CHARACTERIZATION OF A STRAIN OF *Herbaspirillum seropedicae* MUTANT IN GENE *glnE*

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Abstract

The bacterium *Herbaspirillum seropedicae* is an endophytic nitrogen-fixing bacterium. Among the plants with which *H. seropedicae* is associated, grasses of economic interest are included, such as sugarcane, wheat and corn. Nitrogen fixed by *H. seropedicae* is incorporated into the plant's host biomass and can also induce plant growth through the production of phytohormones. The metabolic flux through the GS pathway is regulated by a sensing enzyme, GlnE. GlnE is a bifunctional enzyme, capable of catalyzing reversible adenylylation in a conserved tyrosine residue of GS, decreasing its biosynthetic activity at high concentrations of ammonium. At low concentrations of ammonium, GlnE promotes the death of GS, activating it [1]. For a better understanding of the regulatory mechanism of GlnE, a strain of *H. seropedicae* was constructed with a phase deletion of the *glnE* gene, called ELΔglnE, and we started its characterization. We verified the activity and expression of GS in wild and ELΔglnE strains in high ammonium and low glutamate .

Introduction

The bacterium *Herbaspirillum seropedicae* is an endophytic diazotrophic organism, that is, it is capable of reducing atmospheric nitrogen to ammonium without harming the host [2]. Among the plants with which it is associated, grasses with economic interest are included, such as sugar cane, wheat, corn and rice. Thus, this bacterium has a high potential to replace nitrogen fertilization, which, in addition to being very expensive, is aggressive to the environment. Therefore, *H. seropedicae* has stood out as a diazotrophic organism model for the study of nitrogen metabolism. Nitrogen assimilation can occur in two ways, one of which is via Glutamine synthetase (GS). Its activity is strongly regulated in multiple ways, one of which is post-translational modification by GlnE. GlnE has adenylyl transferase (ATase) and adenylyl removal (AR) activities on GS, so the balance between ATase and AR activities allows to increase or reduce nitrogen assimilation by the cell.

The Ntr system comprises a set of proteins responsible for sensing fixed nitrogen levels and allows the bacteria to adapt to the available nitrogen level. In this system we have GS, GlnE and GlnK (PII protein). In *E. coli*, it is known that under low nitrogen conditions, GlnK is found uridylylated (UMP), interacts with GlnE promoting GS deadenylylation and favoring its activity and therefore increasing nitrogen assimilation. The opposite is also true, at high nitrogen concentrations, GlnK is deuridylylated and interacts with GlnE promoting GS adenylylation, favoring lower nitrogen assimilation.

To verify the effect of the *glnE* gene deletion we evaluated the biosynthetic activity of GS *in vivo* in high ammonium (20mM) and low glutamate (5mM). In addition to the biosynthetic activity, GS has a reverse reaction activity, γ-glutamyl transferase. This activity was evaluated because studies show that GS-AMP has γ-GT activity inhibited by Mg^{+2} ions, thus it is possible to know the adenylylation state of the enzyme. We verified the expression of GS *in vivo* by Western Blotting, in high ammonium and low glutamate. We then checked GlnK expression by RT-PCR in wild-type and mutant strains under these two conditions as well.

Materials and methods

The wild and mutant strains were cultivated in Nfb-Malate medium until they reached an OD600 0.9. Cell were lysed by sonication and then protein quantified by using the Bradford colorimetric method. After protein quantification, enzymatic activity was carried out as previously described [3]. Protein separation was performed by SDS-PAGE and the gel proteins were transferred to the membrane by Western Blotting and incubated with primary antibody specific for GS 1:1000 and for the secondary anti-rabbit HRP antibody was used. Protein expression was visualized by chemiluminescence. The TRIzol LS Reagent (ThermoFisher) protocol was used for RNA extraction, followed by the protocol with DNase (Invitrogen) and then the protocol for RT-PCR (Applied Biosystems) with specific primers for *glnK* and *rpoA* (α-subunit of RNA polymerase).

Results

We verified the GS activity of wild and ELΔglnE mutant strains in high ammonium (20mM) and low glutamate (5mM).

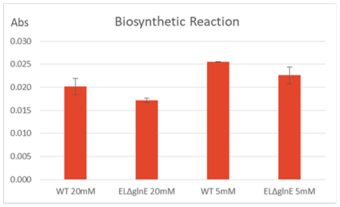


Fig. 1 Biosynthetic activity of GS in wild (WT) and mutant (ELΔglnE) strains grown in high ammonium (20mM) and low glutamate (5mM).

In both wild type and ELΔglnE strains the biosynthetic reaction was detected and regulated by nitrogen availability, with higher activity in 5mM glutamate.

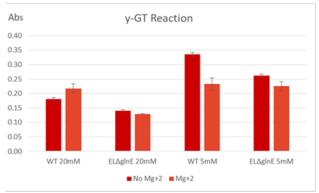


Fig. 2 γ-GT activity of GS in wild (WT) and mutant (ELΔglnE) strains grown in high ammonium (20mM) and low glutamate (5mM).

The γ-GT activity is inhibited by Mg^{+2} ions, so the result suggest that GS is not adenylylated, as the activity was maintained, even after the addition of Mg^{+2}. Furthermore, there is a noticeable difference in the activities of the WT 5mM and the mutant 5mM.

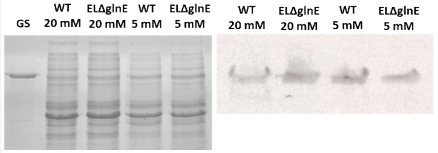
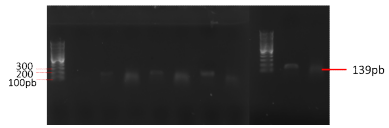


Fig. 3 SDS-PAGE gel and western blotting of protein extracts from WT and ELΔglnE cultures grown in 20mM ammonium and 5mM glutamate. We used purified GS as a marker, with around 52kDa.

The result shown in fig. 3 suggest that GS is being expressed in all strains and seems not to have been much affected by the different growth conditions. This could mean that the enzyme is undergoing other regulations in its activity than adenylylation, such as allosteric regulations, under high ammonium conditions.

Table 1 RT-PCR enumerated samples. Two primers were used for amplification. The *glnK* gene primer to verify its expression *in vivo* and the *rpoA* gene primer as a way to control the reaction.

RT-PCR samples									
***glnK* primer (139pb)**	**1**	**2**	**3**	**4**	**5**	**6**	**7**	**8**	**9**
	No Template	cDNA WT 20	RNA WT 20	cDNA Δ20	RNA Δ20	cDNA WT 5	RNA WT 5	cDNA Δ 5	RNA Δ5
***rpoA* primer (control) (129pb)**	**10**	**11**	**12**	**13**	**14**	**15**	**16**	**17**	**18**
	No template	cDNA WT 20	RNA WT 20	cDNA Δ20	RNA Δ20	cDNA WT 5	RNA WT 5	cDNA Δ 5	RNA Δ5



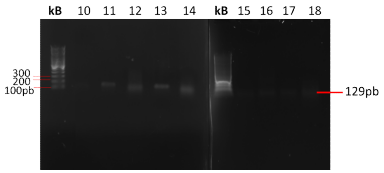


Fig. 4 RT-PCR result with *glnK* primer (139bp) and the *rpoA* gene (129pb) (positive control).

Fig. 4 shows that RT-PCR worked, as well-defined bands formed and the size consistent with the expected fragment. Furthermore, in lanes that contained only RNA, they did not form well-defined bands, showing that the samples were neither contaminated nor contained chromosomal DNA. The characterization of the ELΔglnE will continue to be carried out in the future.

Conclusion

In the present work we noticed that the activity of both strains is better with 5mM glutamate, showing that the absence of GlnE can interfere with the bacteria activity in high ammonium. According to the γ-GT reaction, the GS of the mutant strain is not adenylylated, promoting an increase in glutamine and glutamate levels. This profile in the wild, leads to desuridylylation of GlnK, which interacts with GlnE inactivating GS, but as the mutant strain does not have GlnE, further tests are needed to understand what happens to its metabolism in this case.

Recommendations

1Eisenberg, D., Gill, H. S., Pfluegl, G. M. U., & Rotstein, S. H. Protein Structure and Molecular Enzymology, 1477 (2000).

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3Bender, R. A., Janssen, K. A., Resnick, A. D., Blumenberg, M., Foor, F., & Magasanik, B. (1977). Biochemical parameters of glutamine synthetase from Klebsiella aerogenes. Journal of Bacteriology, 129(2), 1001–1009.

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