Research Notes

The glnA Gene of Rhizobium leguminosarum bv. phaseoli and Its Role In Symbiosis

Soledad Moreno¹, Roberto Meza¹, Josefina Guzman¹, Alfonso Carabez², and Guadalupe Espin¹

¹ Centro de Investigacion sobre Ingenieria Genetica Biotecnologia and ² Instituto de Fisiologia Celular Universidad Nacional Autonoma de Mexico, Apartado Postal 510-3, Cuernavaca, Mor. 62271 Mexico.

Received April 30 1991. Accepted 9 July 1991.

We have determined glutamine synthetase (GS) activity in bacteroids isolated from nodules of Phaseolus vulgaris induced by Rhizobium leguminosarum biovar phaseoli CE3 wild type strain. GSI but not GSII activity was detected. No GS activity was detected in bacteroids of strain SM58, which carries a glnA::Km insertion mutation. Acetylene reduction activity of nodules induced by strain SM58 was about 50% of that found in nodules induced by the wild type. Microscopic analysis of nodules induced by strain SM58 showed that the number of bacteroids within the infected cells was reduced as compared to the number of bacteroids present in the wild type-induced nodules. In addition, SM58 bacteroids contained high amounts of poly-β-hydroxybutyrate granules.

Additional keywords: glutamine synthetase in bacteroids, Rhizobium gln genes, Rhizobium leguminosarum bv. phaseoli-Phaseolus vulgaris symbiosis.

Rhizobium species assimilate ammonia for growth in the free-living state through the action of the GSI, GSII, and glutamate synthase (GOGAT) enzymes. In nodules, the nitrogen-fixing bacteroids export the ammonia produced by the nitorgenase to the plant cells (Bergersen and Turner 1967; O’Gara and Shanmugam 1976), where it is assimilated by the plant glutamine synthetase (GS).

The rhizobial GSI and GSII are products of the glnA and glnII genes, respectively (Somerville and Kahn 1983; Carlson et al. 1985; Carlson and Chelm 1986; Colonna-Romano et al. 1987; Espin et al. 1988; de Bruijn et al. 1989). glnT, a locus first identified in Agrobacterium tumefaciens (Rossbach et al. 1988) and Rhizobium meliloti (de Bruijn et al. 1989) that complements Escherichia coli glutamine auxotrophs and is neither glnA nor glnII, has been cloned from Rhizobium leguminosarum bv. phaseoli Jordan and shown to code for a glutamine synthetase activity (GSIII) (Espin et al. 1990). However, the physiological conditions for GSIII expression or activity in Rhizobium have not been found yet.

Rhizobial GSI is similar to the GS of enteric bacteria: it is a dodecameric protein, and its activity is regulated by an adenylation-deadenylation mechanism (Darrow 1980; Ludwig 1980; Rossi et al. 1989). GSII is an octameric enzyme, is heat sensitive, and is similar to eukaryotic GSs (Darrow 1980; Carlson and Chelm 1986). Transcription of glnA is not significantly regulated by the nitrogen source in the medium (Carlson et al. 1987; Szeto et al. 1987; Rossi et al. 1989; Chiurazzi and Iaccarino 1990). In contrast, the structural gene for GSII, glnII, is regulated in response to the nitrogen and oxygen availability (Adams and Chelm 1988; Martin et al. 1988; Rossi et al. 1989; Shatters et al. 1989; de Bruijn et al. 1989). The presence of GS activity in bacteroids of several Rhizobium species was demonstrated earlier by Brown and Dilworth (1975). However, at that time, the presence of two GS isoenzymes in these bacteria was unknown. In that study, the bacteroid GS activity was found to be significantly lower as compared with that of the plant GS (Brown and Dilworth 1975). In a recent study, no GSII protein was detected in crude extracts of alfalfa, pea, or soybean nodules using an anti-GSII-specific antiseraum (Shatters et al. 1989). Taken together these data suggest that GSI but not GSII might be present in bacteroids of Rhizobium and Bradyrhizobium species.

In regard to the role of the R. l. bv. phaseoli GSI and GSII in symbiosis, we have previously reported a mutant lacking GSII activity that has a Nad⁻ Fix⁺ phenotype (Morett et al. 1985). To investigate the role of R. l. bv. phaseoli GSs in nitrogen metabolism and in symbiosis, we have cloned the glnA gene and constructed by site-directed mutagenesis a strain carrying a glnA::Km mutation.

A cosmid gene library of R. l. bv. phaseoli CE3 strain (Cevallos et al. 1989) was transferred by conjugation into the glnA mutant of Klebsiella pneumoniae (Schroeter) Trevisan UNF1811 (Espin et al. 1981) A transconjugant UNF1811(pSM240) able to grow in the absence of glutamine was identified and shown to contain a plasmid (pSM240). Two EcoRI fragments of 2.8 and 1.2 kb of pSM240 were subcloned into plasmid pSUP202 (Simon et al. 1983). The resultant plasmid, pSM245, complemented the deficiency in GS activity of UNF1811. The two EcoRI fragments of pSM245 were subcloned separately in both orientations into pSUP202 vector, neither fragment alone complemented strain UNF1811, suggesting that the complementing sequence lies within these two EcoRI sites. This suggestion was confirmed by constructing a derivative of pSM245 that had the kan gene from plasmid pGV97 (Leemans et al. 1982) inserted into the EcoRI site that separates the two inserts. The resulting kanamycin resistant plasmid, pSM58, failed to complement strain UNF1811.
To confirm that the complementing plasmid pSM245 encoded for a GS gene, and to determine which R. l. bv. phaseoli gene had been cloned, we compared the electrophoretic mobility of the GS activity of strain UNF1811 (pSM245 with those of GS1 and GSII of R. l. bv. phaseoli and with the K. pneumoniae GS. Strain UNF1811 (pSM245) showed a heat stable activity that comigrated in polyacrylamide gels with GS1 (Fig 1).

The glnA Km mutation created in the construction of pSM58 was used to replace a genomic glnA + allele in CE3. Plasmid pSM58, which cannot replicate in Rhizobium, was transferred by conjugation into strain CE3 for gene replacement. Strain SM58, a CE3 kanamycin resistant derivative, was isolated and confirmed by Southern blot hybridization experiments to carry the glnA Km insertion mutation (data not shown).

We determined the growth and the GS activities of the CE3 and SM58 strains. When CE3 is grown on minimal medium with glutamate as the sole nitrogen source GSII activity is about 20-fold higher than when this strain is grown on ammonia. The level of GSII activity is about twofold higher in glutamate (Table 1). No GSII activity was detected in strain SM58 (Table 1; Fig 1.), and GSII activity was found higher than in the wild type (Table 1). However, although different overall GS levels can be measured, no effect on the growth rate of mutant and wild type strains can be observed (Table 1).

GS activity was also determined in bacteroids of strains CE3 and SM58, Phaseolus vulgaris L. seedlings were inoculated with strains CE3 or SM58. Inoculated plants were grown in a Conviron plant growth chamber at 25 °C with 75% relative humidity and 12-hr light-dark periods. After 20 days bacteroids were isolated as described by Fortin et al. (1985). The heat resistant GSII, but not the heat labile GSII activity, was present in bacteroids of strain CE3 (Table 1). No GS activity was detected in bacteroids of strain SM58.

Acetylene reduction activity of 20-day-old nodules produced by strains CE3 and SM58 was determined. This activity was reduced in nodules induced by SM58 (Table 1). We therefore explored the histological organization and subcellular ultrastructure of nodules induced by the SM58 mutant. As Figure 2 shows, 20 days after inoculation, infected cells filled with bacteroids were observed in nodules induced by strain CE3, whereas fewer bacteroids were observed in nodules induced by strain SM58. In contrast to the wild type, bacteroids of the SM58 contained pronounced deposits of poly-β-hydroxybutyrate (PHB).

In this study, we determined GS activity of nitrogen-fixing R. l. bv. phaseoli bacteroids of the wild type CE3. We found, under the conditions tested, that GS1 is the unique GS activity that is present. This was confirmed by the fact that no GS activity was detected in bacteroids of the GSII mutant. We also found that nodules induced by this strain have a reduced capacity to fix nitrogen. In contrast to the situation in R. l. bv. phaseoli, Bradyrhizobium japonicum glnA strains induced higher numbers of nodules per plant and higher acetylene reduction activity than did the wild type (Carlson et al. 1987). A similar symbiotic phenotype was reported for B. japonicum glnII mutants (Carlson et al. 1987). Nodulation and nitrogen fixation in glnA, and glnII mutants of R. meliloti were reported to be normal (Somerville and Kahn 1983; Somerville et al. 1989; de Bruijn et al. 1989), suggesting

![Table 1. Growth, glutamine synthetase, and acetylene reduction activities of CE3 and SM58 strains of Rhizobium leguminosarum bv. phaseoli]*](image)

<table>
<thead>
<tr>
<th>Strain</th>
<th>Medium</th>
<th>DT* (min)</th>
<th>GSI Transferase</th>
<th>GSII Transferase</th>
<th>GSI Biosynthetic</th>
<th>GSII Biosynthetic</th>
<th>Acetylene reduction*</th>
</tr>
</thead>
<tbody>
<tr>
<td>CE3</td>
<td>MM + glutamate</td>
<td>150</td>
<td>1.38</td>
<td>2.29</td>
<td>0.07</td>
<td>0.08</td>
<td>...</td>
</tr>
<tr>
<td>SMS58</td>
<td>MM + glutamate</td>
<td>150</td>
<td>&lt;0.01</td>
<td>2.56</td>
<td>&lt;0.01</td>
<td>0.09</td>
<td>...</td>
</tr>
<tr>
<td>CE3</td>
<td>MM + NH₄⁺</td>
<td>143</td>
<td>0.63</td>
<td>0.12</td>
<td>&lt;0.01</td>
<td>&lt;0.01</td>
<td>...</td>
</tr>
<tr>
<td>SMS58</td>
<td>MM + NH₄⁺</td>
<td>150</td>
<td>&lt;0.01</td>
<td>0.33</td>
<td>&lt;0.01</td>
<td>&lt;0.01</td>
<td>...</td>
</tr>
<tr>
<td>CE3</td>
<td>Bacteroids</td>
<td>250</td>
<td>0.35</td>
<td>&lt;0.01</td>
<td>0.02</td>
<td>0.02</td>
<td>706 (57)*</td>
</tr>
<tr>
<td>SMS58</td>
<td>Bacteroids</td>
<td>250</td>
<td>&lt;0.01</td>
<td>&lt;0.01</td>
<td>&lt;0.01</td>
<td>&lt;0.01</td>
<td>370 (26)</td>
</tr>
</tbody>
</table>

*Glutamine synthetase activity was determined to whole cells as previously described (Morett et al. 1985). Specific activity is expressed as micromoles of product formed per minute per milligram of protein.

*Duplicate time.

*Acetylene reduction was determined as described previously (Morett et al. 1988). Specific activity is given as arbitrary units of ethylene produced per hour per weight (g) of nodules. Values are the mean of 45 determinations.

*Number in parenthesis are standard deviations. The Student t test showed that the difference between media was highly significant (P < 0.01).
that neither GSI or GSII are essential for the symbiosis. However, glutamine synthesis seems to be important at several stages in the symbiosis, because a B. japonicum glnA,glnII double mutant is a glutamine auxotroph and is defective in nodulation (Carlson et al. 1987), whereas in R. meliloti a glnA,glnII mutant, which under certain conditions is not a glutamine auxotroph, has a Nod+ Fix+ phenotype (Somerville et al. 1989). It is generally assumed that bacteroids do not assimilate ammonia, still it is likely that in R. l. bv. phaseoli bacteroids some glutamine is synthesized, because the utilization of its amide moiety by the glutamine amidotransferase leads to the synthesis of the pyridine nucleotide coenzymes (Meister 1980) that are essential for the nitrogenase activity. Glutamine synthesis might also be important at some stage in the symbiosis before nitrogen fixation is started, such as bacteroid proliferation or differentiation. Reduction of nitrogen fixation in nodules induced by strain SM58 is likely to be related to the reduction in the number of bacteroids seen in the infected cells. Accumulation of PHB in bacteroids of strain SM58 is a phenotype similar to that of nifH mutants of B. japonicum (Hahn et al. 1984). Thus it might be related to the reduction in nitrogen fixation. PHB accumulation could be the result of an increase in its synthesis or an inhibition of its degradation or both. Catabolism of PHB proceeds through the action of a PHB depolymerase and of a d(-)-3-hydroxybutyrate dehydrogenase. These enzymes have been identified in Rhizobium bacteroids (Wong and Evans 1971; Dawes and Senior 1973; Karr et al. 1984); the later enzyme is inhibited by 2-oxoglutarate in Azotobacter vinelandii (Dawes and Senior 1973). Glutamine and 2-oxoglutarate are the initial substrates in the GOGAT/GS pathway; therefore there is a possibility that in the SM58 bacteroids, 2-oxoglutarate accumulates and thus inhibits PHB degradation.

The experiments presented here indicate that in R. l. bv. phaseoli GSI is the unique isoenzyme present in nitrogen-fixing bacteroids; when it is removed by mutation, the effectiveness of the nodule is impaired.

ACKNOWLEDGMENTS

This study was supported by EEC collaborative grant CII-0410-ME/ CII-0406-I. We thank M. Iaccarino for discussions and A. Gonzalez and E. Merino for reviewing the manuscript.

LITERATURE CITED


![Fig. 2. Electron micrographs of thin sections of nodules from Phaseolus vulgaris beans 20 days after inoculation with A, CE3 and B, SM58 strains. Structural features are abbreviated as follows: B, bacteroids; PM, peribacteroid membrane; PHB, poly-beta-hydroxybutyrate granules; CW, plant cell wall. The magnification is 12,000-fold; a 1-μm scale is included. Microscopic examination was carried out as described by Soberon et al. 1990.](image-url)


