NOTES

Introduction of the *Escherichia coli* gdhA Gene into *Rhizobium phaseoli*: Effect on Nitrogen Fixation

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*Rhizobium phaseoli* lacks glutamate dehydrogenase (GDH) and assimilates ammonium by the glutamine synthetase-glutamate synthase pathway. A strain of *R. phaseoli* harboring the *Escherichia coli* GDH structural gene (gdhA) was constructed. GDH activity was expressed in *R. phaseoli* in the free-living state and in symbiosis. Nodules with bacteroids that expressed GDH activity had severe impairment of nitrogen fixation. Also, *R. phaseoli* cells that lost GDH activity and assimilated ammonium by the glutamine synthetase-glutamate synthase pathway preferentially nodulated *Phaseolus vulgaris*.

Bacteria of the genus *Rhizobium* have a symbiotic interaction with leguminous plants. They are able to fix nitrogen in root nodules, in which the bacteria differentiate into bacteroids (27). Nodule formation and bacterial differentiation are multistep processes which require complex interactions of the symbiont with its host plant. At present, little is known about the biochemical events that allow *Rhizobium* spp. to engage in effective symbiosis.

The *Rhizobium* enzymes involved in ammonium assimilation are switched off during symbiotic nitrogen fixation, allowing fixed nitrogen to be excreted and assimilated by the plant (6, 12, 25). However, free-living bacteria are able to assimilate ammonium. It has been reported that in several *Rhizobium* species, ammonium assimilation proceeds mainly through the glutamine synthetase (GS)-glutamate dehydrogenase (GDH) pathway. All glutamate auxotrophs that have been isolated from *Rhizobium* spp. are affected in their GOGAT activity (8, 11, 15, 21, 22), and no mutants have been found with altered glutamate dehydrogenase (GDH) activity. We found that in *Rhizobium phaseoli*, ammonium is assimilated by the GS-GOGAT pathway, since no GDH activity was detected (5), and the *Escherichia coli* GDH structural gene did not hybridize with total DNA from *R. phaseoli* (unpublished data).

It may be questioned why *Rhizobium* species do not assimilate ammonium through the GDH-GS pathway. Is the GS-GOGAT pathway in *Rhizobium* species a necessary condition for establishment of effective symbiosis with a plant? To answer these questions, we introduced the *E. coli* *gdha* gene into an *R. phaseoli* wild-type strain and studied its expression and its effects in culture and during symbiosis.

The *E. coli* *gdha* structural gene contained in plasmid pSAE4 (26) was subcloned into the plasmid vector pRK404 (7) (Fig. 1). Plasmid pSAE4 was cleaved with HindIII, PstI, and *Pvu*II, and the fragments were separated by agarose gel electrophoresis. The band corresponding to the 3.6-kilobase HindIII-PstI fragment was purified by electroelution and ligated (16) into the pRK404 plasmid previously digested with HindIII-PstI. After transformation (16) into *E. coli* PA340, which lacks GDH and GOGAT activities (2), clones harboring a recombinant plasmid were selected by tetracycline (10 μg/ml) resistance in complex LB medium (18) and by growth on minimal M9 medium (3) containing 10 mM NH₄Cl as the nitrogen source. This construction, pAB17, was mobilized from *E. coli* to *R. phaseoli* CFN4242 (23) in a triparental mating, with *E. coli* HB101(pRK2013) (4) as the donor or helper plasmid (10). The *Rhizobium* transconjugants were selected on complex PY medium (19) supplemented with tetracycline and nalidixic acid (100 μg/ml). Strain CFN4250 harbors plasmid pAB17, which contains the GDH gene, and strain CFN4251, harboring the pRK404 plasmid, was used as the control strain.

Further analysis of strain CFN4250 revealed that it harbored a 14-kilobase plasmid containing a 3.6-kilobase insert which corresponded to the fragment isolated from plasmid pSAE4 (data not shown).

To determine the stability of plasmids pAB17 and pRK404 in *R. phaseoli*, the respective strains were grown in nonselective (PY) medium during three growth cycles of 36 h each, and every 4 h the cells were plated onto agar plates of PY medium with or without tetracycline. There were 10 cell doublings per growth cycle, and the plasmid loss was 1% per cell doubling for both plasmids. The percentage of plasmid loss was determined from the difference between cells grown on PY and those grown on PY plus tetracycline.

To ensure that we had cloned the *gdha* structural gene, we determined the GDH activity of strains CFN4250 and CFN4251(pRK404). The cells were harvested by centrifugation at 4°C, suspended in the extraction solutions, and ruptured with Braun cell homogenizer MSK type 853030 and 0.1-mm (diameter) glass beads. GDH activity was extracted with 50 mM Tris hydrochloride–10 mM mercaptoethanol (pH 7.6). GDH and GOGAT activities were detected by monitoring NADPH oxidation at 340 nm. GOGAT activity was extracted and determined as described elsewhere (5), and GDH activity was measured in a 1-ml reaction mixture containing 50 mM Tris (pH 7.6), 5 mM 2-oxoglutarate, 0.25 mM NADPH, 40 mM NH₄Cl, and 0.05 ml of cell extract.
GDH activity was also measured by determining the glutamate formed, as described elsewhere (5).

Only strain CFN4250 was able to express GDH activity, and at the same level as in *E. coli* PA340(pAB17), (Table 1). The *E. coli* GDH activity in *R. phaseoli* CFN4250 was expressed in a constitutive manner in media containing different nitrogen sources (data not shown), in contrast to the repression by glutamate observed in *E. coli* (14).

The presence of GDH activity in *R. phaseoli* affected the intracellular concentrations of its substrate and product. The 2-oxoglutarate content was lower and the glutamate content was higher in the strain with GDH activity (Table 1), even though GOGAT activity was lower than in the strain lacking GDH activity.

Further evidence for the operations of the GDH-GS pathway in strain CFN4250 was its capacity to grow on minimal medium containing NH₄Cl as the sole nitrogen source and l-methionine sulfoxime (MSF; Fig. 2A), an inhibitor of GOGAT activity (Fig. 2B; 17).

The results presented indicate that the *E. coli* GDH activity expressed in *R. phaseoli* is able to produce the glutamate needed to support growth when GOGAT is inactivated by MSF. This was also reflected by an increase in glutamate (Table 1), which possibly leads to GOGAT repression (5; Table 1) and a consequent decrease in 2-oxoglutarate.

To determine the symbiotic properties of strain CFN4250, *Phaseolus vulgaris* was inoculated with this strain and CFN4251. The seeds were surface sterilized in 20% hypochlorite and germinated on moist, sterile filter paper (19). Three-day-old seedlings were transferred to plastic growth pots, inoculated with a bacterial suspension in PY medium, and grown on nitrogen-free salts solution in a greenhouse (24). Three weeks after inoculation, all plants infected by these strains were nodulated normally. We removed the nodules from the plants to look for the presence of the plasmid in the isolated bacteria. Recovery of bacteria from nodules was done as previously described (20). Plants were preferentially nodulated by rhizobia that had lost the plasmid that harbors the *E. coli* *gdhA* gene. Only 21% of the nodules formed by strain CFN4250 (GDH⁺) were Tc⁵. This is in

![Diagram](image1.png)

**FIG. 1.** Diagrammatic representation of the construction of the pAB17 derivative. Tc, Tetracycline.

![Graph](image2.png)

**FIG. 2.** Growth of *R. phaseoli* strains in the presence of MSF and in vitro inhibition of GOGAT activity by this metabolite. (A) Cells were grown on minimal medium with 10 mM NH₄Cl as the nitrogen source in the presence of 5 mM MSF. Symbols: ○, CFN4250; ●, CFN4251. (B) In vitro inhibition of GOGAT activity from *R. phaseoli* CFN42 by MSF.

<table>
<thead>
<tr>
<th>Strain</th>
<th>GDH Sp act (μmol/min per mg of protein) of:</th>
<th>GOGAT Conc (nmol/mg of protein) of:</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>NADPH oxidized</td>
<td>Glutamate produced</td>
</tr>
<tr>
<td>CFN4250</td>
<td>0.265</td>
<td>0.250</td>
</tr>
<tr>
<td>CFN4251</td>
<td>0.004</td>
<td>0.002</td>
</tr>
<tr>
<td>PA340(pAB17)</td>
<td>0.331</td>
<td>ND</td>
</tr>
</tbody>
</table>

* Cells were grown for 12 h on minimal medium supplemented with 10 mM NH₄Cl as the nitrogen source. ND, Not determined.

**TABLE 1.** Relationships among the presence of GDH and GOGAT activities, 2-oxoglutarate, and glutamate content in *R. phaseoli* strains*
TABLE 2. Acetylene reduction and GDH and GOGAT activities in nodules and bacteroids from plants inoculated with a strain harboring the E. coli gdhA gene

<table>
<thead>
<tr>
<th>Strain (phenotype)</th>
<th>Mean ± SEM % Tc⁺ nodules</th>
<th>Mean ± SEM % nitrogenase activity</th>
<th>Sp act (µmol of NADPH/min per mg of protein)</th>
</tr>
</thead>
<tbody>
<tr>
<td>CFN4250 (GDH⁺)</td>
<td>21 ± 8</td>
<td>15 ± 3</td>
<td>0.320</td>
</tr>
<tr>
<td>CFN4251 (GDH⁻)</td>
<td>75 ± 10</td>
<td>100 ± 15</td>
<td>0.001</td>
</tr>
</tbody>
</table>

* Five seeds were inoculated for each experiment, and the data are from five different experiments. All of the nodules present in each plant were crushed to test the bacteriological phenotype.

Nitrogenase activity was determined by measurement of acetylene reduction in detached nodules. The nodules were transferred to tubes with rubber seal stoppers, acetylene was injected to a final concentration of 10% of the gas phase, and ethylene production was determined by gas chromatography with a Packard model 430 chromatograph. The activity found in detached Tc⁺ nodules was only 15% of that found in nodules from plants inoculated with CFN4251 (GDH⁺) or CFN4250, which had lost the plasmid (Tc⁺) (Table 2). Finally, to verify that the nodules formed by strain CFN4250 really expressed GDH activity, bacteroids were isolated as described by Awonaike et al. (1) and GDH activity was determined. Bacteroids from Tc⁺ nodules with GDH⁺ strains expressed activity at the same level as the free-living bacteria harboring the E. coli gdhA gene (Table 2). However, bacteroids from nodules with GDH⁻ strains, originating from infection with Tc⁻ strains CFN4250 and CFN4251, had an almost undetectable amount of GDH (Table 2). The GOGAT activity of the isolated bacteroids was lower in those that harbored the E. coli gdhA gene (Table 2). We found that 90% of the bacteria isolated from Tc⁺ nodules are Tc⁺. A correlation can be established among the Tc⁺ bacteria found in nodules inoculated with strain CFN4250, the presence of GDH activity, and the 85% decrease in the nitrogenase activity of these nodules. On the other hand, nodules with Tc⁻ bacteria lacked GDH activity and had optimal nitrogenase activity.

The presence of GOGAT activity in bacteroids does not interfere in the formation of an effective symbiosis between Rhizobium spp. and plants. However, symbiotic nitrogen fixation seems to be affected by the presence of GDH activity in R. phaseoli; detached nodules infected by strain CFN4250 had a sixfold diminution in acetylene reduction (Table 2). These data agree with the previous report of Osborne and Signer (22) that GOGAT⁻ phenotype revertants isolated from plant nodules which expressed high levels of GDH activity are able to nodulate clover but do not fix nitrogen. Recently, Lane et al. have reported the introduction of the E. coli gdhA gene in R. japonicum (13). The recipient strain used was an Asm⁻ mutant strain of R. japonicum which was unable to grow with NH₄Cl as the nitrogen source, lacked GOGAT activity, and did not express nitrogenase in the nodules. In this genetic background, they found no effect of GDH activity on symbiotic properties.

Nodules formed by bacteria with GDH activity have a severe reduction in nitrogen fixation. An active GDH enzyme in bacteroids could probably interfere with N₂ fixation by draining carbon skeletons and reductive power or by formation of its product, glutamate. There is evidence that 2-oxoglutarate is needed to support nitrogen fixation, since a mutant lacking 2-oxoglutarate dehydrogenase is impaired in nitrogen fixation (9).

As presented in this paper, R. phaseoli bacteroids were not able to switch off E. coli GDH activity (Table 2). Plants were preferentially nodulated by strains that had lost the plasmid harboring the gdhA gene (Table 2). It seems that plants are preferentially nodulated with rhizobia capable of ammonium assimilation by the GS-GOGAT pathway, which leads to effective symbiosis. We are cloning the gdhA gene in a stable plasmid to determine whether plants can preferentially select rhizobia with a specific ammonium assimilation pathway.

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LITERATURE CITED


