JOURNAL OF BACTERIOLOGY, Feb. 2001, p. 854–864 Vol. 183, No. 3
0021-9193/01/$04.00+0 DOI: 10.1128/JB.183.3.854–864.2001


# Enhanced Symbiotic Performance by *Rhizobium tropici* Glycogen Synthase Mutants

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Received 28 July 2000/Accepted 7 November 2000

**We isolated a Tn*5*-induced *Rhizobium tropici* mutant that has enhanced capacity to oxidize *N,N*-dimethyl-*p*-phenylendiamine (DMPD) and therefore has enhanced respiration via cytochrome oxidase. The mutant had increased levels of the cytochromes $c_1$ and CycM and a small increase in the amount of cytochrome $aa_3$. In plant tests, the mutant increased the dry weight of *Phaseolus vulgaris* plants by 20 to 38% compared with the control strain, thus showing significantly enhanced symbiotic performance. The predicted product of the mutated gene is homologous to glycogen synthases from several bacteria, and the mutant lacked glycogen. The DNA sequence of the adjacent gene region revealed six genes predicted to encode products homologous to the following gene products from *Escherichia coli*: glycogen phosphorylase (*glgP*), glycogen branching enzyme (*glgB*), ADP glucose pyrophosphorylase (*glgC*), glycogen synthase (*glgA*), phosphoglucomutase (*pgm*), and glycogen debranching enzyme (*glgX*). All six genes are transcribed in the same direction, and analysis with *lacZ* gene fusions suggests that the first five genes are organized in one operon, although *pgm* appears to have an additional promoter; *glgX* is transcribed independently. Surprisingly, the *glgA* mutant had decreased levels of high-molecular-weight exopolysaccharide after growth on glucose, but levels were normal after growth on galactose. A deletion mutant was constructed in order to generate a nonpolar mutation in *glgA*. This mutant had a phenotype similar to that of the Tn*5* mutant, indicating that the enhanced respiration and symbiotic nitrogen fixation and decreased exopolysaccharide were due to mutation of *glgA* and not to a polar effect on a downstream gene.**

*Rhizobium tropici* induces nitrogen-fixing nodules on several unrelated tropical legume plants, including species of *Phaseolus, Leucaena*, and *Macroptilium* (30). Bacterial respiration is essential for symbiotic nitrogen fixation in two ways: the ATP necessary for nitrogen fixation is derived from oxidative phosphorylation, and respiration removes oxygen, thereby preventing inactivation of nitrogenase by oxygen.

Rhizobia, like many other bacteria, possess branched respiratory chains with three or more terminal oxidases. The electrons derived from different sources are channeled to the quinone pool in the cytoplasmic membrane and from there are transferred directly to quinol oxidases or, via the cytochrome $bc_1$ complex and cytochrome *c*, to cytochrome *c* oxidases. The respiratory chains of *Bradyrhizobium japonicum, Rhizobium leguminosarum, Rhizobium etli*, and *Azorhizobium caulinodans* have been studied (1, 10, 18, 20, 28, 44). They all possess a cytochrome *c* oxidase of the $aa_3$ type, which is a major component of the respiratory chain in aerobiosis. This type of oxidase has also been described in *R. tropici* (14). Alternative cytochrome *c* oxidases or quinol oxidases may also contribute to aerobic respiration. In nodules, where oxygen levels are low, a cytochrome *c* oxidase of the $cbb_3$ type with a high affinity for oxygen facilitates respiration. The subunits of this oxidase are encoded by the *fixNOQP* genes, which have been described in most rhizobia (10, 18).

*R. etli*, like the related species *R. tropici*, can nodulate *Phaseolus* beans. Mutants of *R. etli* with increased respiration (33, 44, 45, 46) were isolated on the basis of their enhanced capacities to oxidize *N,N,N′,N′*-tetramethyl-*p*-phenylendiamine (TMPD) by using the Nadi cytochrome oxidase test (29). This test is based on the reaction of TMPD and α-naphthol in the presence of cytochrome *c* and cytochrome *c* oxidase to produce indophenol blue. *Rhizobium* mutants affecting formation of cytochrome $bc_1$, CycM, or cytochrome $aa_3$ are Nadi$^-$ (10), whereas mutants with increased respiration via cytochrome *c* stain more strongly (Nadi$^{++}$). Two *R. etli* Nadi$^{++}$ mutants had increased respiration due to induction of the *fixNOQP* genes under free-living conditions (34, 46). The genes affected in both mutants are linked to the purine biosynthetic pathway, and it was proposed that the intermediary metabolite 5-amino-4-imidazolecarboxamide ribonucleotide could act as a negative effector of cytochrome $cbb_3$ production in *R. etli* (46). One of these mutants and two uncharacterized mutants increased the nitrogen content of *Phaseolus vulgaris* plants by 22 to 25% compared with the wild-type strain (33, 44, 45). Mutants of *Sinorhizobium meliloti* with increased respiration and symbiotic performance have also been described (54). The aim of this study was to isolate and characterize *R. tropici* mutants with enhanced respiration and symbiotic nitrogen fixation on *P. vulgaris* plants.

## MATERIALS AND METHODS

**Microbiological techniques.** The bacterial strains and plasmids used are listed in Table 1. *R. tropici* and *Agrobacterium tumefaciens* strains were grown at 28°C in TY medium (2) or Y minimal medium (43) supplemented with 10 mM

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TABLE 1. Bacterial strains and plasmids

| Strain or plasmid | Genotype or relevant characteristics | Source or reference |
|---|---|---|
| CIAT899 | Wild-type; Rif$^r$ Cm$^r$ Ap$^r$ | 30 |
| A554 | *glgA1*::Tn*5*; Nadi$^{++}$; Km$^r$ | This work |
| A639 | *glgA1*::Tn*5* obtained by homologous recombination | This work |
| A656 | *glgA*Δ*Pst*I | This work |
| *A. tumefaciens* | | |
| A348 | Wild-type; pTi$^-$ Cm$^r$ | 49 |
| A5129 | *pgm*::Tn*5* Km$^r$ | 49 |
| *E. coli* | | |
| DH5α | F$^-$ *supE44* Δ*lacU169* (ϕ80*lacZ*ΔM15) *hsdR17 recA1 endA1 gyrA96 thi-1 relA1* | 41 |
| LCB499 | *glgB* derivative of PA601 | 7 |
| RH97 | *glgC*::Tn*10* derivative of MC4100 | R. Hengge-Aronis |
| RH98 | *glgA*::Tn*10* derivative of MC4100 | R. Hengge-Aronis |
| Plasmids | | |
| pBluescript II KS | pUC derivative; F1 replication origin; Ap$^r$ | Stratagene Cloning Systems (La Jolla, Calif.) |
| pMP220 | Derivative of pKT214 carrying *lacZ* without a promoter; Tc$^r$ | 47 |
| pJQ200KS | *sacB* Lac Gm$^r$ | 39 |
| pIJ1891 | pLAFR3 derivative; Lac Tc$^r$ | A. Mavridou |
| pJB4JI | Suicide plasmid for Tn*5* mutagenesis of *Rhizobium* | 3 |
| pRK2013 | Helper plasmid that provides plasmid transfer functions Km$^r$ | 11 |
| pIJ7719 | Overlapping cosmids from CIAT899 DNA library which complement A554 and A656 | This work |
| pIJ7720 | | |
| pIJ9158 | | |
| pIJ7741 | 6.6-kb *Hin*dIII fragment from pIJ7720 carrying *glgA*, *pgm*, and *glgX* in pBluescript II KS | This work |
| pIJ7746 | 6.6-kb *Hin*dIII fragment from pIJ7720 in pIJ1891 complementing A554 and A656 | This work |
| pIJ7796 | *glgA*::Tn*5* in pJQ200KS | This work |
| pIJ7814 | 1.6-kb *Eco*RI deletion of pIJ7746 | This work |
| pIJ7844 | 4.3-kb *Sac*I fragment from pIJ7720 carrying *glgC* and *glgA* in pBluescript II KS | This work |
| pIJ7883 | 2.2-kb *Cla*I-*Sac*I fragment carrying *glgA* into pBluescript II KS | This work |
| pIJ7884 | 624-bp *Pst*I deletion of *glgA* in pIJ7883 | This work |
| pIJ7899 | *glgA*Δ*Pst*I in pJQ200KS | This work |
| pIJ7959 | 4.3-kb *Sac*I subclone of pIJ7720 in pIJ1891 complementing A554 and A656 | This work |
| pIJ9011 | 0.8-kb *Xba*I-*Pst*I fragment in pMP220; *glgX-lacZ* | This work |
| pIJ9014 | 2.4-kb *Hin*dIII fragment in pMP220; *glgC-lacZ* | This work |
| pIJ9015 | 1.5-kb *Eco*RI-*Xba*I fragment in pMP220; *pgm-lacZ* | This work |
| pIJ9016 | 0.7-kb *Eco*RI-*Xba*I fragment in pMP220; *glgA-lacZ* | This work |
| pIJ9034 | *pgm-lacZ* but carrying Tn*5* of A554 | This work |
| pIJ9044 | *pgm-lacZ* but carrying *Pst*I deletion | This work |
| pIJ9147 | 1.3-kb *Hin*dIII fragment in pMP220; *glgB-lacZ* | This work |
| pIJ9160 | 8-kb *Hin*dIII fragment in pMP220; *glgP-lacZ* | This work |

ammonium chloride and 0.2% (wt/vol) succinate, glucose, galactose, or mannitol. *Escherichia coli* strains were grown at 37°C in L medium (31), to which maltose (0.5% [wt/vol]) was added for experiments involving detection of glycogen. Antibiotics were added as appropriate to the following final concentrations (micrograms per milliliter): ampicillin, 400; gentamicin (GEN), 10; kanamycin (KAN), 20; rifampin, 20; spectinomycin, 100; and tetracycline, 10. Sucrose, when present, was added at 5% (wt/vol).

**Genetic techniques.** Plasmids were transferred from *E. coli* DH5α to *R. tropici* by triparental matings with the helper plasmid pRK2013 (11). *R. tropici* CIAT899 was mutagenized with Tn*5* using pJB4JI (3), selecting for mutants on Y-succinate medium supplemented with rifampin and KAN. Colonies were screened using the Nadi cytochrome oxidase test (29), which measures cytochrome *c* oxidase activity based on the reaction of *N*,*N*-dimethyl-*p*-phenylendiamine or TMPD and α-naphthol, in the presence of bacterial cytochrome *c* and cytochrome *c* oxidase, to produce indophenol blue. Mutants with enhanced activity (Nadi$^{++}$) were isolated as colonies staining more strongly. The approximately 13-kb *Eco*RI fragment carrying the Tn*5* insertion from the Nadi$^{++}$ mutant A554 was cloned into the *Eco*RI site in the multiple cloning site of partially digested pJQ200KS (39). The resulting plasmid (pIJ7796) was introduced into CIAT899, and selection was made for sucrose-resistant GEN-sensitive recombinants. A639 was one such recombinant and was confirmed by DNA hybridization to contain Tn*5* in the appropriate location. The *glgA* deletion mutant A656 was generated by a reciprocal crossover, exchanging a deletion derivative of *glgA* with the Tn*5* in A554. Plasmid pIJ7883 carries *glgA* in a 2.2-kb *Cla*I-*Sac*I insert. An in-frame deletion of *glgA* was constructed by excising a 624-bp *Pst*I fragment to form pIJ7884, and then the remaining 1.6-kb fragment was subcloned as a *Sal*I-*Sac*I fragment into pJQ200KS to form pIJ7899. This plasmid was conjugated into A554 and sucrose-resistant, GEN and KAN-sensitive recombinants were isolated. One such isolate, A656, was confirmed by DNA hybridization to have the appropriate pattern of DNA fragments. The fusions of the glycogen metabolism genes to the *E. coli lacZ* gene were generated by subcloning the following DNA fragments into pMP220 (47) in the correct orientation, resulting in the plasmids indicated in parentheses: 8-kb *Hin*dIII (*glgP-lacZ*; pIJ9160), 1.3-kb *Hin*dIII (*glgB-lacZ*; pIJ9147), 2.4-kb *Hin*dIII (*glgC-lacZ*; pIJ9014), 0.7-kb *Eco*RI-*Xba*I (*glgA-lacZ*; pIJ9016), 1.5-kb *Eco*RI-*Xba*I (*pgm-lacZ*; pIJ9015), 7.3-kb *Eco*RI-*Xba*I (*glgA*::Tn*5*-*pgm-lacZ*; pIJ9034), 1.0-kb *Pst*I-*Eco*RI (*glgA*Δ*Pst*I-*pgm-lacZ*; pIJ9044), and 0.8-kb *Xba*I-*Pst*I (*glg-lacZ*; pIJ9011) (Fig. 1B). β-Galactosidase activity was assayed essentially as described by Rossen et al. (40) with cells grown in Y medium supplemented with glucose or galactose.

To confirm that the *pgm* gene in pIJ7814 was expressed, we transferred pIJ7814 to strain A5129 (a *pgm* mutant of *A. tumefaciens*) (49) and observed

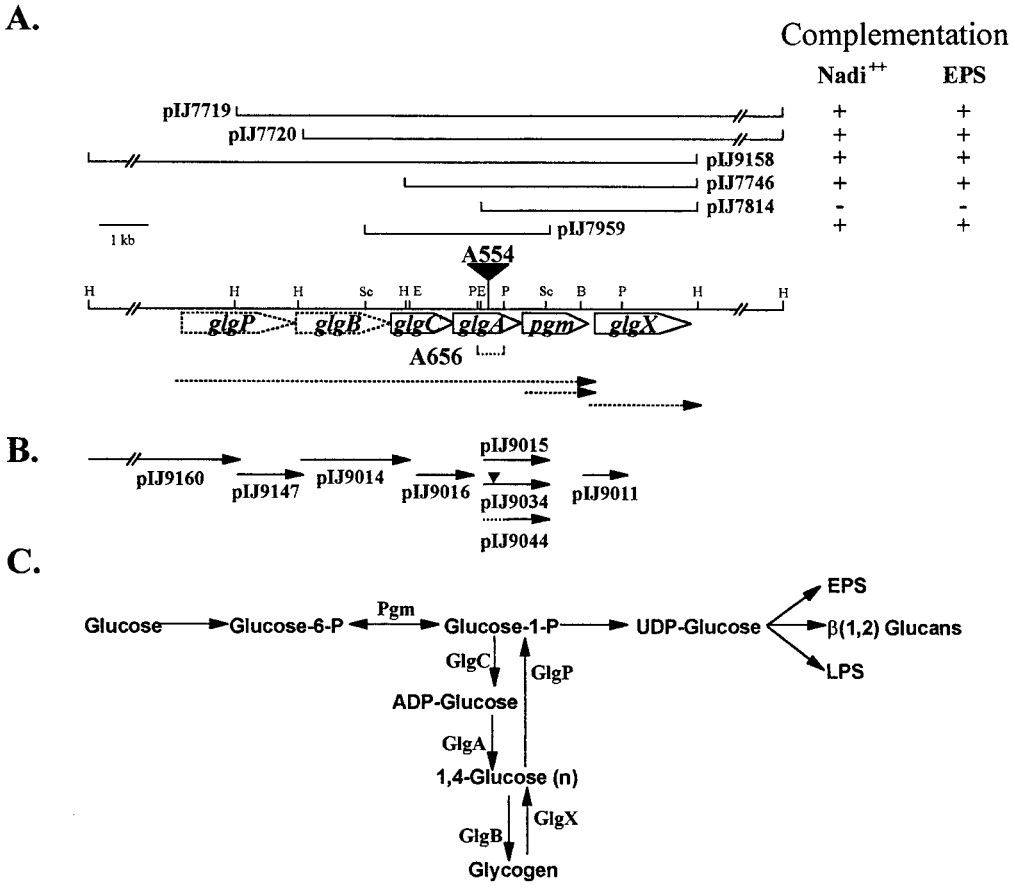


FIG. 1. (A) Physical and genetic map of the *R. tropici* glycogen region and complementation of the Nadi$^{2+}$ and low-EPS phenotypes of mutants A554 and A656 by different *R. tropici* cosmids. +, complementation; −, no complementation. The open arrows represent the glycogen metabolism genes and the direction of their transcription. The broken lines indicate that the genes have not been sequenced completely. The position of Tn*5* in mutant A554 is indicated by an inverted triangle, and the fragment of *glgA* deleted in mutant A656 is represented by a dotted segment. The dotted arrows indicate the putative transcriptional units. B, *Bam*HI; E, *Eco*RI; H, *Hin*dIII; P, *Pst*I; Sc, *Sac*I. (B) Transcriptional fusions of the glycogen metabolism genes to *E. coli lacZ*. The direction of transcription is indicated by the arrows. The dotted segment of pIJ9044 indicates the region deleted. (C) Biochemical reactions involved in glycogen metabolism in *A. tumefaciens* (adapted from reference 49). The gene products indicated are proposed to catalyze the corresponding reactions.

complementation of the exopolysaccharide-minus (EPS$^-$) phenotype on Y-mannitol plates.

**DNA manipulations.** Various operations were carried out according to the method of Sambrook et al. (41). DNA hybridizations were done at 65°C in 5× SSC (1× SSC is 0.15 M NaCl plus 0.015 M sodium citrate), and washes were done in 1× SSC–0.1% sodium dodecyl sulfate (SDS) at 65°C. Double-stranded DNA was sequenced with a Thermo Sequenase Dye Terminator cycle sequencing kit (Amersham) and the automatic sequencer ABI377 (Perkin-Elmer). The *glgC, glgA, pgm*, and *glgX* genes were sequenced on both strands; only parts of *glgP* and *glgB* were sequenced on both strands. In addition to the universal and reverse primers, the following primers were used: 5′-GAAGTCAGATCCTGG AAAACGGGAA-3′ (to sequence one strand from the end of Tn*5*), 5′-GGAT ACGTCGGATTTCATGCCCTG-3′ (to sequence the *Pst*I deletion used to generate mutant A656), and 5′-CGTTGTCCTGGCAATAGGCA-3′ (to complete the sequence of the *glgX* gene). The nucleotide sequence was analyzed with the Genetics Computer Group version 8.1 package.

**Cell fractionation, protein gel electrophoresis, heme staining, and immunostaining.** *R. tropici* cells grown on succinate were fractionated into membranes and soluble fractions essentially as described previously (9). Protein concentrations were estimated by the method of Bradford (5) using bovine serum albumin as a standard. The membrane and soluble fractions were suspended in loading buffer (124 mM Tris [pH 7.0] 20% [vol/vol] glycerol, 4.6% [wt/vol] SDS), incubated at 42°C for 20 min, separated by SDS-polyacrylamide gel electrophoresis (PAGE), and transferred to a nitrocellulose filter. Proteins containing covalently bound heme were stained by chemiluminescence as described by Vargas et al. (51).

For detection of phosphoglucomutase, cells were grown for 48 h on glucose minimal medium. The cells were washed with 30 mM Tris-HCl (pH 8.0)–3 mM EDTA and resuspended in the same buffer containing 20% (wt/vol) sucrose, 200 μg of lysozyme/ml, and 1 mM phenylmethanesulfonyl fluoride. After 1 h on ice, the cells were centrifuged at 12,000 × *g* for 30 min and resuspended in 50 mM Tris-HCl (pH 8.0), 10 mM $MgCl_2$, 20% sucrose, 1 mM phenylmethanesulfonyl fluoride, and 10 mg of DNase I/ml. The cells were broken by two passages through a French pressure cell, and the extract was centrifuged at 3,000 × *g* for 30 min to remove unbroken cells and then at 100,000 × *g* for 3 h to remove membranes. Soluble proteins were precipitated with 10% trichloroacetic acid, and the pellet was washed with acetone. Samples were separated by SDS-PAGE, transferred to nitrocellulose, incubated with a 1-in-500 dilution of antiserum to the *Agrobacterium* phosphoglucomutase (48), and stained with goat antirabbit immunoglobulin conjugated to alkaline phosphatase (Sigma, St. Louis., Mo.).

**Spectra and respiratory activities.** For spectra, *R. tropici* cells were washed in fresh Y-succinate medium, diluted to an optical density of 0.05 at 540 nm, and grown at 30°C for 12 h. The cells were harvested by centrifugation, washed, and suspended to 30% (wt/vol) in 0.1 M phosphate buffer (pH 7.4) with 40% glycerol. Cytochrome spectra were recorded using an SLM Aminco Midan II spectrophotometer. The samples were reduced with a few granules of dithionite or oxidized with ammonium persulfate. Carbon monoxide difference spectra (reduced + CO minus reduced) were obtained by bubbling CO for 2 min through a dithionite-

reduced cell suspension, and the spectra were recorded against a reduced sample. The spectra were measured at room temperature in 1.0-cm-diameter light path cuvettes. For the determination of oxygen uptake, the cells were harvested after 48 h of growth at 28°C in Y-succinate medium and resuspended in 1 ml of 25 mM potassium phosphate buffer (pH 7.0). The oxygen uptake was measured with a Hansatech electrode at 25°C after addition of 2 mM TMPD and 10 mM sodium ascorbate (final concentrations).

**Glycogen determination.** The glycogen content of *R. tropici* strains was determined as described by Krisman (22) using *R. tropici* cells grown for 3 days at 28°C in 40 ml of Y-glucose medium containing 3.5 instead of 10 mM $NH_4Cl$. The amount of glycogen present was calculated from the absorbance at 540 nm in relation to standard spectra recorded using rabbit glycogen (concentration range, 20 to 300 μg/ml) as a standard.

**Quantitative analysis of EPS.** The strains were grown for 4 days in Y medium supplemented with either glucose or galactose at 28°C. The cultures were centrifuged at 10,000 × *g* for 60 min, and the supernatant was collected, diluted 1:1 with distilled water, and centrifuged again in order to eliminate residual cells. The high-molecular-weight EPS (HMW-EPS) was precipitated from the supernatant by adding sodium chloride (to 0.3 M) and 2.5 volumes of ethanol. After 16 h at 4°C, the precipitated HMW-EPS was spooled with a glass rod and left to dry at 37°C in a preweighed petri dish. The ethanol-precipitable material was measured by weight difference. The structure of *R. tropici* CIAT899 EPS was previously determined (15).

**Plant tests.** *P. vulgaris* cv. Negro Jamapa plants (black beans) were grown in a greenhouse in Leonard jars with a nitrogen-free medium for an average of 45 days, as described by Vincent (52). At least four replicates were used per strain. Three parameters were measured: the dry weight of the aerial part of the plant, the number of nodules formed, and the dry weight of nodules per plant. The results were analyzed statistically by analysis of variance.

**Nucluotide sequence accession number.** The DNA sequence was submitted to the EMBL database and has been assigned accession number AJ291603.

## RESULTS

**Isolation and characterization of an *R. tropici* Nadi$^{++}$ mutant.** Tn*5*-induced mutants of *R. tropici* CIAT899 were screened on succinate minimal medium using the Nadi test (29). One mutant, A554, which stained darker blue (Nadi$^{++}$) than the wild type was chosen for detailed study because it had enhanced levels of symbiotic nitrogen fixation (see below). This mutant had a higher rate of TMPD oxidation than CIAT899 (21 versus 15 ng-atom of $O_2$/min/mg [dry weight] of cells). The Tn*5* and flanking DNA from A554 was cloned on an *Eco*RI fragment into the suicide vector pJQ200KS (39) to form pIJ7796, and the Tn*5* was recombined into CIAT899. The recombinant, A639, had the same Nadi$^{++}$ phenotype as A554, confirming that the Tn*5* insertion was responsible for the phenotype. DNA hybridizations with DNA from A554 and A639 demonstrated that each carried only one copy of Tn*5* and that it was inserted in the same position in both strains.

Three overlapping cosmids were isolated from an *R. tropici* CIAT899 library by colony hybridization, using DNA adjacent to the Tn*5* insertion as a probe. The three cosmids, pIJ7719, pIJ7720, and pIJ9158 (Fig. 1A), contained a 6.6-kb *Hin*dIII band which hybridized to the same probe. All three restored the Nadi$^{++}$ phenotype of A554 and A639 to normal (Fig. 1A) but had no effect on the Nadi phenotype of CIAT899. The cloned 6.6-kb *Hin*dIII fragment (pIJ7746) also complemented A554 and A639 to Nadi$^{+}$ (Fig. 1A).

**Cytochrome composition of A554.** The Nadi$^{++}$ phenotype of A554 suggested that the mutation may affect the expression of respiratory chain components, so these were analyzed. Heme staining of soluble and membrane fractions of CIAT899 (Fig. 2) revealed a major soluble component of approximately 14 kDa, probably corresponding to periplasmic cytochrome *c*, and two membrane components of about 20 and 31 kDa, probably corresponding to CycM and cytochrome $c_1$. In other work, mutations affecting the genes encoding the cytochrome $bc_1$ complex have been shown to abolish the formation of the 31-kDa component (unpublished results). The heme staining of A554 is different from that of CIAT899 in that staining of the soluble 14-kDa component is less intense (Fig. 2A) and the membrane components of 31 (cytochrome $c_1$) and 20 (CycM) kDa stained slightly more strongly (Fig. 2B). This suggests higher levels of cytochrome $c_1$ and CycM in the mutant. Both of these cytochromes are essential for a positive Nadi reaction in *Rhizobium* strains (10). The increases in cytochrome $c_1$ and CycM could account for the enhanced Nadi staining in A554 compared with CIAT899.

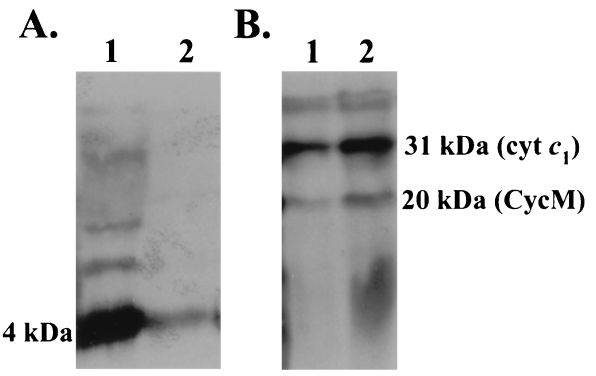


FIG. 2. C-heme stains of soluble (A) and membrane (B) proteins from *R. tropici* CIAT899 (lanes 1) and A554 (lanes 2) after SDS-PAGE. Each lane was loaded with 50 (A) or 30 (B) μg of protein. The molecular masses of the main bands are indicated and were estimated using prestained molecular mass markers (New England Biolabs) that do not show up on the heme-stained gel.

The reduced *minus* oxidized difference spectra (Fig. 3A) of the wild-type strain revealed peaks characteristic of cytochrome *c* (maximum at 553 nm), cytochrome *b* (maximum at 562 nm), and cytochrome $aa_3$ (maximum at 603 nm). A554 has a similar spectrum, although the ratio of cytochrome *b* to *c* seems to be higher than in CIAT899 and there is a somewhat increased level of cytochrome $aa_3$. The relatively small change in the cytochrome *c* region of the spectrum may be due to a decrease in absorption due to the soluble cytochrome *c* (Fig. 2A) being compensated for by increased levels of cytochrome $c_1$ and CycM (Fig. 2B). The reduced + CO *minus* reduced difference spectra (Fig. 3B) confirmed the presence of cytochrome $aa_3$ (peak at 419 nm; troughs at 443 and 610 nm) and revealed spectroscopic features characteristic of cytochrome *o* or a high-spin cytochrome *b* (which are indistinguishable by this technique) (peaks at 417 and 544 nm; troughs at 430 and 559 nm). The CO spectrum of A554 is similar to that of CIAT899, except that it reveals a slightly increased amount of cytochrome $aa_3$ (troughs at 610 and 443 nm).

**A554 has enhanced symbiotic performance.** *P. vulgaris* cv. Negro Jamapa beans were grown under nitrogen limitation and inoculated with the wild-type CIAT899 or A554. Uninoculated control plants grew very poorly, and CIAT899 strongly stimulated growth (Table 2). The average dry weights of plants inoculated with the mutant A554 were 38 and 20% greater than those observed with CIAT899 in experiments conducted in two separate growing seasons (Table 2). The yield difference

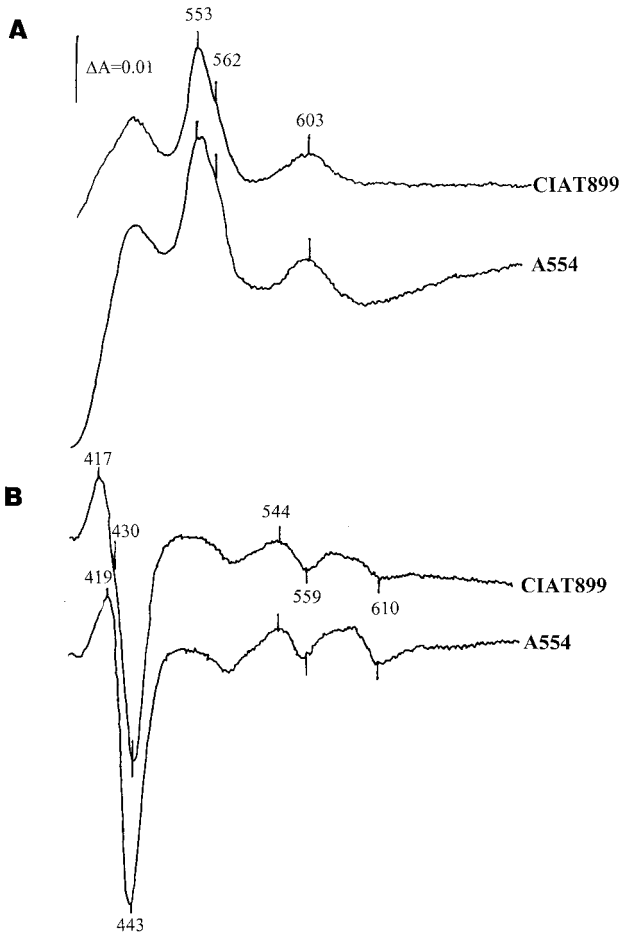


FIG. 3. (A) Reduced *minus* oxidized difference spectra of *R. tropici* wild-type (CIAT899) and glycogen synthase mutant (A554) strains. (B) Reduced + CO *minus* reduced difference spectra of strains CIAT899 and A554. The numbers represent the wavelengths (in nanometers) at which the main peaks and troughs are detected. The cells were grown in Y-succinate minimal medium for 12 h. The protein concentrations were as follows: CIAT899, 15.2 mg/ml; A554, 17.0 mg/ml.

between plants inoculated with A554 and CIAT899 in the first experiment was significant at the 95% confidence level, although the difference in the second experiment was not significant at the 95% confidence level. However, using a two-way analysis of variance combining the data from both experiments, A554 was found to give a significantly enhanced yield compared with the wild type (confidence level, 98%).

The increased symbiotic performance might be due to enhanced nitrogen fixation by individual nodules or to increased nodulation. Those plants with increased nitrogen fixation also have an increased number of nodules and increased nodule mass (Table 2). The same trend is evident from both experiments. Therefore, we conclude that the enhanced symbiotic performance is probably due to increased numbers of nitrogen-fixing nodules.

**Characterization of the gene region affected in A554.** The DNA sequence of the 6.6-kb *Hin*dIII fragment that complemented the Nadi$^{++}$ phenotype of A554 (Fig. 1A) revealed the presence of four open reading frames transcribed in the same direction. Three adjacent *Hin*dIII fragments of 2.4, 1.3, and 8.0 kb (Fig. 1A) were also subcloned and partially sequenced, revealing two additional open reading frames also transcribed in the same direction. All six of the genes were found to encode proteins with sequences similar to those of the products of *E. coli* genes involved in glycogen metabolism (37): *glgP* (encoding glycogen phosphorylase), *glgB* (glycogen branching enzyme), *glgC* (ADP glucose pyrophosphorylase), *glgA* (glycogen synthase), *pgm* (phosphoglucomutase), and *glgX* (glycogen debranching enzyme). The roles of these six proteins in the synthesis and degradation of glycogen in *E. coli* are illustrated in Fig. 1C. In *E. coli* and many other bacteria, glycogen synthesis proceeds as follows (37). Phosphoglucomutase (Pgm) isomerizes glucose-6-P to glucose-1-P, which is used to form ADP-glucose in a reaction catalyzed by ADP-glucose pyrophosphorylase (GlgC). This activated form of glucose is polymerized via α-1,4 linkages by glycogen synthase (GlgA). Subsequently, the branching enzyme (GlgB) forms α-1,6-glycosidic linkages. Glycogen is degraded by debranching enzyme (GlgX), which removes the α-1, 6 linkages, and by glycogen phosphorylase (GlgP), which degrades the α-1,4 linkages, giving rise to glucose-1-P. The organization of the glycogen metabolism genes in *R. tropici* is similar to that reported for *A. tumefaciens* (48, 50), although the *glgX* gene was not described in *A. tumefaciens*.

**(i) *glgP*.** The *R. tropici glgP*-like gene was only partially sequenced. A 621-bp fragment showed 91% similarity at the protein level with *A. tumefaciens* GlgP. The 3′ end of the *R. tropici glgP* gene was also sequenced (77% similarity at the protein level with *A. tumefaciens* GlgP in a 294-bp fragment), displaying an overlap between the predicted *glgP* termination codon (TGA) and the *glgB* initiation codon (ATGA), suggesting translational coupling (21).

**(ii) *glgB*.** The 5′ and 3′ ends of *R. tropici glgB* were sequenced, showing high similarity with *A. tumefaciens glgB* (66% in 285 bp and 89% in 405 bp at the protein level, respectively), which was proposed to encode glycogen branching enzyme based on sequence similarity with the *E. coli* gene (48).

TABLE 2. Symbiotic phenotype of *R. tropici* wild type and *glgA* mutant strains on *P. vulgaris* cv. Negro Jamapa plants[a]

| Strain | Expt 1 | | | Expt 2 | | |
|---|---|---|---|---|---|---|
| | Plant dry wt (g) | No. of nodules | Nodule dry wt (mg) | Plant dry wt (g) | No. of nodules | Nodule dry wt (mg) |
| Uninoculated control | 0.355 a | 0 d | 0 f | 0.563 i | 0 l | 0 o |
| CIAT899 | 3.181 b | 366 e | 239 g | 4.335 j | 557 m | 272 p |
| A554 | 4.394 c | 416 e | 290 h | 5.219 jk | 764 n | 439 q |
| A656 | | | | 5.403 k | 689 mn | 401 q |

[a] Values followed by different letters are statistically significantly different from each other ($P < 0.05$) within each experiment based on analysis of variance.

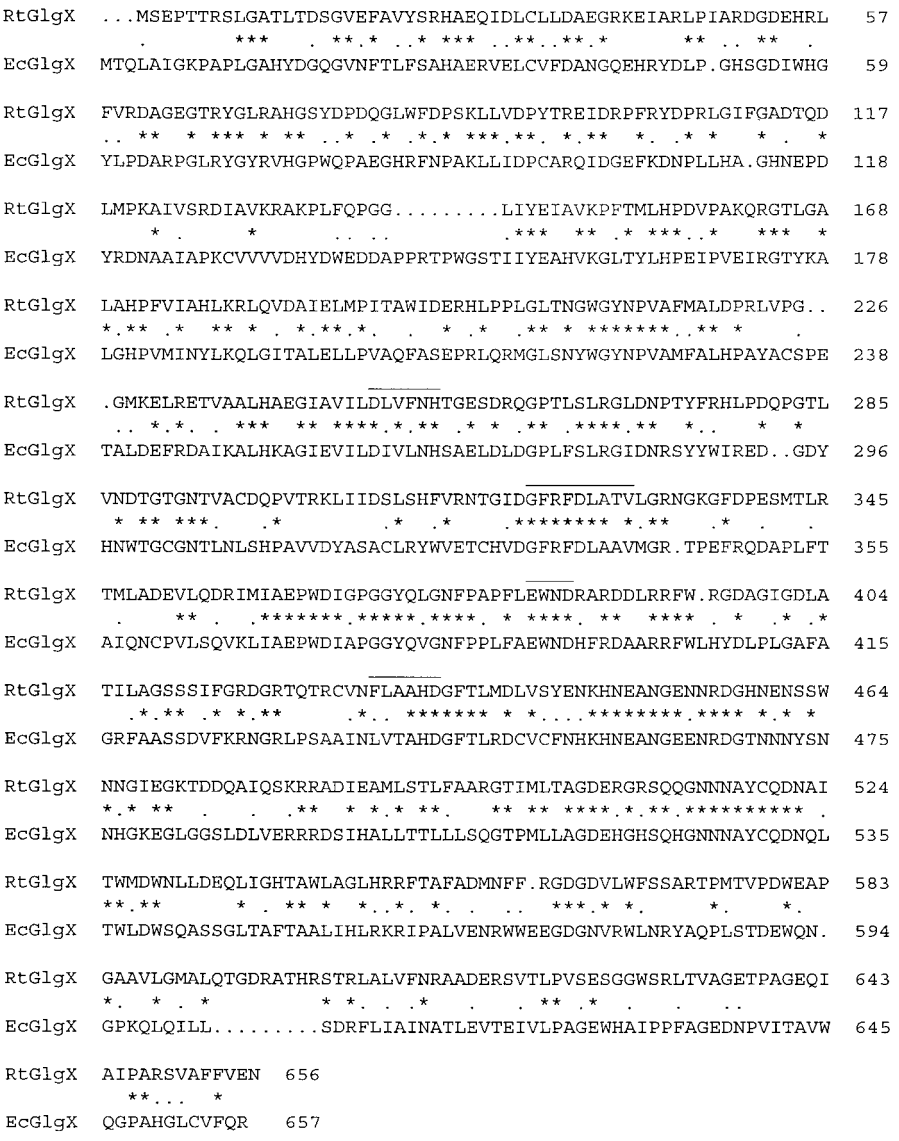


FIG. 4. Protein sequence comparison of the glycogen debranching enzymes (GlgX) of *R. tropici* and *E. coli* (similarity, 61%; identity, 41%). The asterisks represent identical amino acid residues. The dots indicate conserved substitutions. Important residues conserved between *R. tropici* and *E. coli* proteins are marked with lines.

**(iii) *glgC*.** The *glgC* gene is 26 bp downstream of *glgB* and is preceded by an A/G-rich region, part of which could constitute a ribosome-binding site. The deduced protein (420 residues) is homologous to GlgC from *A. tumefaciens* and *E. coli* (95 and 73% similarity, respectively).

**(iv) *glgA*.** The *glgA* gene is 3 bp downstream of *glgC* and does not have an obvious ribosome-binding site. The deduced protein (480 residues) is 90 and 66% homologous with GlgA from *A. tumefaciens* and *E. coli*, respectively. In *E. coli* GlgA, two important sites have been described, and they are conserved in the *R. tropici* glycogen synthase: the lysine at position 15, which forms part of the motif KXGG (where X represents any amino acid) and is involved in the binding of the ADP-glucose substrate (12), and the arginine at position 277 (lysine in the *E. coli* protein), which constitutes part of the proposed active site (13). DNA sequencing of a 3.5-kb *Eco*RI-*Bam*HI fragment carrying part of the Tn*5* cloned from A554 revealed that the transposon is inserted within *glgA* at a position corresponding to amino acid residue 304. This mutation was called *glgA1*::Tn*5*.

**(v) *pgm*.** A gene encoding a phosphoglucomutase homolog is included in the glycogen region of *R. tropici*, as is found in *A. tumefaciens* (50), but in *E. coli* it is elsewhere in the genome (27). The *R. tropici pgm* gene homolog is 4 bp downstream of the *glgA* stop codon and is preceded by a possible ribosome-binding site, GAGAGG, 11 bp from the ATG. The deduced Pgm protein (542 residues) is 88 and 50% similar to the *A. tumefaciens* and *E. coli* Pgms, respectively.

Downstream of *pgm* in *R. tropici* is a 211-bp noncoding region that has two inverted-repeat segments spanning 27 and 26 bp, respectively, which could constitute a rho-independent transcription terminator. A similar region was identified elsewhere in *R. tropici* (sequence U47030 from the EMBL gene bank) (36).

TABLE 3. Expression of *glg*- and *pgm-lacZ* transcriptional fusions in *R. tropici*

| Plasmid | β-Galactosidase activity[a] | |
|---|---|---|
| | Glucose | Galactose |
| pMP220 | 150 ± 1 | 165 ± 20 |
| pIJ9160 (*glgP-lacZ*) | 1,425 ± 96 | 746 ± 54 |
| pIJ9147 (*glgB-lacZ*) | 54 ± 2 | ND |
| pIJ9014 (*glgC-lacZ*) | 97 ± 2 | ND |
| pIJ9016 (*glgA-lacZ*) | 60 ± 2 | ND |
| pIJ9015 (*pgm-lacZ*) | 1,787 ± 156 | 1,303 ± 30 |
| pIJ9011 (*glgX-lacZ*) | 283 ± 49 | 172 ± 13 |

[a] Activity is expressed in Miller units ± standard error. The cells were grown in minimal medium supplemented with glucose or galactose for 24 h. The results are averages of at least three experiments. ND, not determined.

**(vi) *glgX*.** There is an *R. tropici glgX* homolog 211 bp downstream of *pgm*, and it is preceded by a possible ribosome-binding site, GGAAAG, 10 bp from the predicted ATG. The deduced protein (656 residues) is homologous to the predicted debranching enzymes of many bacteria, including *E. coli* (656 amino acids; 61% similarity) (53) (Fig. 4). *R. tropici* GlgX also shows 56% similarity with the *Flavobacterium* sp. isoamylase enzyme, which degrades α-1,6 glycosidic bonds (23). This similarity is primarily around the four domains (indicated in Fig. 4) common to amylolytic enzymes (19).

**The *glgA* mutant lacks glycogen.** The glycogen contents of strains CIAT899, A554 (*glgA1*::Tn*5*), and the complemented strain A554/pIJ7720 were analyzed. CIAT899 and the complemented strain had similar levels of glycogen (1.7 and 1.6 ng/$10^6$ CFU, respectively), whereas A554 had essentially no glycogen (<0.01 ng/$10^6$ CFU). This confirms that the transposon inserted in the glycogen synthase gene of A554 prevents glycogen formation and that the mutation is complemented by cosmid pIJ7720 carrying the *glgA* region.

The cloned gene region from *R. tropici* was tested for its ability to complement *E. coli* mutants with affected glycogen synthesis. Colonies of glycogen-deficient mutants of *E. coli* can be distinguished from the wild type by iodine staining (16). This assay was used to analyze complementation of the *E. coli glgA, glgB*, and *glgC* mutants (RH98, LCB499, and RH97, respectively [Table 1]) by *R. tropici* plasmids carrying *glg* genes. pIJ7741 partially complemented RH98, pIJ7844 complemented RH98 and partially complemented RH97, and pIJ9158 complemented LCB499. We conclude that the glycogen production of these mutants was fully or partially restored by the *R. tropici glgA, glgB*, and *glgC* genes.

**Analysis of operon structure using *lacZ* fusions.** Transcriptional fusions of all six genes were constructed using the *lacZ* reporter plasmid pMP220 (47) (Fig. 1B). The resulting plasmids were introduced into CIAT899, and the β-galactosidase activity was measured (Table 3). Significant levels of activity were detected for the *glgP-lacZ* (pIJ9160) and *pgm-lacZ* (pIJ9015) gene fusions, whereas the *glgB-lacZ* (pIJ9147), *glgC-lacZ* (pIJ9014), and *glgA-lacZ* (pIJ9016) fusions had very low levels of activity. The *glgX-lacZ* fusion (pIJ9011) had β-galactosidase activity, but the level observed was low. These results indicate that there is an operon composed of *glgP, glgB, glgC*, and *glgA*. The *pgm* gene appears to have a separate promoter, although the presence of only 4 bp between the predicted coding regions of *glgA* and *pgm* suggests that *pgm* is also expressed within the *glgPBCA* operon. Such a potential dual control of *pgm* expression seems reasonable in view of the fact that *pgm* has an important role in metabolism independent of glycogen biosynthesis (Fig. 1C). In *A. tumefaciens*, the *glgP, glgB, glgC, glgA*, and *pgm* genes are transcribed from a promoter located upstream of *glgP* (48). The *A. tumefaciens pgm* gene can also be transcribed as a shorter product from a promoter immediately upstream of an internal translation initiation codon within the *pgm* gene to yield a shorter Pgm protein (48). DNA sequence comparisons revealed an in-frame ATG within the *R. tropici pgm* gene in a context almost identical to the sequence found in *A. tumefaciens*. Therefore, *pgm* in *R. tropici* may be transcribed independently, as is seen in *A. tumefaciens*.

The *glgX* gene appears to be in a separate transcriptional unit, based on the observations that there is a potential transcription terminator between *pgm* and *glgX* and that the *glgX-lacZ* fusion is expressed.

**Analysis of a nonpolar *glgA* deletion mutant.** To determine if the pleiotropic phenotypes of the *glgA* mutant A554 are due to polarity of the Tn*5*, an in-frame deletion mutation in *glgA* was constructed in vitro and recombined into the genome of *R. tropici* to form A656. This mutant was similar to A554 in that it had a $Nadi^{++}$ phenotype on Y-succinate plates and had increased levels of TMPD oxidation (23 ng-atom of $O_2$/min/mg [dry weight]) compared with CIAT899 (15 ng-atom of $O_2$/min/mg [dry weight]). Heme staining revealed that the cytochrome *c* content was essentially the same as that seen with A554, in that the content of cytochrome $c_1$ and CycM was increased and the soluble cytochrome *c* content was decreased (data not shown).

The growth of *P. vulgaris* plants inoculated with the *glgA* deletion mutant A656 was determined in parallel with one of the tests of the *glgA*::Tn*5* mutant. As shown in Table 2, A656 performed significantly better (25%; $P < 0.05$) than the control strain, CIAT899. It behaved indistinguishably from A554, and if the results from the three independent tests of the two *glgA* mutants are pooled in a two-way analysis of variance, it is evident that mutation of *glgA* induces significantly increased growth ($P < 0.02$) compared with CIAT899.

The gene downstream of *glgA* is *pgm*. The *A. tumefaciens* phosphoglucomutase has been purified, and an antiserum has been prepared (48). To confirm that the deletion mutation in *glgA* does not have a polar effect on *pgm*, we used the antiserum produced against the *A. tumefaciens* Pgm to determine the amount of Pgm protein in A656 and the wild type, CIAT899 (Fig. 5). It is evident that the level of staining in the mutant is indistinguishable from that seen with the wild type, confirming that the deletion mutation does not cause a polar effect on *pgm* expression. Similar results were seen in three independent analyses. In *A. tumefaciens*, two different-size Pgm proteins are produced as a result of different transcription and translation start sites in the *pgm* gene (48). In wild-type *R. tropici*, there appear to be two bands recognized by the antiserum, and these are also present in the mutant (Fig. 5A). The estimated size of the products is about 60 kDa, which is close to the molecular mass (58.4 kDa) predicted from the DNA sequence. The difference between the two products is estimated to be about 2 kDa. These experiments do not distinguish

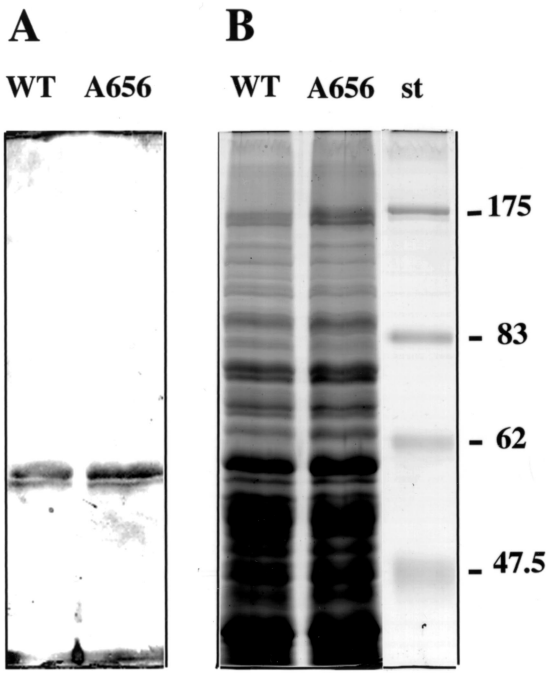


FIG. 5. Immunostaining of phosphoglucomutase in A656 (*glgA*Δ*Pst*I). Soluble proteins from the *glgA* deletion mutant A656 and the wild type, (wt), CIAT899, were separated by SDS-PAGE and either stained with Coomassie blue (B) or immunostained with antiserum to phosphoglucomutase (A). The same amount of protein (10 μg) was loaded in each lane. The sizes (in kilodaltons) of the prestained standards (st) are indicated to the right of the gel.

between two distinct translation products and processing of a single product.

***glgA* mutants have reduced EPS content.** The glycogen synthase mutant A554 was originally identified following growth on succinate minimal medium plates, on which it grows normally. It also forms normal-size colonies on galactose or mannitol minimal medium or on complete medium (TY). However, on glucose or maltose the colony size was reduced significantly and the colonies appeared to have less EPS. In liquid glucose minimal medium, the mutants had a somewhat extended lag phase, but the growth rates were normal.

Quantitative analysis of HMW-EPS produced in liquid cultures (Table 4) confirmed that A554 produces less HMW-EPS than CIAT899 after growth on glucose but produces similar amounts of HMW-EPS following growth on galactose. The mutation is complemented by pIJ9158, which restores normal levels of EPS production (Table 4). The relative amounts of HMW-EPS shown in Table 4 are based on the yield of EPS per milligram (dry weight) of cells; similar reductions in the HMW-EPS yield were seen in other preparations of the mutant grown on glucose if the yield was calculated relative to the number of CFU (data not shown). It seemed unlikely that mutation of *glgA* would directly cause a decrease in HMW-EPS levels. A more likely explanation was that the Tn*5* insertion in A554 might have a polar effect on the downstream *pgm* gene, which is involved in the formation of glucose-1-P, which is a precursor for both glycogen and HMW-EPS (Fig. 1C). To test this, we analyzed HMW-EPS production by the nonpolar *glgA* deletion mutant A656. Surprisingly, A656 has a phenotype similar to that of A554, producing a low level of HMW-EPS following growth on glucose but not galactose (Table 4). Colonies of A656 were, like A554, normal on mannitol and galactose minimal medium but were smaller on glucose minimal medium. This suggests that the *glgA* mutation itself influences the level of HMW-EPS produced on glucose medium.

TABLE 4. EPS produced by *R. tropici* wild-type and *glgA* mutant strains[a]

| Strain | HMW-EPS (mg/mg [dry wt] of cells ± SE) | |
|---|---|---|
| | Glucose | Galactose |
| CIAT899 | 1.8 ± 0.3 | 1.8 ± 0.3 |
| CIAT899/pIJ9158 | 1.6 ± 0.2 | 1.9 ± 0.3 |
| A554 (*glgA*::Tn*5*) | 0.7 ± 0.1 | 1.6 ± 0.3 |
| A554/pIJ9158 | 1.5 ± 0.2 | 1.8 ± 0.3 |
| A656 (*glgA*Δ*Pst*I) | 0.5 ± 0.1 | 2.0 ± 0.3 |
| A656/pIJ9158 | 2.2 ± 0.3 | 2.0 ± 0.4 |

[a] Experiments were repeated at least three times. On each occasion, the HMW-EPS production by the *glgA* mutants grown on glucose was less than 40% that of controls, and no significant difference was seen with galactose-grown cells.

To further confirm that it is loss of *glgA* that causes a decrease in HMW-EPS production (rather than polar effects of the mutations on *pgm*), we introduced into the *glgA* mutants different plasmids carrying *glgA* or *pgm* (Fig. 1A). On glucose plates, a plasmid (pIJ7959) carrying *glgA* (but not *pgm*) restored the sizes of colonies to normal. Conversely, pIJ7814, which expresses *pgm* but lacks *glgA*, had no effect on A554 or A656 colony size on glucose medium. These complementation results have the same pattern as those obtained using the Nadi test (Fig. 1A), demonstrating that these two phenotypes are correlated. The complementation of HMW-EPS production was confirmed by quantitative analysis of HMW-EPS produced in liquid culture using the two *glgA* mutants A554 and A656 carrying the various plasmids. Those strains with restored $EPS^+$ colony morphology (Fig. 1A) all formed amounts of HMW-EPS similar to those observed when pIJ9158 was present (data not shown). The level of HMW-EPS produced by the *glgA* mutants carrying pIJ7814 was similar to that seen (Table 4) with A554 or A656 lacking plasmid (data not shown).

We also measured the level of *pgm-lacZ* expression in plasmid constructs carrying the *glgA1*::Tn*5* or *glgA* deletion (pIJ9034 and pIJ9044, respectively [Fig. 1B]). In both cases, the level of *pgm* expression was similar to that observed previously with the *pgm-lacZ* fusion on pIJ9015 (Table 3).

On the basis of all of these results, we conclude that mutation of the *glgA* gene in some way decreases EPS production in *R. tropici* grown in glucose and that this is unlikely to be due to a polar effect on *pgm*.

## DISCUSSION

It had been reported previously that some rhizobial mutants which had increased respiratory capacities had enhanced symbiotic nitrogen fixation (33, 44, 45), and it was our aim to identify mutants of *R. tropici* that had increased respiration and hence symbiotic nitrogen fixation. The mutant strain A554 was investigated in detail because it fulfilled both of these criteria, and it was somewhat unexpected to find that the mutation

affected glycogen synthesis. Mutation of the glycogen synthase *(glgA)* gene of *R. tropici* induces pleiotropic effects in addition to the expected block of glycogen formation. It is not immediately obvious why mutation of *glgA* should also lead to (i) alteration in cytochromes, presumably causing increased ability to oxidize TMPD; (ii) decreased EPS production during growth on glucose (but not galactose or mannitol); and (iii) increased nodulation, apparently resulting in enhanced symbiotic performance with the *Phaseolus* bean. Indeed, we cannot be sure that these phenotypes are all direct consequences of the inability to form glycogen or which, if any, of these phenotypes causes the other. Thus, for example, we do not know if enhanced symbiotic performance is due to the absence of glycogen, increased respiratory potential, or an alteration in the level of HMW-EPS. It may be of particular significance that an *R. etli* mutant defective for storage of the other major source of stored carbon (poly-β-hydroxybutyrate) causes enhanced symbiotic performance (8).

In *E. coli*, glycogen metabolism is highly regulated at a metabolic level and is also under complex genetic control (37). Several metabolites have positive or negative control over glycogen gene expression. Cyclic AMP (cAMP) receptor protein-cAMP and ppGpp are the main positive regulators of the expression of the *glgCAP* operon in *E. coli*. cAMP receptor protein-cAMP also stimulates expression of another gene involved in glycogen biosynthesis, *glgS*, whose function is unknown (17). The stationary-phase sigma factor $\sigma^s$ (24) has a positive control over *glgS*. On the other hand, three negative effectors have been described: one that acts in *cis* (*glgR*) over the *glgCAP* operon, one that acts in *trans* (*glgQ*) over the *glgBX* and *glgCAP* genes (37), and the carbon storage regulator *csrA*, which negatively affects all glycogen metabolism genes (53) by specifically destabilizing mRNA (26).

It is possible that mutating *glgA* in *R. tropici* may result in a change in the levels of glucose phosphates and/or sugar nucleotides, such as ADP-glucose, and that this may affect aspects of metabolism other than glycogen synthesis. Interestingly, mutations of the *S. meliloti cya3* gene encoding an adenyl cyclase-like protein also enhanced symbiotic effectiveness (42). In *E. coli*, intracellular UDP-glucose has regulatory effects via RpoS (4), and it is possible that some such regulatory effect could occur in *R. tropici*. Accumulation of sugar nucleotides or glucose phosphates might account for the pleiotropic phenotypes, but this needs to be tested experimentally. One way to test the effects of accumulation of ADP-glucose would be to generate a nonpolar *glgC* mutant. This should be defective for glycogen synthesis but would be predicted not to accumulate ADP-glucose (Fig. 1C) and so could be used to distinguish the effects of the absence of glycogen from possible accumulation of ADP-glucose. Glycogen and EPS biosynthesis are connected at the level of glucose-1-P (Fig. 1C), and the glucose-dependent repression of HMW-EPS biosynthesis in *glgA* mutants may be a consequence of increased levels of glucose phosphates, which could have some kind of inhibitory feedback effect (directly or indirectly) on enzymes of HMW-EPS biosynthesis. A key enzyme common to both pathways is phosphoglucomutase; when a mutation in this gene was first identified in *A. tumefaciens* and *S. meliloti*, the gene was originally called *exoC* (6) because of the strong effect on EPS formation. Later, the *exoC* gene was renamed *pgm* (48) when its function was more clearly understood. The observation that *pgm* is immediately downstream of *glgA* led us to suspect that the decreased level of HMW-EPS in the *glgA* Tn*5* mutant was due to a polar effect on *pgm*, but we found no evidence for such an effect.

The observation that the HMW-EPS-reduced phenotype of *glgA* mutants is specific for growth on glucose but not other sugars, such as galactose, also argues against a simple polar effect on *pgm*. A *pgm* mutant of *Agrobaterium* lacks EPS on both galactose and glucose media, due to its inability to form UDP-glucose (49), and it is likely that a similar situation would occur in *Rhizobium*. The normal level of production of HMW-EPS by the *R. tropici glgA* mutant on galactose therefore implies that *pgm* is expressed in the *glgA* mutant. Our analysis of gene expression indicated that the *pgm* gene is expressed under a promoter separate from that of *glgA*, although its location immediately downstream of *glgA* would suggest it is also transcribed along with *glgA*.

A putative *pgm* mutant of *R. tropici* CIAT899 was reported previously (32). This mutant had reduced levels of EPS (although the reduction was not as great as in the *R. tropici exo* mutants characterized in the same work). The mutant was normal with respect to motility and a symbiotic phenotype on bean. This is different from *S. meliloti* or *A. tumefaciens pgm* (*exoC*) mutants, which lack EPS, have altered LPS, are non motile, and are defective for symbiosis or avirulent, respectively (6, 25). The reported phenotype of the *R. tropici exoC* mutant is similar to that of the glycogen synthase mutant described here. The insertion of Tn*5* in the *pgm* gene was not demonstrated (32), and it was assumed to be a *pgm* (*exoC*) mutant based on complementation of an *S. meliloti exoC* mutant by a cosmid that carried other genes (32). We suspected that this previously described mutation might be in *glgA* and tried to construct a *pgm* mutant of *R. tropici*. A spectinomycin-resistant interposon (38) was cloned at two different sites of *pgm* in two different constructs. We integrated the *pgm*::Ω alleles on a *sacB*-based suicide-selection plasmid into the genome but could not generate a second recombination event to make a *pgm* mutant unless a complementing clone carrying *pgm* was introduced. This suggests that the *pgm* gene may be essential for growth in *R. tropici*. During the course of our work, we had tried to introduce the cloned *glgA* genes from *R. tropici* or *A. tumefaciens* into a *pgm* mutant of *A. tumefaciens*, and in both cases we failed to obtain transconjugants with the *pgm* mutant even though rates of conjugation into the wild-type strain were normal. This suggests that in an *A. tumefaciens pgm* mutant background, increased expression of *glgA* may be lethal. Possibly our inability to generate a *pgm* mutant of *R. tropici* is a related effect.

The increase in plant growth stimulated by *R. tropici glgA* mutants could in principle be due to enhanced efficiency of nitrogen fixation by bacteroids, increased nodulation, or both. The primary effect seems to be due to enhanced nodulation. The increased nodulation seems unlikely to be due to altered respiration, but this cannot be formally ruled out. A decrease in EPS formation could in principle affect nodulation, since EPS plays a signaling role during nodulation (35), and indeed, mutations affecting the levels of HMW-EPS in *S. meliloti* also enhanced symbiotic performance (42). Alternatively, it is possible that the inability to store glycogen in some way causes more efficient infection. However, in general, the plant tends

to control the numbers of nodules formed, and this is related to the levels of fixed nitrogen supplied by nitrogen-fixing bacteroids to the plant. Thus, in plants inoculated with rhizobia defective for nitrogen fixation, nodulation is often increased. In view of such observations, one speculative model for the enhanced symbiotic performance of *glgA* mutants is that increased symbiotic performance may occur because the mutants are initially somewhat impaired in symbiotic nitrogen fixation. This might occur, for example, if the onset of nitrogen fixation is delayed. Alternatively, it could be envisaged that glycogen in bacteroids acts as a carbon store that buffers periods of deficiency in carbon supply from the plant. With *glgA* mutants, a lack of plant-supplied carbon could rapidly lead to cessation of nitrogen fixation, and this could in principle induce the plant to initiate the formation of additional nodules, and the net effect could be an overall increase in symbiotic performance. Clearly, experimental evidence would be required to test such models. However, the identification of such a bacterial mutant does suggest that there are opportunities for enhancing symbiotic nitrogen fixation in field environments.

## ACKNOWLEDGMENTS

We thank D. N. Rodríguez-Navarro and C. Morera for skilled assistance with experimental work, M. J. Delgado and S. Bhatt for advice and help with analysis of cytochromes, E. Arthur for help with statistical analysis, and A. Davies for help with bacterial strains. We thank R. Ugalde and V. Lepek for providing strain A5129 and antiserum to phosphoglucomutase, A. Smith for advice on glycogen analysis, and E. Rathbun for advice on immunostaining.

This work was supported by E. U. project CI1*CT94-0042 and the BBSRC, and A.Z. was supported by CONICET Argentina.

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