Analysis of *Mesorhizobium loti* Glycogen Operon: Effect of Phosphoglucosyltransferase (*pgm*) and Glycogen Synthase (*glgA*) Null Mutants on Nodulation of *Lotus tenuis*

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The phosphoglucosyltransferase (*pgm*) gene codes for a key enzyme required for the formation of UDP-glucose and ADP-glucose, the sugar donors for the biosynthesis of glucose containing polysaccharides. A *Mesorhizobium loti* *pgm* null mutant obtained in this study contains an altered form of lipopolysaccharide (LPS), lacks exopolysaccharide (EPS), β cyclic glucan, and glycogen and is unable to nodulate *Lotus tenuis*. The nonnodulating phenotype of the *pgm* mutant was not due to the absence of glycogen, since a glycogen synthase (*glgA*) null mutant effectively nodulates this legume. In *M. loti*, *pgm* is part of the glycogen metabolism gene cluster formed by *GlgP* (glycogen phosphorylase), *glgB* (glycogen branching), *glgC* (ADP-glucose pyrophosphorylase), *glgA*, *pgm*, and *glgX* (glycogen debranching). The genes are transcribed as a single transcript from *glgP* to at least *pgm* under the control of a strong promoter (promoter I) upstream of *glgP*. An alternative promoter (promoter II), mapping in a 154-bp DNA fragment spanning 85 bp upstream of the *glgA* start codon and the first 69 bp of the *glgA* coding region, controls the expression of *glgA* and *pgm*, independently of the rest of the upstream genes. Primer extension experiments showed that transcription starts 19 bp upstream of the *glgA* start codon.

Additional keywords: inverted repeats, promoter activity.

The reactions that lead to the synthesis of glycogen in bacteria have been extensively studied (Preiss 1984; Preiss and Romeo 1989). The enzymes involved in glycogen metabolism are ADP-glucose pyrophosphorylase (EC 2.7.7.27), ADP-glucose glycogen synthase (EC 2.4.1.21), glycogen branching enzyme (EC 2.4.1.18), glycogen phosphorylase (EC 4.4.1.1), and glycogen debranching enzyme (EC 3.2.1.). The genetic organi-

zation of the glycogen (*glg*) operon has been studied in *Escherichia coli* (Romeo et al. 1988; Yang et al. 1996), *Bacillus stearothermophilus* (Takata et al. 1997), *Bacillus subtilis* (Kiel et al. 1994), *Agrobacterium tumefaciens* (Ugalde et al. 1998; Uttaro et al. 1994), and *Rhizobium tropici* (Marroquí et al. 2001). Significant differences were found between them. The *A. tumefaciens* and *R. tropici* *glg* operon is formed by at least five open reading frames (ORF) in a single transcript *glgPBCApgm* (Marroquí et al. 2001; Ugalde et al. 1998). *Pgm*, present only in the *A. tumefaciens* and *R. tropici* glycogen operon, encodes the phosphoglucosyltransferase (*pgm*) enzyme, which isomerizes glucose-6-P to glucose-1-P.

The genetic organization of the *glg* operon in *A. tumefaciens* and *R. tropici* imposes restrictions for the expression of *pgm* under metabolic conditions that may turn off the operon. In *A. tumefaciens* and *R. tropici*, the presence of an alternative promoter for *pgm* allows the expression of this gene independently from the rest of the *glg* operon (Marroquí et al. 2001; Ugalde et al. 1998). *Pgm* is required, not only for the formation of ADP-glucose (the sugar donor for the synthesis of glycogen) but also for the formation of UDP-glucose (the sugar donor for the synthesis of other polysaccharides containing glucose). The presence of *pgm* in the glycogen operons of rhizobia is quite interesting, considering the key role of this gene in the synthesis of some polysaccharides required in *Rhizobium*-legume interactions leading to the formation of nodules. Recently, it was proposed that *pgm* may be an essential gene in *R. tropici*, and thus, mutants are nonviable (Marroquí et al. 2001). On the other hand, in *S. meliloti* and *A. tumefaciens*, *pgm* mutants were obtained; moreover, these mutants were described as either defective for symbiosis or avirulent, respectively (Cangelosi et al. 1987; Leigh et al. 1985).

M. loti forms on *Lotus tenuis*, *L. corniculatus*, and *L. pedunculatus* determinate nitrogen-fixing nodules and on *Leucaena leucocephala* indeterminate-type nodules. Different polysaccharides have been described as being important for the successful *Rhizobium*-legume interaction that leads to the formation of nitrogen-fixing nodules (Geremía et al. 1987; Hotter and Scott 1997; Leigh and Walker 1994; Pellock et al. 2000). *M. loti* exopolysaccharide (EPS) may be required for effective symbiosis on legumes that form indeterminate nodules but may not play any role in the induction of determinate nodules (Hotter and Scott 1991). Studies carried out with *Rhizobium leguminosarum*, *Bradyrhizobium japonicum*, and *Rhizobium etli* suggest that lipopolysaccharide (LPS) might

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Nucleotide sequence data reported for *M. loti* *glgC*, *glgA*, *pgm*, and part of the glycogen debranching (*glgX*) are available in the GenBank database under the accession number AF268969.

be important for effective induction of determinate nodules (Cava et al. 1989; Noel et al. 2000; Stacey et al. 1991). Little is known about the role that glycogen could play during nodulation of legumes. Recently, it was found that, in *R. tropici*, glycogen synthase (*glgA*) mutants have increased respiratory capacities and enhanced symbiotic performance (Marroquí et al. 2001).

We focused our work on studying the role of polysaccharides in the formation of determinate nodules in *M. loti*-*L. tenuis* symbiosis. The curious position of *pgm* in the *glg* operon and the participation of this enzyme in the synthesis of polysaccharides that may be involved in nodulation encouraged us to study the *glg* operon in *M. loti*. Here, we describe the genetic and transcriptional organization of the *M. loti glg* operon. We demonstrate that in *M. loti*, as in other bacteria belonging to the *Rhizobiaceae* family, *pgm* expression is controlled by two promoters but in a slightly different way from that reported for *A. tumefaciens* and *R. tropici*. We also describe the nodulating phenotype of glycogen synthase and Pgm null mutants. The essential symbiotic role of Pgm and its transcriptional control through two different promoters would justify further studies on its regulation under free-living lifestyle and symbiosis.

RESULTS

Genetic organization of *M. loti* glycogen genes.

A gene library of *M. loti* strain Ayac 1 BII was mass conjugated into *A. tumefaciens* *pgm* mutant A5129, and exconjugants were screened for restoration of fluorescence. By this procedure, cosmid pPRC1 was isolated. Restoration of wild-type fluorescence and mucoid phenotype in the mutant suggested that pPRC1 contains an active *M. loti pgm* gene that is expressed in the *A. tumefaciens* background. The nucleotide sequence of the 3.9-kb cosmid DNA insert revealed the presence of three contiguous ORF. The deduced amino acid sequence of

two of them showed a 59 and 75% identity to *glgA* and *pgm* of *A. tumefaciens*, respectively. The third ORF, although incomplete, showed a significant identity to the *A. tumefaciens glgC* (Uttaro and Ugalde 1994). The *M. loti* strain Ayac 1 BII gene library was screened by colony hybridization with a probe corresponding to the 3' end of *glgC*. Cosmid pPRC2 was isolated and partial sequence analysis of the insert allowed the complete sequence of an ORF that is 82% identical to *A. tumefaciens glgC*. Upstream of *glgC*, sequences with homology to *A. tumefaciens glgP* and *glgB* were identified (Fig. 1). Downstream of the *pgm* gene, an ORF with homology to *glgX* of *E. coli* is present. The organization of *M. loti glg* genes and the direction of transcription are shown in Figure 1. The genetic organization is identical to that observed for the glycogen operon in *A. tumefaciens* (Ugalde et al. 1998), *R. tropici* (Marroquí et al. 2001), and *M. loti* strain MAFF 303099 (Kaneko et al. 2000a, 2000b).

The intergenic region between *glgC* and *glgA* (Fig. 2) contains an element of 117 bp formed by two direct repeats, each one of them having two nearly perfect palindromic sequences. This sequence is highly homologous to a palindromic DNA Bru-RS2 element present in the genome of *Brucella* spp. (Halling and Bricker 1994). The GC content of this region is 45%, while the flanking genes *glgC* and *glgA* are 63 and 65%, respectively, thus suggesting lateral acquisition during evolution. Computational analysis of the secondary structure indicated that it has the potential to form stem-loops. Comparison with the recently published complete genome of *M. loti* strain MAFF303099 revealed that, in both strains, this region is highly conserved, but strain MAFF303099 contains only one of the two directed repeats between *glgC* and *glgA* (Kaneko et al. 2000).

Complementation of *A. tumefaciens* mutants.

Plasmids pPRC27, pPRC218, and pPRC220 were derivatives of pPRC2 harboring insertions of *Tn3HoHo1* in *pgm*,

Fig. 1. Schematic representation of *Mesorhizobium loti* glycogen cluster. **A**, Scheme of the genomic arrangement of *M. loti* glycogen genes. The genes boxed with continuous lines were sequenced on both strands of the DNA (accession number AF268969). Genes boxed with dotted lines were partially sequenced and their sizes are tentative. **B**, *M. loti* DNA genomic fragments cloned into pLAFR3 to generate pPRC1 and pPRC2 and mapping of *Tn3HoHo1* insertions in the pPRC2 cosmid (arrowheads) used for complementation of *Agrobacterium tumefaciens* mutant. The cosmid name is indicated under the arrows. **C**, Restriction map and location of the gentamicin cassette inserted for the construction of the *glgA M. loti* mutant. **D**, Fragments cloned in the expression vector pMP220 are indicated. These fragments were screened for promoter activity. Arrows indicate the orientation towards *lacZ* of pMP220.

glgA, and *glgB*, respectively (Table 1 and Fig. 1). Complementation of *A. tumefaciens* A5129 *pgm* mutant with the selected plasmids was assessed by observation of fluorescent colonies under UV light on calcofluor Luria-Bertani (LB) plates. pPRC2 and pPRC220 restored fluorescence, while negative results were obtained with pPRC27 and pPRC218. Enzymatic determination of Pgm revealed that pPRC220 restored only 50% of the activity compared to the control (*A. tumefaciens* A5129 harboring wild-type plasmid pPRC2), showing that a polar insertion in *glgB* did not completely abolish the expression of the downstream *pgm* gene. The fact that 50% of the activity was conserved suggested that downstream *glgB*, a region promoting the expression of downstream genes might be present. Plasmid pPRC220 also restored glycogen synthase activity of the *A. tumefaciens* A1120 *glgB* polar mutant (data not shown). These results support the idea that in *M. loti*, *glgA* and *pgm* are part of the *glg* operon but that they may also be transcribed from an alternative promoter located upstream *glgA*.

Identification of regions with promoter activity using *lacZ*-fusions.

The analysis of the transcriptional organization of the *glg* cluster was carried out using the pMP220 reporter system as described below. Figure 1 shows the localization of the DNA regions used for the construction of transcriptional *lacZ* fusions. Strains and plasmids are described in Table 1. Table 2 shows that plasmid pMPA1 containing the first 200 bp of *glgP* and 1.6 kb corresponding to the upstream region of the *glg* operon displayed the highest β -galactosidase activity when cloned in the direction of transcription. However, this DNA fragment cloned in the inverse orientation had no activity (data not shown), thus suggesting that this region contains the promoter of the *M. loti glg* operon (promoter I). The other fusion with significant β -galactosidase activity was pMPD10, containing the intergenic region between *glgC* and *glgA* (Table 2), indicating that this region contains a second promoter (promoter II). This region displayed promoter activity independent of the orientation of the fragment (data not shown). This could be a consequence of the presence of the two repeated palindromic DNA elements (discussed above). In order to define the localization of promoter II, different regions of pMPD10 were subcloned in plasmid pMP220 (Fig. 3). The resulting plasmids were introduced into *M. loti* wild type and subjected to β -galactosidase activity assays. Table 3 shows that the minimal region with β -galactosidase activity is contained in plasmid pMD104, which harbors a 154-bp DNA fragment from nucleotides 1,385 to 1,539. This region extends from 85 bp upstream of the *glgA* start codon to base 69 of the *glgA* coding region and contains only one of the inverted repeated sequences (Fig 2).

Primer extension and reverse transcription-polymerase chain reaction (RT-PCR) experiments.

In order to identify the transcription start site of the mRNA transcribed from promoter II, a primer extension was carried out as described below. Figure 4 shows that transcription starts 19 bp upstream of the *glgA* start codon, a region that is contained in plasmid pMPD104. This result indicates that promoter II may be functional for the transcription of *glgA* and *pgm*, which is consistent with the *lacZ*-fusion experiments.

A RT-PCR experiment was carried out to ascertain that promoter II is an alternative promoter and not the unique promoter for *pgm* expression. The expected 710-bp fragment spanning the region between *glgC* and *glgA* (Fig. 5) was amplified, demonstrating transcriptional coupling between both genes and showing that the palindromic repeated element homologous to *Brucella* Bru-RS2 present in this intergenic region does not function as a transcriptional terminator. These results confirm that *glgA* and *pgm* are part of the *glg* operon transcribed under the control of promoter I.

Phenotype of *M. loti pgm* and *glgA* mutants.

Strains *M. loti* M1128 *pgm* and M1124 *glgA* mutants are described in Table 1. The M1124 *glgA* mutant does not accumulate glycogen due to lack of glycogen synthase activity but contains normal EPS, cyclic glucan, and LPS (Table 4). On the other hand, the M1128 *pgm* mutant has no *pgm* activity but has wild-type glycogen synthase activity. Due to the inability to synthesize ADP-glucose and UDP-glucose, the M1128 *pgm* mutant displays a pleiotropic effect on the synthesis of all polysaccharides containing glucose. Consequently, M1128 mutant does not accumulate glycogen, exopolysaccharide, or cyclic glucan (Table 4). Polyacrylamide gel electrophoresis (PAGE) analysis (Fig. 6) shows that the mutant accumulates an altered form of LPS. The mutant, although having a slow-growing phenotype compared to the wild type, is able to achieve the same cell density as the wild type at the late stationary phase of growth (data not shown).

Nodulation of *Lotus tenuis*

by *R. loti glgA* and *pgm* mutants.

A *M. loti glgA* nonpolar mutant (strain M1124, Table 1) formed on *L. tenuis* normal nodules. After 4 weeks, plants remained healthy and green and reached the same size as those nodulated by the wild-type parental strain. No significant differences were observed in the number of nodules induced by either strain. This result suggests that *M. loti* glycogen has no relevant role as a carbon and energy source during nodulation and nitrogen fixation under the laboratory nodulation conditions. Conversely, the *M. loti pgm* mutant (strain M1128) was

Fig. 2. Nucleotide sequence of the intergenic region between *glgC* and *glgA*. A putative ribosome binding site (RBS) is underlined. The start codon is shown with an angled arrow and the stop codon is shown with an asterisk. The short arrows above the sequence indicate the terminal and the internal repeats. The long arrows mark the long, almost perfect direct repeats. The transcriptional start site is shown with an open circle. The oligonucleotide PE2P2 is indicated by an arrow below the sequence.

unable to form nodules on *L. tenuis*. Complementation of M1128 mutant with pPRC1 restored the ability to form normal nodules, indicating that in *M. loti*, Pgm is absolutely required for the induction of nodules in *L. tenuis*.

DISCUSSION

Studies on the glycogen metabolism gene cluster of *M. loti* revealed that its genetic and transcriptional organization is highly similar to that described in *A. tumefaciens* (Ugalde et al. 1998; Uttaro and Ugalde 1994) and *R. tropici* (Marroquí et al. 2001) but different from *E. coli* (Romeo et al. 1988; Yang et al. 1996), *Bacillus stearothermophilus* (Takata et al. 1997), and *Bacillus subtilis* (Kiel et al. 1994). Glycogen metabolism genes form a single operon *glgPBCapgm*, transcribed from *glgP* to *pgm*. Downstream of *pgm* is an ORF highly similar to the *E. coli glgX* debranching enzyme (Romeo et al. 1988). No experiments were carried out to determine whether or not *glgX* belongs to the *glg* operon. The presence of a *pgm* gene in the operon of three species belonging to the α -2 proteobacteria family constitutes an important difference from the glycogen metabolism gene clusters described so far in other bacteria. As in *A. tumefaciens* and *R. tropici*, the operon in *M. loti* is transcribed under the control of a strong promoter upstream from *glgP* (promoter I). A second alternative promoter (promoter II)

present between *glgC* and *glgA* suggests that *glgA* and *pgm* could be transcribed under the control of one or both of the promoters. These results are consistent with the observation that cosmid pPRC220, with a polar mutation in *glgB*, restored 50% of the *pgm* activity of an *A. tumefaciens pgm* mutant, while cosmid pPRC218, with a polar mutation in *glgA*, did not. Structure and localization of the alternative promoter constitutes a difference with *A. tumefaciens* and *R. tropici*, in which the second promoter mapped inside the *pgm* coding region and leads to the translation of a protein product shorter than the one generated by the polycistronic mRNA (Ugalde et al. 1998, Marroquí et al. 2001). It seems that these bacteria use different strategies to regulate *pgm* expression. We demonstrate that *M. loti pgm* is an essential nodulation gene and that its activity is required for the formation of EPS, glycogen, cyclic glucan, and normal LPS. It is conceivable that, due to the organization of the *M. loti glg* operon, a mechanism may be necessary to express Pgm under different growth or metabolic conditions in order to maintain an intracellular pool of UDP-glucose. Further experiments will be required to demonstrate the physiological role of promoter II.

The region between *glgC* and *glgA*, where Promotor II maps, has a palindromic structure with a different percentage of GC from the surrounding genes, suggesting that it may have been acquired by lateral transfer during evolution. In other bacteria,

Table 1. Bacterial strains and plasmids used in this study

Strain or plasmid	Genotype or characteristics	Reference or source
<i>Escherichia coli</i> Strains		
DH5 α -F'IQ	Host for cloning	Sambrook et al. 1989
XL1-Blue	Host for cloning	Bullock et al. 1987
<i>Agrobacterium tumefaciens</i> Strains		
A348	Wild type, pTiA6 Rif ^r Cm ^r	Garfinkel et al. 1981
A5129	<i>pgm</i> ::Tn5	Uttaro and Ugalde, 1994
A1120	<i>glgB</i> ::Km ^r	Ugalde et al. 1998
<i>Mesorhizobium loti</i> Strains		
Ayac 1 BII	Wild type, Nod ⁺	Lepek. et al. 1990
M1124	<i>glgA</i> ::Gm ^r	This study
M1128	<i>pgm</i> ::Tn3HoHo1	This study
Plasmids		
pBC SK (+)	pUC derivative, F1 replication origin, Cm ^r	Stratagene Cloning Systems (La Jolla, CA, U.S.A.)
pPH1JI	Inc P Gm ^r , Sp ^r	Hirsch and Beringer 1983
pHoHo1	Plasmid carrying Tn3- <i>lacZ</i> transposon, Ap ^r	Stachel et al. 1985
pSshe	Cm ^r , pACYC184 containing <i>tnpA</i>	Stachel et al. 1985
pLAFR3	pLAFR1 containing <i>Hae</i> II fragment of pUC8 with the Plac promoter (expression vector).	Staskowicz et al. 1987
pPRC1	pLAFR3 derivative carrying a 3.9-kb fragment of genomic DNA of <i>M. loti</i> Ayac 1 BII that complements a <i>pgm</i> mutant	This study
pPRC2	pLAFR3 derivative carrying DNA of <i>M. loti</i> Ayac 1 BII containing <i>glgP</i> , <i>glgB</i> , <i>glgC</i> , <i>glgA</i> , <i>pgm</i> , and <i>glgX</i>	This study
pERL4	A 3.9-kb fragment of <i>M. loti</i> genomic DNA from pPRC1, cloned in pBC SK (+)	This study
pESRL4	pERL4 digested with <i>Sac</i> II and religated	This study
pESRL4G	pESRL4; <i>glgA</i> ::Gm	This study
pMP220	IncP broad-host-range <i>lacZ</i> fusion vector, Tc ^r	Spaink et al. 1987.
pMPA1	1.8-kb fragment containing the 5' end of <i>glgP</i> and 1.6 kb upstream cloned in pMP220 orientated toward <i>lacZ</i>	This study
pMPB1	2.8-kb <i>Pst</i> II/ <i>Eco</i> RI fragment containing 3' end <i>glgP</i> and 5' end <i>glgB</i> cloned in pMP220 orientated toward <i>lacZ</i>	This study
pMPC8	2.2-kb <i>Bam</i> HI/ <i>Bam</i> HI fragment containing 3' end <i>glgB</i> and 5' end <i>glgC</i> cloned in pMP220 orientated toward <i>lacZ</i>	This study
pMPD10	989 pb <i>Bam</i> HI/ <i>Bam</i> HI fragment, containing the 3' end of <i>glgC</i> and the 5' end of <i>glgA</i> , cloned into pMP220 orientated toward <i>lacZ</i>	This study
pMPD101	320-bp fragment from pMPD10 cloned into pMP220 orientated toward <i>lacZ</i>	This study
pMPD102	274-bp fragment from pMPD10 cloned into pMP220 orientated toward <i>lacZ</i>	This study
pMPD103	232-bp fragment from pMPD10 cloned into pMP220 orientated toward <i>lacZ</i>	This study
pMPD104	154-bp fragment from pMPD10 cloned into pMP220 orientated toward <i>lacZ</i>	This study
pMPD105	88-bp fragment from pMPD10 cloned into pMP220 orientated toward <i>lacZ</i>	This study
pMPE5	1.5-kb <i>Bam</i> HI/ <i>Bam</i> HI fragment, comprising the 3' end of <i>glgA</i> and the 5' end of <i>pgm</i> , cloned into pMP220 orientated toward <i>lacZ</i>	This study
pPRC27	pPRC2 with Tn3HoHo1 insertion in <i>pgm</i> .	This study
pPRC218	pPRC2 with Tn3HoHo1 insertion in <i>glgA</i>	This study
pPRC220	pPRC2 with Tn3HoHo1 insertion in <i>glgB</i>	This study

intergenic polycistronic regions with inverted repeats have been described as upstream mRNA stabilizers (Newbury et al. 1987), as motifs that may represent recognition elements for DNA-regulation interactions (Karimova and Ullmann 1997), or as translational regulators (Goupil-Feuillerat et al. 2000). *LacZ*-fusion experiments showed that the minimum region with full promoter activity is contained in a 154-bp DNA fragment spanning from 85 bp upstream to 69 bp downstream of the *glgA* ATG start codon (Table 3, plasmid pMPD104 and Fig. 2). Primer extension experiments show a transcription start site 19 bp upstream of the *glgA* start codon within the 154-bp DNA fragment. A conserved ribosome binding site motif is present between transcriptional and translational starts. It is interesting to note that promoter activity fell 60% when the 69-bp fragment of the *glgA* coding region was reduced by 29 bp from the 3' end (Table 3, plasmid pMPD102). Recently, a new class of prokaryotic activator with a binding site downstream of the transcription start site was described (Munson and Scott 2000).

We show that full activity of promoter II requires only one of the two direct repeat sequences present between *glgC* and *glgA*. The palindromic structure of this region could explain the fact that promoter activity is independent of any orientation when the insert is cloned into plasmid pMPD10.

A *M. loti glgA* null mutant does not synthesize glycogen but does form nodules of normal structure and function. This result

shows that glycogen is not necessary as a source of carbon and energy during nodule induction and invasion. Recently *R. tropici glgA* mutants were shown to enhance symbiotic performance (Marroquí et al. 2001). As this mutation induced pleiotropic effects, it is not clear if the increased nodulation was due to the inability to store glycogen (Marroquí et al. 2001).

In *R. tropici*, a mutation in *pgm* was reported to be lethal (Marroquí et al. 2001). This is not the case in *M. loti*, although the mutant displays a slower growth rate than the wild type. The mutant lacks glycogen, EPS, and cyclic $\beta(1-2)$ glucan, all polysaccharides containing glucose and requiring ADP-glucose or UDP-glucose as sugar donors. The mutant also presents an LPS with altered mobility in PAGE analysis. It was shown by chemical characterization that *M. loti* LPS contains glucose, glucuronic acid, galactose, and galacturonic acids (Russa et al. 1995), all sugars requiring UDP-glucose as precursors in *R. meliloti* and *A. tumefaciens* (Uttaro et al. 1993).

Pgm mutant fails to nodulate *L. tenuis*. Genetic evidence indicates that *Rhizobium* EPSs are required for the development of indeterminate nodules but not for determinate nodules (Leigh and Coplin 1992). *M. loti exo* mutants nodulate normally when tested on a determinate nodulating host (Hotter and Scott 1991, 1997). Since *L. tenuis* is a determinate nodulating host, the nodulation defect of *M. loti pgm* mutants might be due to the lack of other glucose-containing polysaccharide, like cyclic $\beta(1-2)$ glucan (Lepek et al. 1990) or LPS (Russa et al. 1995), and not to the lack of EPS. Studies carried out with *R. leguminosarum* and *Bradyrhizobium japonicum* suggest that LPS may be important in early stages of induction of determinate nodules (Cava et al. 1989 Stacey et al. 1991). The lack of cyclic $\beta(1-2)$ glucan in *S. meliloti* (Geremía et al. 1987) results in the formation of pseudonodules. It is possible that the absence of nodules in *M. loti pgm* mutants might be the result of either a defective LPS or a combination of defective LPS and lack of cyclic glucan. Further analysis will be required to distinguish between these possibilities.

Table 2. β -Galactosidase activity of fusions containing different regions of *Mesorhizobium loti* glycogen operon

Plasmid ^a	β -galactosidase activity ^b
pMPA1 (<i>glgP-lacZ</i>)	1792 $\pm$ 32
pMPB1 (<i>glgB-lacZ</i>)	10 $\pm$ 5
pMPC8 (<i>glgC-lacZ</i>)	0
pMPD10 (<i>glgA-lacZ</i>)	307 $\pm$ 25
pMPE5 (<i>pgm-lacZ</i>)	0

^a Plasmids were introduced into *M. loti* wild type strains.

^b $\Delta A_{420} \text{ nm} \times 1000/A_{600} \text{ nm} \cdot \text{min}^{-1} \pm$ standard error. The results are averages of three experiments. $\Delta A_{420} \text{ nm}$ was calculated by subtraction of *lacZ* expression in *M. loti* wild type containing pMP220 vector that was 98 ± 1 .

Fig. 3. Map of *glgC*, *glgA*, and the intergenic region. The DNA cloned to form the different *lacZ* fusion plasmids is shown. Numbers below bars indicate nucleotide position (accession number AF268969). Not drawn to scale.

MATERIALS AND METHODS

Bacterial strains, growth media, and plasmids.

M. loti, *A. tumefaciens*, and *E. coli* strains and plasmids used in this study are listed in Table 1. In triparental mating, *E. coli* RK2073 or RK2013 were used as helper.

M. loti and *A. tumefaciens* were grown in AB minimal medium (Douglas et al. 1985) or M9 minimal medium (Sambrook et al. 1989) supplemented with sucrose (0.5% wt/vol). Other media used were tryptone yeast (Lepek et al. 1990) for *M. loti*, and Luria-Bertani for *A. tumefaciens* and *E. coli*. Antibiotics were used at the following concentrations: 50 μg of kanamycin (Km) per ml, 100 μg of ampicillin per ml, 20 μg of

Table 3. β -Galactosidase activity of fusions containing different pMPD10 subclones^a

Plasmid	β -galactosidase activity ^b
pMPD10	155 $\pm$ 13
pMPD101	169 $\pm$ 2
pMPD102	63 $\pm$ 5
pMPD103	20 $\pm$ 3
pMPD104	238 $\pm$ 15
pMPD105	0

^a A series of additional *lacZ*-fusions were constructed by cloning different fragments from pMPD10 into pMP220 in the direction of transcription and introduced into *Mesorhizobium loti* wild-type strain.

^b $\Delta A_{420} \text{ nm} \times 1000/A_{600} \text{ nm} \cdot \text{min}^{-1} \pm$ standard error. The results are averages of three experiments. $\Delta A_{420} \text{ nm}$ was calculated by subtraction of *lacZ* expression in *M. loti* wild type containing pMP220 vector. The background value for pMP220 was 52 ± 9 .

chloramphenicol per ml, 20 µg of gentamicin per ml, 100 µg of carbenicillin per ml, 50 µg of nalidixic acid per ml, 10 µg of tetracycline per ml, and 30 µg of trimethoprim per ml. For the selection of *Tn3*HoHo1-harboring strains, ampicillin or carbenicillin was used in *E. coli* and *M. loti* and carbenicillin was used in *A. tumefaciens*.

Cloning and sequencing.

A *M. loti* genomic library was prepared in pLAFR3 vector as described by Staskowicz and associates (1987). The library was mass conjugated into *A. tumefaciens* A5129 *pgm* mutant. Transconjugants were selected on minimal medium supplemented with sucrose and tetracycline and screened for mucous phenotype and fluorescence under UV light when plated on LB-calcofluor medium (Uttaro and Ugalde 1994). A cosmid,

named pPRC1, containing a DNA fragment of approximately 3.9 kb was isolated by this procedure. Cosmid pPRC2, with a DNA insert of approximately 18 kb, was isolated by screening the *M. loti* genomic library with a probe containing the 3' end of *M. loti glgC* (Sambrook et al. 1989). This probe, extending from nucleotide 661 to 1,236 (GenBank accession number AF268969) was obtained by subcloning the *EcoRI-BamHI* fragment (Fig. 1). DNA inserts were sequenced (Sanger et al. 1977) by subcloning different restriction fragments in pBluescript II KS (+) (Stratagene; La Jolla, CA, U.S.A.).

The 5' end of the glycogen operon was cloned by inverse polymerase chain reaction (PCR). Total chromosomal DNA was digested with *PstI* and ligated and circularized fragments were used as templates for PCR amplification with the following primers: 5'-GCTTTACCCGCGATTCC-3' and 5'-ATCAG-ACCGAGATTGGAAA-3'. A 1.8-kb fragment containing the 5' end of *glgP* and 1.6 kb upstream of *glgP* was obtained, and both strands of several clones were sequenced.

Fig. 4. Mapping of the 5' end of mRNA transcribed by Promoter II. The transcription start site is indicated by an arrow. Total RNA of *Mesorhizobium loti* wild-type strain was used to synthesize cDNA by reverse transcription with avian myeloblastosis virus reverse transcriptase with oligonucleotide PE2P2 as primer and [γ - 32 P]ATP. Products were subjected to polyacrylamide gel electrophoresis in parallel with the sequencing ladder.

Fig. 5. Reverse transcription-polymerase chain reactions (RT-PCR) carried out on total RNA obtained from wild-type *Mesorhizobium loti* Ayac 1 BII strain. RT-PCR experiments were carried out as described by Ugalde and associates (1998). The primers for the RT-PCR experiments were *glgCMD* (5'-GCCAATATCGACCTGACC-3') and *glgAMU* (5'-CGGGAAAGCCTGGAATGAGCGT-3'). One of the reaction primers corresponds to the 3' end of *glgC* (position 898 to 915) and the other to the 5' end of *glgA* (position 1,605 to 1,583). Lane 1, Control reaction carried out without the addition of reverse transcriptase. Lane 2, *glgC* to *glgA* intergenic region. Molecular size markers are also shown.

Table 4. Phenotype of *glgA* and *pgm* *Mesorhizobium loti* mutants^a

<i>M. loti</i> Strain	Enzymatic activity (%) ^b		Polysaccharide accumulation ^c			Lipopolysaccharides (LPS)	Nodulation
	Pgm	Glycogen Synthase	Exopolysaccharides (EPS) ^d	Glycogen	Cyclic β (1-2) glucan		
Wild type	100	100	+	+	+	Normal	Normal
M1128 (<i>pgm</i> ⁻)	0	100	-	-	-	Altered	Absent
M1124 (<i>glgA</i> ⁻)	100	0	+	-	+	Normal	Normal

^a Enzymatic activities, glycogen accumulation, isolation and detection of cyclic β (1-2) glucan, and LPS isolation and examination were carried out as described in text.

^b Enzymatic activities are expressed as percentages of the wild-type strain activity. Wild-type activities (in units per mg of protein) were as follows: Phosphoglucosyltransferase (Pgm), 0.56; glycogen synthase (GlgA), 0.086.

^c + = accumulation, - = lack of accumulation.

^d Visualized as blue bright under UV light when grown on plates with Luria Bertani-calcofluor medium.

Primer extension.

The transcription start site was determined by primer extension analysis as previously described (Ugalde et al. 1998). The oligonucleotide PE2P2 (5'-CGGTCACGTCGGCCAGCCCA-3') complementary to the region between nucleotides 1,539 and 1,520 (GenBank accession number AF268969) was used (Fig. 2).

RT-PCR experiments.

RNA extraction and RT-PCR experiments were carried out as was previously described (Ugalde et al. 1998). The primers for the RT-PCR experiments were *glgCMD* (5'-GCCAATATCGACCTGACC-3', positions 898 to 915) and *glgAMU* (5'-CGGGAAAGCCTGGAATGAGCGT-3', positions 1,605 to 1,583).

Construction of *lacZ* fusions.

Plasmids pMPA1, pMPB1, pMPC8, pMPD10, and pMPE5 were constructed by cloning different *M. loti* DNA fragments obtained from pPRC2 into the self-mobilizable broad-host-range vector plasmid pMP220. Plasmids pMPD101, pMPD102, pMPD103, pMPD104, and pMPD105 were constructed by subcloning PCR and digestion products from pMPD10 into pMP220. All the constructions were sequenced to eliminate any possible mutations due to PCR amplification. Transformation of *M. loti* with these plasmids was carried out by triparental mating. β -Galactosidase activity was determined as described elsewhere (Sambrook et al. 1989).

*Tn3*HoHo1 mutagenesis.

Cosmid pPRC2 was randomly mutagenized with *Tn3*HoHo1 as described (Stachel et al. 1985). Insertions were mapped by standard restriction mapping techniques. pPRC2 and their Cb resistant derivatives were mobilized into the *A. tumefaciens* 5129 mutant, and transconjugants were selected in LB medium with the appropriate antibiotics.

Fig. 6. Polyacrylamide gel electrophoresis-Tricine gel analysis of lipopolysaccharide (LPS) from *Mesorhizobium loti* wild type and *pgm* mutant. Lanes 1 and 3, *M. loti* wild type, and lanes 2 and 4, *M. loti* M1128 mutant. The volume of LPS solution running was: 5 μ l (lanes 1 and 2) and 10 μ l (lanes 3 and 4). LPS was stained with silver as described in text.

Construction of *M. loti glgA* and *pgm* mutants and nodulation assays.

The 3.9-kb DNA insert of pPRC1 was cloned in pBC SK (+) plasmid (Stratagene), and the resulting plasmid pERL4 was digested with *Sac*II and religated in order to eliminate the *Pst*I site of the polylinker (pESRL4). A nonpolar gentamicin cassette (Ugalde et al. 2000) was introduced into the unique *Pst*I site of the insert (Fig. 1). The resulting plasmid, named pESRL4G, was electroporated in *M. loti*, and gentamicin-resistant clones were isolated. Double recombination events were obtained by testing sensitivity to chloramphenicol. On this basis, a mutant named *M. loti* M1124 was selected. PCR analysis showed that the mutant resulted from a double crossover event. To obtain the *M. loti* M1128 *pgm* mutant, plasmid pPRC27 was mobilized into *M. loti* and the *pgm-lacZ* fusion was forced to integrate into the chromosome by allelic exchange, using the mobilizable plasmid pH1JI, which belongs to the same incompatibility group (Hirsch and Beringer 1983). Analysis by DNA hybridization using the 474-bp *Sall*-*Bam*HI fragment of the *pgm* gene as a probe (Fig. 1) showed that pM1128 is the result of a double crossover event. Nodulation assays were carried out as described elsewhere (Olivares et al. 1980).

Determination of enzymatic activities, of glycogen accumulation, and of the presence of β (1-2) glucan and LPS.

For enzymatic activity determination, cell extracts were obtained as described (Ugalde et al. 1998) with modifications. *A. tumefaciens* cells were disrupted by sonication and *M. loti* by osmotic shock.

Phosphoglucosyltransferase and glycogen synthase activities were determined as described elsewhere (Ugalde et al. 1998). One unit of activity is defined as follows: for Pgm, the amount of enzyme that converted 1.0 μ mole of α -D-glucose-1-phosphate to α -D-glucose-6-phosphate per minute; for GlgA, the amount of enzyme that catalyzed the incorporation of 1 μ mole glucose into insoluble glycogen per minute. Glycogen accumulation was determined by the iodine method (Krisman 1962). Isolation and detection of β (1-2) glucan was carried out as described elsewhere (Iñon de Iannino et al. 1998). The sample preparation for determination of LPS was carried out as previously described by Hitchcock and Brown (1983). Tricine-gel analysis of the samples in 10% polyacrylamide gels was performed as described by Schagger and von Jagow (1987).

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