**RESEARCH LETTER**

**Glycogen phosphorylase is involved in stress endurance and biofilm formation in Azospirillum brasilense Sp7**

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**Keywords**

Azospirillum brasilense; glycogen; glycogen phosphorylase; stress endurance; biofilm.

**Abstract**

Here we report the identification of a glycogen phosphorylase (glgP) gene in the plant growth-promoting rhizobacterium *Azospirillum brasilense*, Sp7, and the characterization of a glgP marker exchange mutant of this strain. The glgP mutant showed a twofold reduction of glycogen phosphorylase activity and an increased glycogen accumulation as compared with wild-type Sp7, indicating that the identified gene indeed encodes a protein with glycogen phosphorylase activity. Interestingly, the glgP mutant had higher survival rates than the wild type after exposure to starvation, desiccation, and osmotic pressure. The mutant was shown to be compromised in its biofilm formation ability. Analysis of the exopolysaccharide sugar composition of the glgP mutant revealed a decrease in the amount of glucose, accompanied by increases in rhamnose, fucose and ribose, as compared with the Sp7 exopolysaccharide. To the best of our knowledge, this is the first study that demonstrates GlgP activity in *A. brasilense*, and shows that glycogen accumulation may play an important role in the stress endurance of this bacterium.

**Introduction**

The *Azospirillum* genus comprises plant growth-promoting rhizobacteria that fix atmospheric nitrogen and promote root development, leading to improved water and mineral uptake of diverse crops (Burdman *et al*., 2002; Dobbelare *et al*., 2002). Attachment of *Azospirillum* to the plant root is essential for the establishment of a successful plant–bacterium association, with bacterial extracellular polysaccharides being an important player in this interaction (Del Gallo *et al*., 1989; Michiels *et al*., 1990, 1991; Burdman *et al*., 2000b).

Several studies determined the monosaccharide composition of the exopolysaccharides of *Azospirillum brasilense* (Del Gallo & Haegi, 1990; Burdman *et al*., 2000a, b; Bahat-Samet *et al*., 2004). It was shown that exopolysaccharide composition varies during bacterial growth, with glucose and arabinose being the dominant monosaccharides during the exponential and stationary growth phases, respectively (Bahat-Samet *et al*., 2004).

The reserve material poly-β-hydroxybutyrate (PHB) is also known to play an important role in the association of *A. brasilense* with plant roots as well as in the stress endurance and cell survival of this bacterium (Kadouri *et al*., 2002, 2003a, b). Another reserve material occurring in bacteria is glycogen. The occurrence of glycogen has already been reported in *A. brasilense* cyst-like cells (Caiola *et al*., 2004). However, in contrast to PHB, the ecological relevance of glycogen accumulation in *A. brasilense* has not been investigated. During a search for ribose-5-phosphate isomerase (*rpi*) genes of *A. brasilense* Sp7, we identified a PCR product that matched significantly with glgP, which encodes glycogen phosphorylase (GlgP; EC 2.4.1.1). GlgP, which was not reported previously in *A. brasilense*, is involved in the breakdown of glycogen (Alonso-Casajús *et al*., 2006).
The above background motivated us to generate and characterize a glgP mutant of *A. brasilense* Sp7. Findings from this study provide evidence supporting a role of glycogen accumulation in stress endurance, extracellular polysaccharide production and biofilm formation in this bacterium.

**Materials and methods**

**Bacterial strains, plasmids and media**

The strains and plasmids used in this study are listed in Table 1. *Azospirillum brasilense* Sp7 and the glgP mutant were routinely grown at 30 °C in Luria–Bertani (LB) medium (Difco), or in high or low carbon-to-nitrogen ratio (C:N) fructose minimal medium (Burdman *et al*., 1999). *Escherichia coli* strains were cultured in LB medium at 37 °C. Kanamycin (Km, 25 μg/mL) and trimetoprim (25 μg/mL) antibiotics were used as described (Vanstockem *et al*., 1987).

**DNA manipulations and sequence analyses**

Cloning, transformation and Southern blot procedures were performed using standard methods (Sambrook *et al*., 1989). Detection of hybridized products was performed using the DIG DNA Labeling kit (Roche Diagnostics Corp.). Sequence analyses were performed using BLASTN/BLASTX/BLASTP (http://www.ncbi.nlm.nih.gov; Altschul *et al*., 1997) and PFAM (http://pfam.sanger.ac.uk/). Sequences were aligned using the BLAST function tbl2seq (http://www.ncbi.nlm.nih.gov/). The glgP sequence of *A. brasilense* Sp7 was submitted to GenBank (accession number FJ696410).

**Construction of an *A. brasilense* Sp7 glgP::Km mutant**

A 749-bp internal fragment of the glgP coding region was PCR-amplified using primers glgP-28F (5'–CGCTATCTG CAGCACA-3') and glgP-777R (5'–GACCTGCAACGTC GAACA-3'). The PCR product was purified and cloned into pGEM-T-easy vector (Promega) to generate plasmid pGLG that was transformed into *E. coli* DH5α. Subsequently, the 1.2-kb HincII Km resistance cassette from pUCA800 was excised and inserted into the unique PfMI site of glgP in pGLG, to yield pGLG-Km. Then, the 2-kb glgP::Km fragment from pGLG-Km was excised using EcoRI and cloned into the suicide vector pSUP202 to yield pSUP-GLG-Km. This vector was transformed into *E. coli* S17.1, which was further used to mobilize it into *A. brasilense* Sp7 through triparental mating (using *E. coli* HB101 carrying the helper vector pRK2073). *Azospirillum brasilense* transconjugants were selected on MMAB (Vanstockem *et al*., 1987) supplemented with kanamycin and trimetoprim, and kanamycin-resistant clones were verified by Southern blot and PCR. One of the confirmed glgP::Km mutants (hereafter, glgP mutant) was used for further analysis.

<table>
<thead>
<tr>
<th>Table 1. Bacterial strains and plasmids used in this study</th>
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<tbody>
<tr>
<td>Strains</td>
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<tr>
<td><em>A. brasilense</em></td>
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<tr>
<td>Sp7</td>
</tr>
<tr>
<td>glgP::Km</td>
</tr>
<tr>
<td><em>Escherichia coli</em></td>
</tr>
<tr>
<td>DH5α</td>
</tr>
<tr>
<td>HB101</td>
</tr>
<tr>
<td>S17.1</td>
</tr>
<tr>
<td>Plasmids</td>
</tr>
<tr>
<td>pGEM-T Easy</td>
</tr>
<tr>
<td>pUCA800</td>
</tr>
<tr>
<td>pRK2073</td>
</tr>
<tr>
<td>pGLG</td>
</tr>
<tr>
<td>pGLG-Km</td>
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<tr>
<td>pSUP-GLG-Km</td>
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Glycogen phosphorylase assays

Bacteria were grown for 48 h in 500 mL high-C:N medium until an OD_{600 nm} of 0.9 (about 10^8 CFU mL^{-1}). Cells were harvested by centrifugation (6000 g, 15 min, 4 °C), washed twice by centrifugation (as above), suspended in 5 mL of 50 mM phosphate buffer (pH 7.0) and then disrupted in an ultrasonic sonicator (MCE) at 1.2 Å for 10 min on ice, with intervals of 30 s after every 1 min of sonication. Cell debris of enzyme that catalyzes the production of 1 unit of enzyme activity (EU) was defined as the amount according to assay A of Alonso-Casajús et al. (2006). One unit of enzyme activity (EU) was defined as the amount of enzyme that catalyzes the production of 1 μmol NADH min^{-1}.

Glycogen visualization and quantification

Intracellular glycogen was visualized by iodine staining as described by Alonso-Casajús et al. (2006), with a few modifications: 1 mL of bacterial cells grown in LB liquid medium for 48 h were diluted to an OD_{600 nm} of 0.5 and centrifuged. Pellets were then washed and suspended in 0.5 mL 50 mM phosphate buffer (pH 7.0). Glycogen was visualized after staining with 10 μL lugol. Stained pellets were photographed after centrifugation (6000 g, 5 min). Glycogen extraction for quantification was performed following the growth of cells in 250 mL of high-C:N medium. Measurements were taken after 24 and 48 h of growth. After estimation of cell concentration by OD_{600 nm} measurement, 30 mL of cultures were centrifuged (12000 g, 30 min, 4 °C). The pellets were collected with 5 mL of doubly distilled water, washed twice by centrifugation (as above) and then hydrolyzed with 15 mL of 30% KOH in boiling water for 90 min, in a bath with boiling water. After cooling for 30 min, the suspensions were centrifuged (20 min, 2000 g, 20 min, 4 °C) to remove cell debris, and the supernatants were collected. Ethanol was added (at 1.2 vol.) and the suspensions were boiled. After cooling, the suspensions were centrifuged (18000 g, 10 min, 4 °C). Glycogen-containing pellets were dissolved with 5 mL of doubly distilled water, and glycogen was purified by two additional alcohol precipitations (following addition of equal volumes of ethanol). Glycogen measurements were performed according to Drelling et al. (1987), with standard curves being produced with Oyster glycogen (Sigma). This experiment was conducted three times.

Phenotypic characterization of the glgP mutant

The following phenotypes were determined as described by Lerner et al. (2009): exopolysaccharide amount and composition; plant growth promotion; root adhesion ability; and survival to different stresses, including desiccation, starvation, and osmotic pressure. Briefly, for desiccation experiments, cells were allowed to dry for 1 h in 0.2-μm membrane filter papers (Whatman) at 25 °C. For starvation experiments, cells were kept in 0.06 M potassium phosphate buffer (pH 6.8) for 12 days at 30 °C. Sensitivity to osmotic pressure was determined by adding 1 vol. of 2 or 4 M fructose solutions to the cultures (yielding final fructose concentrations of 1 and 2 M, respectively) and incubating the suspensions at 30 °C for 24 h. In all cases, bacterial viability was assessed by dilution plating at the beginning and end of the incubation periods. Biofilm formation on 24-well polystyrene plates (Thermo Fisher Scientific) was determined by crystal violet staining according to Uhlich et al. (2006). All experiments were performed at least twice with at least three replicates per treatment. Data were subjected to one-way ANOVA using the JMP IN v 3.2.1 software (SAS Institute Inc.).

Results

Identification of the A. brasilense Sp7 glgP gene

Based on sequences of bacteria phylogenetically related to A. brasilense and on its codon usage, we designed degenerate primers to screen strain Sp7 for rpi genes by PCR. With a primer combination, an ~400-bp product was obtained that showed high similarity to glycogen phosphorylase (glgP) genes in the database. We then generated additional primers to enlarge the sequence of this gene from strain Sp7. These primers yielded an 864-bp fragment (not shown) that showed high identity to glycogen phosphorylase of Rhodospirillum centenum (81% and 77%, by BLASTN and BLASTX, respectively), Rhizobium leguminosarum bv. viciae (79% and 60%) and Mesorhizobium loti (75% and 61%). BLASTP revealed that the encoded protein possesses a conserved domain characteristic of the glycoc_transf_1 superfamily, and Pfam analysis showed this sequence has a phosphorylase domain (PF00343). Sequence analysis of the Sp7 glgP gene and its flanking region revealed that this gene is part of a single-gene locus.

Generation of an A. brasilense glgP mutant and its initial characterization

We used marker exchange recombination to generate a glgP knockout mutant in the background of A. brasilense Sp7. The selected mutant was confirmed by PCR and Southern blot (data not shown). The glgP mutant produced colonies that did not differentiate from those of the wild type by the naked eye, and light microscopy revealed that both possess a similar cell morphology (not shown). No differences in growth ability were observed between the mutant and the wild-type strain in various tested carbon sources (not shown).
Enzymatic assays with cell extracts revealed a significant \( (P=0.05) \) reduction of glycogen phosphorylase specific activity in the \( glgP \) mutant (77 ± 7 EU mg per protein) relative to the wild type (119 ± 20 EU mg per protein). This result confirmed that the mutated gene indeed encodes a protein with glycogen phosphorylase activity, but also suggested that strain Sp7 contains at least one additional gene encoding this activity.

Glycogen accumulation was evaluated by glycogen staining of bacteria with lugol (iodine). Consistently, lugol staining revealed a much darker appearance of \( glgP \) mutant pellets as compared with those of strain Sp7 (Fig. 1a), thus confirming that the mutant accumulated more glycogen than the wild type. This result was supported by quantitative measurements of glycogen content in wild-type and mutant strains. Although differences between strains were not always statistically significant, in three independent experiments, the mutant strain consistently had a higher glycogen content than the wild type after 24 and 48 h of growth (shown in Fig. 1b for 24 h of growth from three different experiments).

**The \( glgP \) mutant has an altered exopolysaccharide and its biofilm formation is affected**

The effect of the \( glgP \) mutation on exopolysaccharide production was evaluated. A trend was observed in which the \( glgP \) mutant produced less exopolysaccharide than the wild type (468.7 ± 75.6 and 601.5 ± 88.2 mg exopolysaccharide g \(^{-1}\) biomass, respectively); however, these differences were not statistically significant \( (P=0.05) \). Analyses of the exopolysaccharide monosaccharide composition of the \( glgP \) mutant revealed a decrease in the concentration of glucose, accompanied by increased levels of rhamnose, fucose and ribose as compared with that of the wild-type strain (Table 2).

As the \( glgP \) mutation was shown to alter the exopolysaccharide composition of Sp7, we asked whether this mutation influences biofilm production by this bacterium. Indeed, our results showed a significant decrease in the ability of the \( glgP \) mutant to produce biofilm on a polystyrene surface as compared with the wild type in all tested media (Fig. 2).

**The \( glgP \) mutant is more resistant under several stresses than the wild type**

We analyzed the ability of the \( glgP \) mutant to survive under different stress conditions in comparison with the wild-type strain. The mutant showed significantly \( (P=0.05) \) higher survival levels than Sp7 to desiccation, starvation and osmotic pressure (Table 3). No differences were found between the strains in their ability to survive after exposure to a high temperature (55 °C) and to UV-radiation, and in their sensitivity to elevated levels of NaCl (data not shown). In addition, under the conditions tested, no significant differences were observed between the \( glgP \) mutant and Sp7.

![Fig. 1. Glycogen accumulation in Azospirillum brasilense Sp7 and the \( glgP \) mutant.](image)

**Table 2. Relative monosaccharide composition of exopolysaccharide extracted from Azospirillum brasilense Sp7 and \( glgP \) mutant strains**

<table>
<thead>
<tr>
<th>Sugar</th>
<th>Sp7</th>
<th>( glgP ) mutant</th>
</tr>
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<tbody>
<tr>
<td>Rhamnose</td>
<td>2.1 ± 0.6</td>
<td>7.7 ± 1.4</td>
</tr>
<tr>
<td>Fucose</td>
<td>1.3 ± 0.5</td>
<td>4.9 ± 0.3</td>
</tr>
<tr>
<td>Ribose</td>
<td>2.5 ± 0.3</td>
<td>4.2 ± 0.5</td>
</tr>
<tr>
<td>Arabinose</td>
<td>1.4 ± 0.2</td>
<td>1.8 ± 0.7</td>
</tr>
<tr>
<td>Xylose</td>
<td>1.9 ± 1.2</td>
<td>1.9 ± 0.4</td>
</tr>
<tr>
<td>Mannose</td>
<td>28.8 ± 1.0</td>
<td>34.9 ± 0.9</td>
</tr>
<tr>
<td>Galactose</td>
<td>15.2 ± 0.1</td>
<td>17.0 ± 5.6</td>
</tr>
<tr>
<td>Glucose</td>
<td>46.8 ± 1.3</td>
<td>27.7 ± 1.3</td>
</tr>
<tr>
<td>Total</td>
<td>100.0</td>
<td>100.0</td>
</tr>
</tbody>
</table>

Results represent averages and SD of the relative presence of each sugar (%) calculated as \((\mu gm L^{-1}) \times 100/total sugars\) from two experiments with similar results.
Azospirillum brasilense glgP

Table 3. Survival rate (%) of Azospirillum brasilense wild-type Sp7 and glgP mutant in response to desiccation, starvation and osmotic pressure

<table>
<thead>
<tr>
<th>Strain</th>
<th>Desiccation</th>
<th>Starvation</th>
<th>Osmotic pressure</th>
</tr>
</thead>
<tbody>
<tr>
<td>Sp7</td>
<td>0.13 ± 0.08</td>
<td>2.36 ± 0.47</td>
<td>0.002 ± 0.002</td>
</tr>
<tr>
<td>glgP mutant</td>
<td>14.48 ± 3.53</td>
<td>9.17 ± 2.50</td>
<td>4.414 ± 0.979</td>
</tr>
</tbody>
</table>

Significant differences (P = 0.05) were found between the strains in the different treatments. Experiments were repeated three times and yielded similar results. Averages and SD from representative experiments are shown.

in their ability to adhere to sweet corn roots and to induce plant growth promotion (data not shown).

Discussion

Glycogen is a reserve glucose polysaccharide occurring in many bacteria, with properties similar to those of animal glycogen (Preiss, 1984, 2006). Its metabolism in bacteria has been extensively studied and involves enzymes such as ADP-glucose pyrophosphorylase, ADP-glucose glycogen synthase, glycogen branching enzyme, glycogen phosphorylase and glycogen debranching enzyme (Lepek et al., 2002). In the present study, a glgP gene, encoding glycogen phosphorylase, was identified in A. brasilense Sp7. GlgP is a glucan-degrading enzyme that catalyzes the production of glucose-1-phosphate by the reversible cleavage of an α-1,4 bond at the nonreducing ends of polyglucans. The A. brasilense Sp7 glgP gene showed high similarity to glgP genes of several rhizobial species.

To gain insights into the ecological relevance of glycogen metabolism in A. brasilense, we generated a glgP mutant in the background of strain Sp7. Enzymatic assays revealed that this mutant is significantly compromised in glycogen phosphorylase activity. In addition, the mutant strain clearly accumulated more glycogen than the wild-type strain. These results confirmed that the mutated gene indeed encodes a protein with GlgP activity. However, glycogen phosphorylase activity was not completely abolished in the mutant strain, suggesting that the A. brasilense Sp7 genome contains at least one additional gene encoding such activity.

Very recently, the draft genome of another A. brasilense strain – Sp245 – became partially available to some research groups, including ours. In the Sp245 draft genome, two genes are annotated as glgP. The first report on the presence of two glgP genes in a given organism was for the cyanobacterium Synechocystis sp. (Kaneko et al., 1996). Comparative analyses revealed that the glgP gene of strain Sp7 studied in this work is 97% and 98% identical to one of the Sp245 glgP genes (named here glgP1sp245) at the nucleic acid and amino acid levels, respectively. As the glgP gene from strain Sp7, glgP1sp245 is part of a single-gene locus. The second glgP gene from Sp245, glgP2sp245, is located in an operon, with the glycogen debranching enzyme glgX. glgP2sp245 showed identity levels of 67% (at the nucleic acid level, for a partial alignment of 560 bp) and 51% (at the amino acid level, for a partial alignment of 288 aa) with the Sp7 glgP. Similar identity levels were found between glgP1sp245 and glgP1sp245.

We generated a phylogenetic tree based on GlgP protein sequences from the database using CLUSTALW (not shown). While the Sp7 GlgP and GlgP1sp245 clustered with typical Glgps from the Alphaproteobacteria, GlgP2sp245 grouped into a distinct cluster that contained Glgps mostly from Cyanobacteria, Deltaproteobacteria and Acidobacteria. In agreement with these and with the low level of identity found between these two kinds of genes, Southern blot analysis with restricted DNA from strain Sp7 and the Sp7...
glycogen fragment as a probe yielded a single signal (not shown). To identify a putative second glgP gene in strain Sp7, PCR was performed using two sets of primers, specifically designed for glgP2Sp245. Despite several attempts, no PCR product could be obtained (not shown). Therefore, the source of the residual GlgP activity observed in the Sp7 glgP mutant, as well as the likely occurrence of a second glgP gene in Sp7, are yet to be elucidated.

PHB and glycogen are major carbon storage compounds in azospirilla (Caiola et al., 2004). The ecological role of PHB in A. brasilense has been investigated in depth (Kadouri et al., 2002, 2003a, b). These studies demonstrated that in A. brasilense Sp7, PHB plays an important role in cell endurance to UV radiation, heat, osmotic pressure, osmotic shock, desiccation and oxidative stresses. In contrast to PHB, the ecological role of glycogen in A. brasilense is poorly understood. Results from our study support the fact that, in addition to PHB, glycogen accumulation also contributes to survival to several stresses in A. brasilense.

The glgP mutant was found to be more resistant than the wild type to starvation, desiccation and osmotic stresses. During starvation, preaccumulated glycogen could play an important role as carbon and energy sources in the glgP mutant. If this is the case, the advantage of the mutant over the wild type is probably due to the presence of an unaffected, second glgP gene that could contribute to glycogen utilization during starvation. Fu & Xu (2006) found different functions for two glgP genes in Synechocystis sp. In this organism, while one of the glgP genes was shown to be essential for growth at a high temperature, the second glgP gene was expressed under CO₂ fixation and a limited supply of glucose.

In agreement with our results on qualitative and quantitative glycogen accumulation in the glgP mutant, early reports suggested a correlation between glycogen accumulation and survival under starvation in E. coli (Strange, 1968) and Streptococcus mitis (Van Houte & Jansen, 1970). Moreover, recent comparative transcriptome analyses revealed upregulation of genes involved in glycogen accumulation during hyperosmotic stress in Sinorhizobium meliloti (Dominguez-Ferreras et al., 2006) and Yersinia pestis (Han et al., 2005). In S. meliloti, during desiccation and osmotic pressure, glycogen could aid in restoring the volume and turgency of the cell cytoplasm (Dominguez-Ferreras et al., 2006). Glycogen accumulation during high-temperature stress was shown to contribute to the survival of Salmonella typhimurium (McMeechan et al., 2005) and Saccharomyces cerevisiae (Kim et al., 2006). In our study, under tested conditions, the wild type and the glgP mutant did not differ in their survival ability following exposure to a high temperature.

Under tested conditions, the glgP mutant and the wild type did not significantly differ in the amount of exopolysaccharide; however, they differed in their exopolysaccharide composition. For instance, glucose was reduced by half in the glgP mutant relative to the wild type. This could be due, at least partially, to a reduced availability of glucose in the mutant because of a decreased glycogen phosphorylase activity.

As polysaccharides are important components of the extracellular matrix that connect cells in a biofilm, the alterations in the exopolysaccharide of the glgP mutant could be associated with its reduced biofilm formation ability relative to the wild type. In addition to connecting cells, the biofilm extracellular matrix yields several other key features, including the ability to protect cells from stress (Hall-Stoodley & Stoodley, 2002; Danhorn & Fuqua, 2007). Thus, it is important to consider that, although under tested conditions the glgP mutant was more resistant than the wild type to several stresses, the reduced biofilm formation ability of the mutant could compromise its fitness in a ‘true’ ecological niche, such as the soil or the rhizosphere.

The relationship between biofilm development and root colonization has been reviewed recently (Danhorn & Fuqua, 2007). Root adhesion and plant growth promotion experiments conducted in this study did not show differences between the glgP mutant and the wild type. These results could suggest that biofilm formation is not critical for the establishment of the plant–A. brasilense association. On the other hand, we cannot exclude the possibility that differences between the mutant and the wild type in these parameters could be detected under other conditions. Moreover, the mechanisms for development of biofilm in biotic and abiotic surfaces by A. brasilense could be different, as it was shown for Pseudomonas putida (Yousef-Coronado et al., 2008).

To summarize, the investigation on the relevance of glycogen metabolism in A. brasilense is in its beginning. Our study revealed that A. brasilense Sp7 might possess at least two genes encoding glycogen phosphorylase activity, suggesting that glycogen metabolism plays an important role in A. brasilense ecology. This hypothesis is supported by the significant phenotypic differences observed between the wild-type strain and a mutant impaired in one of the glgP genes. Although we did not perform complementation assays, it is not likely that these differences are due to the polar effects of the mutation as glgP is part of a single-gene locus and the observed differences were associated with effects on glycogen accumulation and glycogen phosphorylase activity.

Further investigation should focus on the characterization of other genes involved in glycogen metabolism, including glycogen synthesis and utilization. Importantly, research should address the elucidation of the complex interactions between different processes (such as PHB and glycogen metabolism, synthesis of extracellular polysaccharide and
biofilm formation, among others) in conferring a successful ecological performance to this bacterium. This understanding is also critical for applicative purposes, namely, for generation and application of improved inoculants of this bacterium for agriculture.

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References


