Stress resistance in *Azospirillum* strains overexpressing genes involved in poly-β-hydroxybutyrate biosynthesis

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Abstract: Poly-β-hydroxybutyrate (PHB) is a biopolymer produced by bacteria when they are subjected to nutritional stress. The use of PHB under stress is a mechanism that favors their establishment, proliferation, survival, and competition. Biopolymer synthesis involves three chemical reactions by enzymes: β-ketothiolase, acetoacetyl CoA reductase and PHB synthase, encoded by *phb*A, *phb*B and *phb*C genes, respectively. The aims of this research were to quantify PHB production in *A. brasilense* Sp7 strains when *phb*A and *phb*C genes were overexpressed. Also, recent reports suggest a relationship between antioxidant activity and PHB production; therefore, antioxidant enzyme activity (SOD, CAT and APX) was quantified in cultures subjected to osmotic stress induced by NaCl. PHB quantification showed that *phb*A gene overexpression increased around 60% respect WT strain. Instead *phb*C gene overexpression did not alter PHB production compared to the WT. On the other hand, the data obtained by enzymatic quantification infer that *A. brasilense* uses another mechanism to contend with environmental stress. Such a mechanism could be related to the PHB biosynthesis, however studies are necessary to demonstrate our hypothesis.

Keywords: Poly-β-hydroxybutyrate genes ● antioxidant enzyme activity ● *Azospirillum*

Introduction: Many bacteria accumulate granules of poly-β-hydroxybutyrate (PHB). The Gram negative, α-proteobacteria *Azospirillum brasilense* Sp7 accumulates (PHB) up to 75% of the cell dry weight (Tal *et al*., 1990). PHB is synthetized under unbalanced growth conditions: an excess of carbon and a limitation on the nitrogen sources. In bacteria, PHB functions are: carbon and energy reserve, reducing power, encystment, sporulation, stress resistance and desiccation. There are three genes considered to be essential in PHB biosynthetic pathway: *phbA*, *phbB* and *phbC* that encode necessary enzymes to synthetize PHB (β-ketothiolase, acetoacetyl-CoA reductase y PHB synthase, respectively). Biopolymer synthesis begins with the condensation of two acetyl-CoA molecules by β-ketothiolase forming acetoacetyl-CoA, an acetoacetyl-CoA reductase catalyzes the conversion of acetoacetyl-CoA to β-hydroxybutyryl-CoA. Finally, the β-hydroxybutyryl-CoA is polymerized by a PHB synthase (Kadouri *et al*., 2002).
It has been suggested for diverse ecological systems that the accumulation, degradation, and utilization of polyhydroxyalcanoate (PHA) like PHB by several bacteria under stress is a mechanism that favors their establishment, proliferation, survival, and competition, especially in competitive environments where carbon and energy sources are limiting, such as those encountered in the soil and rhizosphere (Okon and Itzigsohn, 1992).

Recently studies suggest an association between PHB accumulation and high stress resistance in bacteria adapted to extreme environments (Ayub *et al*., 2004). Due to this we decided analyze stress tolerance to high NaCl concentrations (150 and 300 mM) in *A. brasilense* strains that overexpressing genes involved in poly-β-hydroxybutyrate biosynthesis. For this, antioxidant enzymes (superoxide dismutase, catalase and ascorbate peroxidase) were quantified.

Materials and Methods:

Bacterial strains, plasmids and growth conditions. Bacterial strains and plasmids are listed in Table 1. For *E. coli* growth Luria Bertani (LB) media was used. *Azospirillum* transconjugants were selected on minimal medium (MMAB).

Table 1. Bacterial strains and plasmids used in this study.

Strain or plasmid	Genotype	Reference
A. brasilense Sp7	Wild-type strain	Nur *et al*. 1982
Ab7A	*A. brasilense* Sp7 overexpressing *phb*A gene	This work
Ab7B	*A. brasilense* Sp7 overexpressing *phb*B gene	This work
Ab7C	*A. brasilense* Sp7 overexpressing *phb*C gene	This work
Ab7-206	*A. brasilense* Sp7 harbouring pMMB206	This work
E. coli DH5α	ΔlacU169 hsdR17 recA1 endA1 gyrA96 thi-l relA1	Gibco-BRL
E. coli S17-1λpyr	Sm^r recA thi pro $hsdR^-$ λpir	Biomedal Lifescience
pMMB206	Expression vector Cm^r	Addgene
p206A	pMMB206 harbouring *phb*A gene	This work
p206B	pMMB206 harbouring *phb*B gene	This work

Cloning. PCR-amplified *phb*A and *phb*C genes were cloned into the broad-host-range expression vector pMMB206. The *phb*A gene was PCR-amplified by using the primers phbA-FEco (5′-AGGAATTCAATGACCTCGGCGCCTTTGACC-3′), with an *Eco* RI site (underlined) located upstream of the start codon, and the primer phbA-RBam (5′-CGGGATCCTCAGACCGCTTCCAGGACC-3′), with a *Bam* HI site (underlined) located downstream of the stop codon. Similarly, the *phb*C gene was amplified by using the primers phbC-FPst (5′-CTGCAGATGGTCGGAGATCTGGGTGTC-3′) and phbC-RPst (5′-CTGCAGTCAGACGATGCGCACCTTGGC-3′) with *Pst* I sites (underlined) located upstream and downstream of the gene. The amplicons were digest with *Eco* RI and *Bam* HI (*phb*A) and *Pst* I (*phb*C) and ligated with the similarly digested expression vector pMMB206. *E. coli* DH5α was transformed with the ligation mix and transformants were selected by blue/white selection on LB plates containing 1 mM IPTG, 80 μg mL^{-1} X-Gal and 35 μg mL^{-1} chloramphenicol. Obtained constructions were named p206A and p206C respectively. Lately constructions were transferred into *E. coli* S17-1 λpyr, and then conjugatively mobilized into *A. brasilense* Sp7. Obtained strains were named Ab7A and Ab7C, respectively. Likewise pMMB206 expression vector was transferred into *E. coli* S17-1 λpyr and conjugatively mobilized into *A. brasilense* Sp7, obtaining Ab7-206 strain. Transconjugants clones were selected in LB agar and minimum medium (MM) with 100 μg mL^{-1} ampicillin and 35 μg mL^{-1} chloramphenicol.

Poly-β-hydroxybutyrate quantification. Estimation of PHB content was determinate as Law and Slepecky (1961) and the amount of PHB produced was calculated from the standard curve

prepared by using PHB (Invitrogen). *A. brasilense* Sp7 strains were growth in MMAB containing malate and NH_4Cl as sole source of carbon and nitrogen, respectively. Cultures were incubated with shaking at 32 °C until OD_{600nm} reached 0.3. At this stage, IPTG was added to 1 mM final concentration to induce the expression of the cloned genes. Bacterial culture was centrifuged at 8,000 rpm al 4 °C for 6 min and the pellet was washed with $MgSO_4$ 10 mM. It was mixed thoroughly in NaClO by vortexing. Mixture was incubated at 37 °C for 90 min and again centrifuged at 8,000 rpm for 10 min to sediment the lipid granules. Supernatant was decanted, and the pellet was washed successively with distilled water, acetone and ethanol and centrifuged at 8,000 rpm for 10 min each wash. Pellet for granules was dissolved in chloroform at 60 °C until all the chloroform gets evaporated. H_2SO_4 was added and the tubes were heated for 20 min in boiling water bath, cooled and mixed thoroughly. Absorbance was read at 235 nm against a H_2SO_4 blank on UV-Vis spectrophotometer. PHB assays were performed for triplicate.

Enzymatic assay. *A. brasilense* Sp7, Ab7A, Ab7C and Ab7-206 strains were cultivated in MM supplemented with NaCl, 0, 150 and 300 mM in order to induce osmotic stress. Crude extracts were prepared as Clara and Knowles (1984). Cell suspensions were disrupted by sonication for a 30-s burst for a total of 10 min. Cellular debris was removed by centrifugation at 8,000 rpm for 20 min. The supernatant was retained for the enzymatic assays. Superoxide dismutase activity (SOD, EC 1.15.1.1) was measured on the basis of SOD´s ability to inhibit the reduction of nitroblue tetrazolium (NBT) by superoxide radicals generated photochemically (Beyer and Fridovich, 1987). Data are presented as U SOD/mg protein. Catalase activity (CAT, EC 1.11.1.6) was measured as described (Aebi, 1984). Consumption of H_2O_2 (extinction coefficient of 39.6 mM^{-1} cm^{-1}) at 240 nm for 1 min was monitored. Data are shown in mKatal/mg protein) Ascorbate peroxidase activity (APX, EC 1.11.1.11) was measured accord Amako *et al*. (1994). Specific activity is presented as mMol min^{-1} mg^{-1} protein.

Total protein (TP). TP was determined using the Bradford method and BSA as a standard (Bradford, 1976).

Statistics. The results are given as mean $\pm$ standard deviation of three independent determinations. Data were analyzed using ANOVA test with a significance level of $P < 0.05$.

Results and Discussion:

Azospirillum, like other microorganisms, synthetizes PHB under unbalanced growth conditions: an excess of carbon and a limitation on the nitrogen sources, in our study *A. brasilense* strains were cultivated in MMAB containing malate and NH_4Cl as sole source of carbon and nitrogen, respectively. And IPTG was added to induce the expression of genes cloned into broad-host-range expression vector. So PHB contend is showed in Table 2.
It has been reported that *A. brasilense* is able to accumulate PHB up to 70% of their cell dry weight in presence of adverse factors like UV radiation, desiccation and osmotic stress, and the polymer-rich cells have better survival (Okon and Itzigsohn, 1992). However our data cannot been compared due to methodology employed in both cases. Nevertheless our results show that in Ab7A strain, *phb*A gen overexpression leads to increased PHB production. This may be due to an increase in β-ketothiolase enzyme activity by raising Acetyl-CoA efflux from TCA cycle to PHB pathway, altering the production of PHB. On the other hand, when *phb*C gen is overexpressed a minor production of PHB in Ab7C can be observed in comparison with *A. brasilense* Sp7. This

can be explained due to a mayor amount of PHB synthase bound to PHB granules. However, is necessary corroborates it, by granule dye and TEM.

Table 2. PHB/mg protein data obtained in *A. brasilense* strains.

Strain	PHB/mg protein
A. brasilense Sp7	617.10 ± 79.91
Ab7A	1007.27 ± 23.04
Ab7B	*
Ab7C	443.96 ± 35.46
Ab7-206	747.76 ± 52.72

* Ab7B status: in process.

In order to evaluate an association between PHB accumulation and stress resistance in bacteria, we decided analyze stress tolerance to high NaCl concentrations (150 and 300 mM) in *A. brasilense* strains used in this study. So that, SOD, CAT and APX enzyme activity were quantified. Figure 1 shown SOD, CAT and APX contents in *A. brasilense* strains.

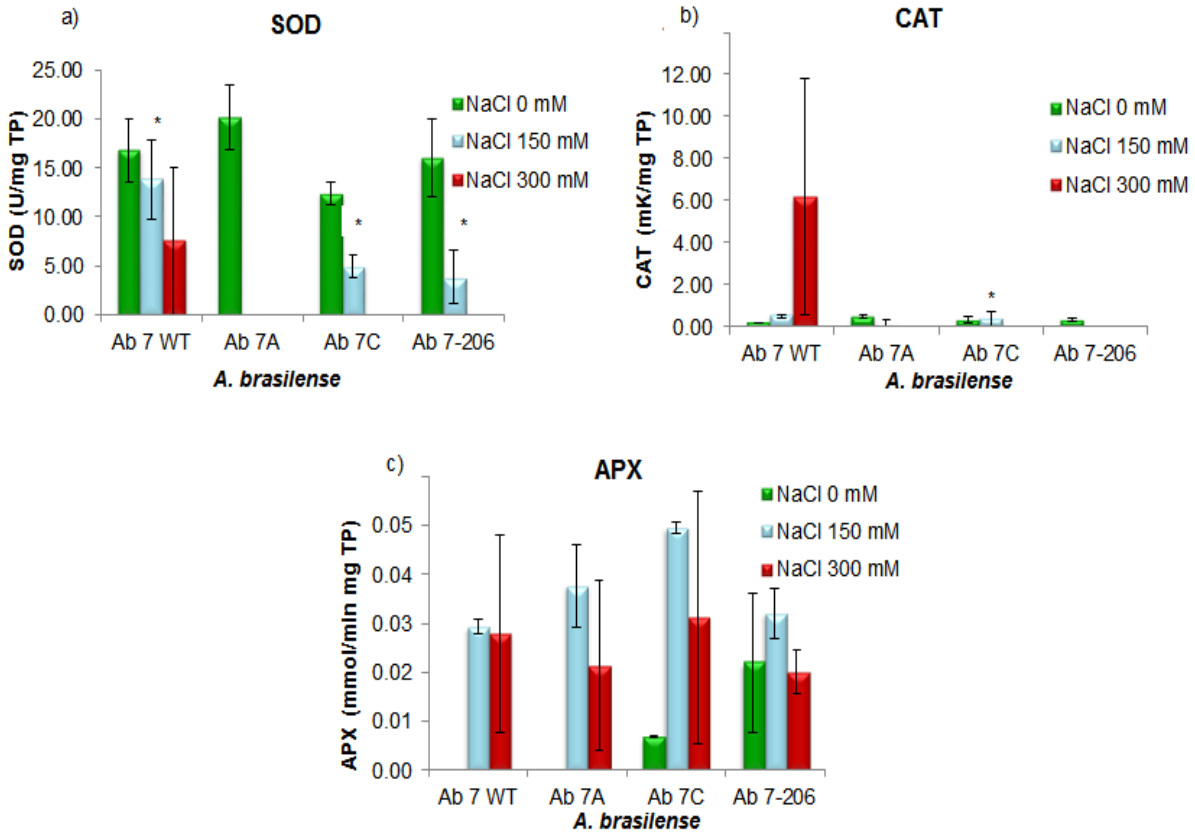

Figure 1. SOD (a), CAT (b) and APX (c) contents in *A. brasilense* strains (**P*<0.05; asterisks represents significant differences between *A. brasilense* Sp7 and genetically modified strains).

SOD and APX production in *A. brasilense* Sp7 was similar that Clara and Knowles (1984) reported. In our study CAT activity was no detected in *A. brasilense* cultures without stress. It was founded that Ab7A produce major SOD levels respect Ab7C in non-stressed cultures. However, when strains are cultivated in NaCl 150 mM, SOD production is null or weakly in all strains except in *A. brasilense* Sp7. In NaCl 300 mM SOD levels were non-detected. Furthermore, CAT was present in all strains from 0 to 1 mK mg^{-1} protein. Nevertheless, in NaCl 300 mM, CAT was only detected in strain Ab7 WT. Regarding the activity APX in non-stressed conditions reported only for Ab7C and Ab7-206 strains. While at NaCl 150 mM an increased APX production was observed, highlighting it in Ab7C and Ab7-206 strains. Finally in NaCl 300 mM, APX production was decreased in all strains. Has been documented that osmotic stress can serve as a direct inducer of the oxidative stress (Aldsworth *et al*., 1999) and *Azospirillum sp.* has high rate of respiratory activity (Bergersen and Turner, 1980) and also, carries enzymes that detoxify O^{2-}, and H_2O_2 (Nur *et al*., 1982). Stouthamer *et al.* (1979) suggested that activity of enzymes of decomposition of O, and H_2O_2 is insufficient to prevent damage to the cell. This may apply also to *A. brasilense*. According to the above, we hypothesize that *A. brasilense* strains employed another defense mechanism to counteract free radicals generated during exposure to NaCl. This can be explained because non-stressed cultures have higher levels of enzyme activity when overexpressed genes involved in PHB biosynthesis (in SOD and CAT). Currently there are reports indicating that PHB production is increased when cultures are subject to osmotic stress induced by NaCl (310 mM). Likewise, there is evidence suggesting the involvement of PHB as an antioxidant response in bacteria (Ayub *et al*. 2004), given that, PHB are carbon and energy reservoirs. So, once obtained PHB production in *A. brasilense* strains overexpressing *phb*A and *phb*C genes, would be useful quantify the PHB production in cultures subjected to osmotic stress.

Conclusions: PHB production was raised upon 60% in Ab7A strain. Nevertheless *phb*C gene overexpression in Ab7C resulted in PHB levels similar to reported by *A. brasilense* Sp7. Considering previous reports that suggest a relationship between antioxidant activity and PHB production, antioxidant enzymatic activity was quantified in the all strains subject to osmotic stress by adding NaCl. The data obtained from enzyme quantification in working strains allow us to inference that other mechanism was used to counteract the effects caused by osmotic stress. That mechanism could be PHB production. However, the metabolism of antioxidant enzyme activities in *A. brasilense* is not completely understood in relation to the ability of these microorganisms to produce PHB. So, once obtained PHB production in *A. brasilense* strains would be useful quantify the PHB production in cultures subjected to osmotic stress.

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