pVikingIb::Tn5- <i>mob-sac</i>	This study
<i>Agrobacterium tumefaciens</i>			
NT-1	(-)	<i>A. tumefaciens</i> NT-1 harboring plasmid pTiC58Tra ^c ::Tn517-52, Km ^r	Beck von Bodman et al. 1989
GMI9023-1		<i>A. tumefaciens</i> GMI9023 harboring plasmid pTiC58Tra ^c ::Tn517-52	This study
C-58	(-)	Vir ⁺ wild-type nopalain strain	Rosenberg and Hugnet 1984
GMI9023	(-)	C-58 cured of its native plasmids	Rosenberg and Hugnet 1984
LBA 4404	(-)	Vir ⁻	Ooms et al. 1982
EHA 101	(-)	Vir ⁺ wild type	Hood et al. 1986

^a Sources: B. G. Rolfe, Plant-Microbe Interaction Group, Australian National University, Canberra; USDA, Beltsville *Rhizobium* Culture Collection, Beltsville Agricultural Research Center, Beltsville, MD; M. F. Hynes, Soil Science Section, Agriculture Canada Research Section, Lethbridge, Alberta, Canada; W. Lotz, Institute für Mikrobiologie und Biochemie der Universität Erlangen-Nürnberg, Staudtstrasse 5, D-8520 Erlangen, Germany; C. A. Wijffelman, Department of Plant Molecular Biology, Leiden University, Nonnensteeg 3, 2311 VJ Leiden, The Netherlands; CIFN, Centro de Investigación sobre Fijación de Nitrógeno, Universidad Nacional Autónoma de México, Cuernavaca, México; CNPq, Centro Nacional de Pesquisa em Biologia do Solo, Seropédica 23851, Rio de Janeiro, Brazil; K. Goethals, Laboratorium Genetika, Universiteit Gent, Ledeganckstraat 35 B-9000 Gent, Belgium.

^b Total DNA from these strains was probed against *R. etli lps*β region, strains in which homologous sequences to *R. etli lps*β were found (+), strains that presented no hybridization signal (-).

^c Abbreviations: Lps, lipopolysaccharide; Ndv, nodule development; Km, kanamycin; Tc, tetracycline; Rif, rifampicin; Sp, spectinomycin; Cb, carbenicillin; Cm, chloramphenicol.

(continued on next page)

visualized by the Eckhardt (Eckhardt 1978) technique, blotted onto nylon membranes, and hybridized similarly. The plasmids used as probes were purified by the alkaline lysis method (Sambrook et al. 1989) and labeled with a [³²P]dCTP by nick translation (Rigby et al. 1976). The *lps*β1 internal fragment used as probe was prepared by PCR amplification of pAGS10 with primers 5'GTATCGGGCGTTTCAATC 3' and 5'CCGTCAGAAACAGGATGC 3'. Reactions were carried out with the GeneAmp PCR reagent kit (Perkin Elmer, Branchburg, NJ).

Nodulation, nitrogen fixation, and virulence assays.

For plant tests, surface-sterilized *P. vulgaris* cv. Negro jamaica seeds were germinated on soft agar plates (8 g/liter). Two-day-old seedlings were transferred to 250-ml Erlenmeyer flasks with Fahraeus agar medium (Fahraeus 1957), without added nitrogen, and inoculated with overnight-grown cultures. Plants were incubated for 15 days at 28°C. Nodulation was scored at day 15, and acetylene reduction was assayed as previously described (Martínez et al. 1987). For *R. trifolii* plant tests, surface-sterilized *Trifolium subterraneum* seeds were

Table 2. (continued from previous page)

Strain	Homology to <i>lps</i> β	Relevant characteristics	Reference or source ^a
<i>R. leguminosarum</i> bv. <i>trifolii</i>			
ANU843	(+)	Wild type	B. G. Rolfe
ANU843-1		pANU843b::Tn5- <i>mob</i> - <i>sac</i>	Stepkowski et al. 1993
ANU843-2	(-)	Cured of plasmid pANU843b Rif ^r	Stepkowski et al. 1993
ANU843-3	(+)	ANU843-2 Rif ^r complemented with <i>R. etli</i> plasmid pCFN42b::Tn5- <i>mob</i>	This study
ANU843-4	(+)	ANU843-2 Rif ^r complemented with <i>R. etli</i> plasmid pTAL 182a::Tn5- <i>mob</i> - <i>sac</i>	This study
ANU843-5	(+)	ANU843-2 Rif ^r complemented with <i>R. leguminosarum</i> plasmid pRleVF39c::Tn5- <i>mob</i> -Sp	This study
TA1	(+)	Wild type	B. G. Rolfe
24	(+)	Wild type	Kowalczyk et al. 1981
USDA1596	(+)	Wild type	USDA
USDA2134	(+)	Wild type	USDA
USDA2152	(+)	Wild type	USDA
<i>R. leguminosarum</i> bv. <i>viciae</i>			
VF39	(+)	Wild type	Hynes and MacGregor 1990
VF39-1		pRleVF39c::Tn5- <i>mob</i> -Sp ^r (RP4-4)	M.F.Hynes
LRS39301	(-)	VF39 cured of pRleVF39c	Hynes and MacGregor 1990
B-10	(+)	Wild type	W. Lotz
RBL1302	(+)	Wild type	C. A. Wijffelman
<i>R. meliloti</i>			
2011	(-)	Wild type	Cassé et al. 1979
1021	(-)	Wild type	Meade et al. 1982
M119	(-)	Wild type	CIFN
cc169	(-)	Wild type	CIFN
311	(-)	Wild type	CIFN
<i>R. tropici</i> IIA			
CFN 299	(-)	Wild type	Martínez et al. 1987
BR 829	(-)	Wild type	CNPBS
BR 10037	(-)	Wild type	CNPBS
<i>R. tropici</i> IIB			
CIAT 899	(-)	Wild type	Graham et al. 1982
BR 848	(-)	Wild type	CNPBS
BR 850	(-)	Wild type	CNPBS
BR 852	(-)	Wild type	CNPBS
<i>R. loti</i>			
NZP2037	(-)	Wild type	Crow et al. 1981
<i>R. fredii</i>			
USDA 191	(-)	Wild type	USDA
<i>Rhizobium</i> spp			
NGR234	(-)	Wild type	B. G. Rolfe
<i>Bradyrhizobium japonicum</i>			
USDA110	(-)	Wild type	USDA
USDA370	(-)	Wild type	USDA
USDA3341	(-)	Wild type	USDA
<i>Azorhizobium caulinodans</i>			
ORS571	(-)	Wild type	K. Goethals
Plasmids			
pDel27		7.5-kb <i>Eco</i> RI fragment containing the <i>lps</i> β region cloned in pLAFR1,Tc ^r	Cava et al. 1989
pAGS10		3.6-kb <i>Eco</i> RI- <i>Bam</i> HI fragment of pDEL27 cloned in pRK7813, Tc ^r	This study
pAGS4		0.718-kb <i>Xho</i> I- <i>Bam</i> HI fragment of pAGS10 cloned in pBluescript II SK ⁻ , Cb ^r	This study
pAGS11		3.2-kb <i>Eco</i> RI- <i>Hind</i> III fragment of pAGS10 cloned in pRK7813	This study
pAGS13		pAGS10 deleted of <i>Xho</i> I- <i>Xho</i> I 0.715-kb fragment	This study
pAGS15		4.3-kb <i>Sal</i> I- <i>Sal</i> I fragment of pDEL27 cloned in pMMB206, Cm ^r	This study
pMMB206		Broad-host-range plasmid vector, IncQ, <i>Pta</i> clac <i>lac</i> Z α, Cm ^r	Morales et al. 1991
pRK7813		Broad-host-range cosmid vector, Mob, IncP, Tc ^r	Jones and Guttererson 1987

inoculated on slants of Fahraeus agar medium in glass tubes (23 × 200 mm). Plants were grown for 7 weeks at 25°C in racks wrapped in aluminum foil to shade the roots and plant dry weight was then used to estimate nitrogen fixation activity. For both hosts, bacteria were isolated from surface-sterilized nodules crushed on PY plates. Single colonies were tested for antibiotic resistance and plasmid pattern. For tu-

morigenicity of *Agrobacterium* or *Rhizobium* strains carrying the Ti plasmid, they were grown on PY medium containing 0.1 mM acetosyringone and 10 mM glucose. Inoculation was carried out by puncturing the stems of *Kalanchoe* plants with a sterile needle and applying the appropriate strain with a toothpick. Plants were maintained at 28°C, and scored weekly for the presence or absence of tumors at each wound site. All plant assays were conducted three times.

DNA sequencing.

Five DNA restriction fragments from pAGS10 (Fig. 1), were cloned into the Bluescript II SK(–) phagemid vector (Stratagene, La Jolla, CA) and transformed into *E. coli* DH5α (Sambrook et al. 1989). An internal fragment of *lpsB1* was obtained by cloning the 250-bp *Sall-Sall* fragment of pAGS10 in the pMOSblue vector (Amersham, Buckinghamshire, UK). Double-stranded DNA was isolated with the Wizard Miniprep DNA purification system (Promega, Madison, WI), and sequenced with a combination of custom-made (Bio-synthesis, Lewisville, TX) and universal oligonucleotide primers with

Fig. 5. Sequences homologous to *Rhizobium etli* CFN42 *lpsβ* region located in plasmids of *R. etli*, *R. leguminosarum* bv. *trifolii*, and *R. leguminosarum* bv. *viciae* strains. Odd-numbered lanes are ethidium bromide-stained plasmid profiles. Even-numbered lanes are Southern blots of each plasmid profile probed with *R. etli* CFN42 *lpsβ1* and *lpsβ2* internal fragments. **A**, *R. etli* strains: 1, 2 CFN42 (pb, 150 kb); 3, 4 TAL182 (pa, 150kb); 5, 6 CFN 285 (pd 600 kb); 7, 8 Viking I (pc, 600 kb). **B**, *R. leguminosarum* bv. *trifolii* strains: 1, 2 ANU843 (pb, 250 kb); 3, 4 USDA 1596 (pa, 250 kb); 5, 6 TA1 (pa, 500 kb). **C**, *R. leguminosarum* bv. *viciae* strains: 1, 2 VF39 (pc, 400 kb); 3, 4 RBL1302 (pc, 400 kb); 5, 6 B10 (pc, 500 kb).

Table 3. Free-living and symbiotic phenotypes of *Rhizobium etli* and *R. leguminosarum* cross-complemented strains

Strain	Relevant genotype	Phenotypes			
		Floc-cula-tion ^a	Mo-til-ity ^b	Nod-ula-tion	N ₂ fixa-tion
<i>R. etli</i>					
CFN42	Wild type	–	+	+	+ ^c
CFNX230	pb [–] Rif ^r	+	–	–	– ^c
CFNX231	pb [–] Rif ^r /pTAL182a	–	+	+	+ ^c
CFNX232	pb [–] Rif ^r /pVikingIb	–	+	+	+ ^c
CFNX233	pb [–] Rif ^r /pANU843b	–	+	+	+ ^c
CFNX234	pb [–] Rif ^r /pVF39c	–	+	+	+ ^c
TAL182	Wild type	–	+	+	+ ^c
TAL182-2	pa [–] Sp ^t	+	–	–	– ^c
TAL182-3	pa [–] Sp ^t /pCFN42b	–	+	+	+ ^c
TAL182-4	pa [–] Sp ^t /pVF39c	–	+	+	+ ^c
<i>R. leguminosarum</i>					
ANU843	Wild type	–	+	+	+ ^d
ANU843-2	pb [–] Rif ^r	+	–	–	– ^d
ANU843-3	pb [–] Rif ^r /pCFN42b	–	+	+	+ ^d
ANU843-4	pb [–] Rif ^r /pTAL182a	–	+	+	+ ^d
ANU843-5	pb [–] Rif ^r /pVF39c	–	+	+	+ ^d

^a Checked in liquid PY meomdium (Noel et al. 1984).

^b Checked in PY with 0.3% agar.

^c Measured by acetylene reduction.

^d Measured by plant dry weight.

Table 4. Tumor induction by derivatives of *Rhizobium etli* CFN42 harboring the Ti-plasmid of *Agrobacterium tumefaciens* NT1

Strain	Relevant characteristics	Tumor induction ^a
<i>A. tumefaciens</i>		
NT1	Source of pTiC58Tra ^c	++
GMI9023-1	pTiC58Tra ^c donated by CFNX228	++
<i>R. etli</i>		
CFN42	Wild type	–
CFNX227	CFN42/pTiC58Tra ^c	+
CFNX183	pb [–] (<i>lpsB</i>)	–
CFNX228	pb [–] (<i>lpsB</i>)/pTiC58Tra ^c	–
CFNX188	pb [–] /pDEL27(<i>lpsB</i>)	–
CFNX229	pb [–] /pDEL27(<i>lpsB</i>)/pTiC58Tra ^c	+

^a Evaluated on *Kalanchoe* stems, 3 weeks after inoculation: ++, large tumors; +, medium size tumors, –, no tumors.

the *Taq* DyeDeoxy Terminator Cycle Sequencing Kit and automatic 373A DNA Sequencing System (Applied Biosystems, Foster City, CA). DNA was sequenced at least twice on both strands.

Analysis of nucleotide and amino acid sequences.

DNA sequence processing, identification of ORFs, deduced amino acid sequence identification, and location of hydrophobic regions (Kyte and Doolittle 1982; with a window of 11 amino acids) were performed with the GeneWorks package (release 2.4; IntelliGenetics, Mountain View, CA). The PSORT program was also used to detect hydrophobic regions. Localization of promoter regions was obtained from a remote search with the Promoter Prediction for Neural Networks program (Reese et al. 1995). Similarities between sequences were obtained from a remote search at NCBI data bases with the BLAST program (Altschul et al. 1990). Figure 2 was obtained with the Publish program; percentage of identity and similarity among sequences was calculated by the BestFit program; multiple sequence alignments were obtained by the PileUp program and displayed with the Pretty program. These programs are from the Wisconsin Package version 8.1 (Genetics Computer Group, Madison, WI).

Localization of the Tn5 insertion in strain CE168.

The IS50 specific primer 5'-GCACGATGAAGAGCAGA AGTT-3' (lower) designed from bases 118 to 98, the 5'-GCGGATGTTTCAGTTGTCTCGG-3' covering bases 2104 to 2084 of *lps*β2 (upper) primer (Fig. 2), and genomic DNA from the LPS mutant CE168 were used to obtain the PCR1 product, from which we determined the DNA sequence of the corresponding right Tn5-*lps*β DNA junction. The same IS50 specific primer (upper) was used together with the 5'-CGACGAATGTGATGGGTG-3' (lower) primer extending from bases 1628 to 1645, to obtain the PCR2 product, from which we determined the DNA sequence of the corresponding left Tn5-*lps*β DNA junction. Both reactions were carried out with the GeneAmp PCR reagent kit (Perkin Elmer, Branchburg, NJ).

ACKNOWLEDGMENTS

We are grateful to Laura Cervantes for technical help, to Patricia Bustos for technical assistance with the automatic sequencer, and to Juan Miranda and María Luisa Tabche for their cooperation in sequence analysis. We thank Hiep Trieu and Dale Noel for sharing unpublished data with us. Also, we thank Lourdes Girard for helpful discussions, and Patrice Dion, Rafael Palacios, Dale Noel, and David Romero for reviewing the manuscript. This work was partially supported by grant no. IN200693 from Dirección General de Asuntos del Personal Académico, Universidad Nacional Autónoma de México.

LITERATURE CITED

Allaway, D., Jeyaretnam, B., Carlson, R. W., and Poole, P. S. 1996. Genetic and chemical characterization of a mutant that disrupts synthesis of the lipopolysaccharide core tetrasaccharide in *Rhizobium leguminosarum*. J. Bacteriol. 178:6403-6406.

Allen, A., and Maskell, D. 1996. The identification, cloning and mutagenesis of a genetic locus required for lipopolysaccharide biosynthesis in *Bordetella pertussis*. Mol. Microbiol. 19:37-52.

Altschul, S. F., Gish, W., Miller, W., Myers, E. W., and Lipman, D. J. 1990. Basic local alignment search tool. J. Mol. Biol. 215:403-410.

Beck von Bodman, S., McCutchan, J. E., and Farrand, S. K. 1989. Characterization of conjugal transfer functions of *Agrobacterium tumefaci-*

ens Ti plasmid pTiC58. J. Bacteriol. 171:5281-5289.

Borthakur, D., Barker, R. F., Latchford, J. W., Rossen, L., and Johnston, A. W. 1988. Analysis of pss genes of *Rhizobium leguminosarum* required for exopolysaccharide synthesis and nodulation of peas: Their primary structure and their interaction with psi and other nodulation genes. Mol. Gen. Genet. 213:155-162.

Boyer, H. W., and Roulland-Dussoix, D. 1969. A complementation analysis of the restriction and modification of DNA in *Escherichia coli*. J. Mol. Biol. 41:459-472.

Breedveld, M. W., Canter-Cremers, H. C. J., Batley, M., Posthumus, M. A., Zevenhuizen, L. P. T. M., Wijffelman, C. A., and Zehnder, A. J. B. 1993. Polysaccharide synthesis in relation to nodulation behavior of *Rhizobium leguminosarum*. J. Bacteriol. 175:750-757.

Brink, B. A., Miller, J., Carlson, R. W., and Noel, K. D. 1990. Expression of *Rhizobium leguminosarum* CFN42 genes for lipopolysaccharide in strains derived from different *R. leguminosarum* soil isolates. J. Bacteriol. 172:548-555.

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

Brom, S., Martínez, E., Dávila, G., and Palacios, R. 1988. Narrow and broad-host-range symbiotic plasmids of *Rhizobium* spp. strains that nodulate *Phaseolus vulgaris*. Appl. Environ. Microbiol. 54:1280-1283.

Buendía, A. M., Enenkel, B., Köplin, R., Niehaus, K., Arnold, W., and Pühler, A. 1991. The *Rhizobium meliloti* *exoZ/exoB* fragment of megaplasmid 2: ExoB functions as a UDP-glucose 4-epimerase and *exoZ* shows homology to NodX of *Rhizobium leguminosarum* biovar *viciae* strain TOM. Mol. Microbiol. 5:1519-1530.

Burrows, L. L., Charter, D. F., and Lam, J. S. 1996. Molecular characterization of the *Pseudomonas aeruginosa* serotype O5 (PAO1) B-band lipopolysaccharide gene cluster. Mol. Microbiol. 22:481-495.

Canter-Cremers, H. C. J., Batley, M., Redmond, J. W., Eydem, L., Breedveld, M. W., Zevenhuizen, L. P. T. M., Pees, E., Wijffelman, C. A., and Lugtenberg, B. J. J. 1990. *Rhizobium leguminosarum* *exoB* mutants are deficient in the synthesis of UDP-glucose 4-epimerase. J. Biol. Chem. 265:21122-21127.

Carlson, R. W., Garci, F., Noel, K. D., and Hollingsworth, R. 1989. The structures of the lipopolysaccharide core components from *Rhizobium leguminosarum* biovar *phaseoli* CE3 and two of its symbiotic mutants, CE109 and CE309. Carbohydr. Res. 195:101-110.

Carlson, R. W., Kalembsa, S., Turowski, D., Pachori, P., and Noel, K. D. 1987. Characterization of the lipopolysaccharide from a *Rhizobium phaseoli* mutant that is defective in infection thread development. J. Bacteriol. 169:4923-4928.

Carlson, R. W., Reuhs, B., Chen, T., Bath, U. R., and Noel, K. D. 1995. Lipopolysaccharide core structure in *Rhizobium etli* and mutants deficient in O-antigen. J. Biol. Chem. 270:11783-11788.

Cassé, F., Boucher, C., Julliot, J. S., Michel, M., and Denarié, J. 1979. Identification and characterization of large plasmids in *Rhizobium meliloti* using agarose gel electrophoresis. J. Bacteriol. 113:229-242.

Cava, J. R., Elias, P. M., Turowski, D. A., and Noel, K. D. 1989. *Rhizobium leguminosarum* CFN42 genetic region encoding lipopolysaccharide structures essential for complete nodule development on bean plants. J. Bacteriol. 171:8-15.

Cava, J. R., Tao, H., and Noel, K. D. 1990. Mapping of complementation groups within a *Rhizobium leguminosarum* CFN42 chromosomal region required for lipopolysaccharide synthesis. Mol. Gen. Genet. 221:125-128.

Coplin, D. L., and Cook, D. 1990. Molecular genetics of extracellular polysaccharide biosynthesis in vascular phytopathogenic bacteria. Mol. Plant-Microbe Interact. 3:271-279.

Crow, V. L., Jarvis, B. D. W., and Greenwood, R. M. 1981. Deoxyribonucleic acid homologies among acid-producing strains of *Rhizobium*. Int. J. Syst. Bacteriol. 31:152-172.

Diebold, R., and Noel, K. D. 1989. *Rhizobium leguminosarum* exopolysaccharide mutants: biochemical and genetic analyses and symbiotic behavior on three hosts. J. Bacteriol. 171:4821-4830.

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, D. H., and Helinski, D. R. 1979. Replication of an origin-containing derivative of plasmid RK2 dependent on a plasmid function provided in trans. *Proc. Natl. Acad. Sci. USA* 76:1648-1652.
- Flores, M., González, V., Brom, S., Martínez, E., Piñero, D., Romero, D., Dávila, G., and Palacios, R. 1987. Reiterated DNA sequences in *Rhizobium* and *Agrobacterium* spp. *J. Bacteriol.* 169:5782-5788.
- García-de los Santos, A., Brom, S., and Romero, D. 1996. *Rhizobium* plasmids in bacteria-legume interactions. *World J. Microbiol. Biotechnol.* 12:119-125.
- Graham, P. H., Viteri, S. E., Mackie, F., Vargas, A. A. T., and Palacios, A. 1982. Variation in acid soil tolerance among strains of *Rhizobium phaseoli*. *Field Crops Res.* 5:121-128.
- Hood, E. E., Helmer, G. L., Fraley, R. T., and Chilton, M. D. 1986. The hypervirulence of *Agrobacterium tumefaciens* A281 is encoded in a region of pTiBo542 outside of T-DNA. *J. Bacteriol.* 168:1291-1301.
- Hooykaas, P. J. J., Klapwijk, P. M., Nuti, M. P., Schilperoort, R. A., and Rörsch, A. 1977. Transfer of the *Agrobacterium tumefaciens* Ti plasmid to avirulent *Agrobacteria* and to *Rhizobium* ex planta. *J. Gen. Microbiol.* 98:477-484.
- Hynes, M. F., and MacGregor, N. F. 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, M. F., Quandt, J., O'Connell, M. P., and Pühler, A. 1989. Direct selection for the curing and deletion of *Rhizobium* plasmids using transposons carrying the *Bacillus subtilis* *sacB* gene. *Gene* 78:11-120.
- Jennings, M. P., Van der Ley, P., Wilks, K. E., Maskell, D. J., Poolman, J. T., and Moxon, E. R. 1993. Cloning and molecular analysis of the *gal E* gene of *Neisseria meningitidis* and its role in lipopolysaccharide biosynthesis. *Mol. Microbiol.* 10:361-369.
- Jiang, X. M., Neal, B., Santiago, F., Lee, S. J., Romana, L. K., and Reeves, P. R. 1991. Structure and sequence of the *rfb* (O antigen) gene cluster of *Salmonella* serovar typhimurium (strain LT2). *Mol. Microbiol.* 5:695-713.
- Jones, J. D. G., and Gutterson, N. 1987. An efficient mobilizable cosmid vector, pRK7813, and its use in a rapid method for marker exchange in *Pseudomonas fluorescens* strain HV37a. *Gene* 61:299-306.
- Kowalczyk, E., Skorupska, A., and Lorkiewicz, Z. 1981. Transfer of nodulation ability in *Rhizobium* using a R68.45 derived plasmid. *Mol. Gen. Genet.* 183:388-391.
- Kyte, J., and Doolittle, R. F. 1982. A simple method for displaying the hydropathic character of a protein. *J. Mol. Biol.* 157:105-132.
- Leigh, J. A., and Lee, C. C. 1988. Characterization of polysaccharides of *Rhizobium meliloti* *exo* mutants that form ineffective nodules. *J. Bacteriol.* 170:3327-3332.
- Leigh, J. A., and Walker, G. C. 1994. Exopolysaccharides of *Rhizobium*: Synthesis, regulation and symbiotic function. *Trends Genet.* 10:63-67.
- López-Lara, I. M., Orgambide, G., Dazzo, F. B., Olivares, J., and Toro, N. 1995. Surface polysaccharide mutants of *Rhizobium* sp. (*Acacia*) strain GRH2: Major requirement of lipopolysaccharide for successful invasion of *Acacia* nodules and host range determination. *Microbiol.* 141:573-581.
- Macpherson, D. F., Manning, P. A., and Morona, R. 1994. Characterization of the dTDP-rhamnose biosynthetic genes encoded in the *rfb* locus of *Shigella flexneri*. *Mol. Microbiol.* 11:281-292.
- Martínez, E., Palacios, R., and Sánchez, F. 1987. Nitrogen-fixing nodules induced by *Agrobacterium tumefaciens* harboring *Rhizobium phaseoli* plasmids. *J. Bacteriol.* 169:2828-2834.
- Martínez, E., Pardo, M. A., Palacios, R., and Cevallos, M. A. 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-Romero, E. 1994. Recent developments in *Rhizobium* taxonomy. *Plant Soil* 161:11-20.
- Meade, H. M., Long, S. R., Ruvkun, G. B., Brown, S. E., and Ausubel, F. M. 1982. Physical and genetic characterization of symbiotic and auxotrophic mutants of *Rhizobium meliloti* induced by transposon mutagenesis. *J. Bacteriol.* 149:114-122.
- Miller, J. H. 1972. Experiments in Molecular Genetics. Cold Spring Harbor Laboratory, Cold Spring Harbor, NY.
- Morales, V. M., Backman, A., and Bagdasarian, M. 1991. A series of wide-host-range low-copy-number vectors that allow direct screening for recombinants. *Gene* 97:39-47.
- Müller, P., Keller, M., Weng, W. M., Quandt, J., Arnold, W., and Pühler, A. 1993. Genetic analysis of the *Rhizobium meliloti* *exoYFQ* operon: *ExoY* is homologous to sugar transferases and *ExoQ* represents a transmembrane protein. *Mol. Plant-Microbe Interact.* 6:55-65.
- Nishiguchi, R., Takanami, M., and Oka, A. 1987. Characterization and sequence determination of the replicator region in the hairy-root-inducing plasmid pRiA4b. *Mol. Gen. Genet.* 206:1-8.
- Noel, K. D. 1992. Rhizobial polysaccharides required in symbiosis with legumes. Pages 324-357 in: *Molecular Signals in Plant-Microbe Communications*. D. P. S. Verma, ed. CRC Press, Boca Raton, FL.
- Noel, K. D., Sánchez, A., Fernandez, L., Leemans, J., and Cevallos, M. 1984. *Rhizobium phaseoli* symbiotic mutants with transposon Tn5 insertions. *J. Bacteriol.* 158:148-155.
- Ooms, G., Hooykaas, P. J. J., Van Venn, R. J. M., Van Beelen, P., Regensburgtunik, T., and Schilperoort, R. A. 1982. Octopine Ti plasmid deletion mutants of *Agrobacterium tumefaciens* with emphasis on the right side of the T-region. *Plasmid* 7:15-19.
- Piñero, D., Martínez, E., and Selander, R. K. 1988. Genetic diversity and relationships among isolates of *Rhizobium leguminosarum* biovar *phaseoli*. *Appl. Environ. Microbiol.* 54:2825-2832.
- Quinto, C., de la Vega, H., Flores, M., Fernández, L., Ballado, T., Soberón, G., and Palacios, R. 1982. Reiteration of nitrogen fixation gene sequences in *Rhizobium phaseoli*. *Nature (London)* 299:724-728.
- Rigby, P. W. J., Dieckman, M., Rhodes, C., and Berg, P. 1976. Labeling deoxyribonucleic acid to high specific activity in vitro by nick translation with DNA polymerase I. *J. Mol. Biol.* 113:237-251.
- Reese, M. G., Harris, N. L., and Eeckman, F. H. 1995. Large scale sequencing specific neural networks for promoter and splice site recognition. Pages 737-738 in: *Pacific Symposium on Biocomputing '96*. L. Hunter and T. Klein, eds. World Scientific Pub., Singapore.
- Rosenberg, C., and Huguet, T. 1984. The pATC58 plasmid of *Agrobacterium tumefaciens* is not essential for tumor induction. *Mol. Gen. Genet.* 196:533-536.
- Sambrook, J., Fritsch, E. F., and Maniatis, T. A. 1989. *Molecular Cloning: A Laboratory Manual*. 2nd ed. Cold Spring Harbor Laboratory, Cold Spring Harbor, NY.
- Sau, S., and Lee, C. Y. 1996. Cloning of type 8 capsule genes and analysis of gene clusters for the production of different capsular polysaccharides in *Staphylococcus aureus*. *J. Bacteriol.* 178:2118-2126.
- Schultze, M., Kondorosi, E., Ratet, P., Buiré, M., and Kondorosi, A. 1994. Cell and molecular biology of *Rhizobium* plant interactions. *Int. Rev. Cytol.* 156:1-75.
- Segovia, L., Young, J. P. W., and 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.
- Skurnik, M., Venho, R., Toivanen, P., and Al-Hendy, A. 1995. A novel locus of *Yersinia enterocolitica* serotype O:3 involved in lipopolysaccharide outer core biosynthesis. *Mol. Microbiol.* 17:575-594.
- Soto, M. J., Zorzano, A., García-Rodríguez, F. M., Mercado-Blanco, J., López-Lara, I. M., Olivares, J., and Toro, N. 1994. Identification of a novel *Rhizobium meliloti* nodulation efficiency *nfe* gene homolog of *Agrobacterium* ornithine cyclodeaminase. *Mol. Plant-Microbe Interact.* 7:703-707.
- Stepkowski, T., Brom, S., García-de los Santos, A., Girard, L., Dávila, G., and Palacios, R. 1993. Plasmid related phenotypes in *Rhizobium leguminosarum* bv. *trifolii* ANU843. Page 653 in: *New Horizons in Nitrogen Fixation*. R. Palacios, J. Mora, and W. E. Newton, eds. Kluwer Acad. Pub., Dordrecht, The Netherlands.
- Turner, S. L., and Young, J. P. 1995. The replicator region of the *Rhizobium leguminosarum* cryptic plasmid pRL8J1. *FEMS Microbiol. Lett.* 133:53-58.
- Wang, S., and Gabriel, O. 1969. Biological mechanisms involved in the formation of deoxy sugars. *J. Biol. Chem.* 244:3430-3437.
- Watson, R. J. 1990. Analysis of the *C₄*-dicarboxylate transport genes of *Rhizobium meliloti*: Nucleotide sequence and deduced products of *dctA*, *dctB*, and *dctD*. *Mol. Plant-Microbe Interact.* 3:174-181.
- Wierenga, R. K., Terpstra, P., and Hol, W. G. J. 1986. Prediction of the occurrence of the ADP-binding $\alpha\beta$ -fold in proteins, using an amino acid sequence fingerprint. *J. Mol. Biol.* 187:101-107.
- Wyk, P., and Reeves, P. 1989. Identification and sequence of the gene for abequose synthase, which confers antigenic specificity on group B *Salmonellae*: Homology with galactose epimerase. *J. Bacteriol.* 171:5687-5693.
- Zevenhuizen, L. P. T. M., and Van Neerven, A. R. W. 1983. Gel-forming capsular polysaccharide of *Rhizobium leguminosarum* and *Rhizobium trifolii*. *Carbohydr. Res.* 124:166-171.