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Characterization of Genes Involved in Poly-β-Hydroxybutyrate Metabolism in *Azospirillum brasilense*

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Abstract

Under suboptimal growth conditions and like many other prokaryotes, rhizobacteria of the genus *Azospirillum* produce high levels of poly-β-hydroxybutyrate (PHB). The two genes, *bdhA* (3-hydroxybutyrate dehydrogenase) and *acsA2* (acetoacetyl-CoA synthetase), which are considered to be involved in the PHB degradation pathway in *Azospirillum brasilense* strain Sp7, were identified, cloned, and sequenced. Additionally, the expression of the bacterial genes *phbA* (β-ketothiolase) and *phbC* (PHB synthase), which are involved in PHB biosynthesis and in the expression of the *acsA2* gene, were studied using GUS fusions. Our results indicate that these genes are constitutively expressed in *Azospirillum brasilense* Sp7 and that the Ntr, P_{II} and P_Z nitrogen regulatory systems, which have been shown to be involved in the regulation of PHB synthesis, do not affect the expression of these genes. Expression of these genes is also shown to occur during association of *A. brasilense* with wheat roots.

Keywords: Poly-β-hydroxybutyrate (PHB), 3-hydroxybutyrate dehydrogenase, acetoacetyl-CoA synthetase, *Azospirillum brasilense*, PHB metabolism

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1. Introduction

A wide variety of microorganisms produce intracellular energy and carbon storage compounds known as poly-β-hydroxybutyrate (PHB) or polyhydroxyalkanoates (PHA) (Steinbuchel and Hein, 2001). It has been suggested that the accumulation, degradation, and utilization of PHAs by several bacteria under stress is a mechanism that favors their establishment, proliferation, survival and competitiveness, especially in environments such as the rhizosphere, where carbon and energy sources are limiting factors (Okon and Itzigsohn, 1992). The biosynthetic pathways of PHAs and their corresponding genes have been widely investigated due to the high potential of PHAs uses in industrial applications. However, PHA degradation and its regulation have only been poorly studied. Like other polymers synthesized by living systems, PHB can be degraded at a later stage by the organism producing it. Hence, the degradation is not simply a reversal of the synthesis (Babel et al., 2001).

Under suboptimal conditions, such as under high C/N ratio, the gram negative nitrogen-fixing rhizobacterium *Azospirillum brasilense* accumulates more than 80% PHB of its cell dry weight. It was shown that the biosynthesis and degradation of PHB in *A. brasilense* involves six enzymes, which were examined in detail (Tal and Okon, 1985; Tal et al., 1990a,b). D-3-hydroxybutyrate dehydrogenase was purified and characterized (Tal et al., 1990a). However, the molecular mechanisms of PHB biosynthesis and degradation in *Azospirillum* and their regulatory systems are still unclear. Recently, the genes *phbA* (β-ketothiolase), *phbB* (acetoacetyl coenzyme A reductase) and *phbC* (PHB synthase), which encoded for enzymes of the PHB biosynthetic pathway in *Azospirillum brasilense* Sp7, were identified (Kadouri et al., 2002).

PHB degradation begins with the depolymerization of PHB to D-β-hydroxybutyrate monomers by PHB depolymerase. A NAD dependent D-β-hydroxybutyrate dehydrogenase performs the oxidation of D-β-hydroxybutyrate to acetoacetate, which can then be converted into acetoacetyl- CoA, by several systems. The transfer of CoA can proceed either directly to acetoacetyl-CoA synthetase or indirectly through succinyl-CoA, catalyzed by the enzyme succinyl-CoA transferase. The activated acetoacetyl-CoA is then hydrolyzed into two molecules of acetyl-CoA, which can then enter the TCA cycle (Aneja and Charles, 1999; Babel et al., 2001; Senior and Dawes, 1973).

In this study, two additional genes, *bdhA* (3-hydroxybutyrate dehydrogenase) and *acsA2* (acetoacetyl-CoA synthetase) from *Azospirillum brasilense* strain Sp7, were identified, cloned and sequenced. The expression of

the bacterial genes *phbA*, *phbC* and *acsA2*, was then studied *in vitro* using the GUS gene fusion system (Jefferson et al., 1986) in the wild type *A. brasilense* Sp7 and its *ntrB*, *ntrC* and *glnBglnZ* mutants. These mutants, which have been shown to be involved in the regulation of PHB synthesis (Sun et al., 2000, 2002), are impaired in the following nitrogen regulatory systems: *ntrB* and *ntrC* mutants are impaired in the Ntr system (Liang et al., 1993) and the mutant *glnBglnZ* is impaired in the P_{II}-P_Z system (De Zamaroczy, 1998). Also, the expression of *phbA*, *phbC* and *acsA2* was monitored during the association of *A. brasilense* with wheat roots.

2. Material and Methods

Bacterial strains

The bacterial strains and plasmids used in this work are listed in Table 1. *Escherichia coli* strains were grown at 37°C in Luria Bertani (LB) medium. A medium with high, normal or low carbon-to-nitrogen (C:N) ratio (Okon et al., 1977) was used for *A. brasilense* growth, at 30°C.

DNA manipulations

Subcloning, transformations and DNA extractions were performed according to standard methods (Sambrook et al., 1989). DNA was sequenced with an ABI Prism 377 DNA sequencer (Applied Biosystem Inc., Foster City, CA). Sequence data were analyzed with the University of Wisconsin Genetic Computer Group Software. Homology searches were performed using the Blast network service (Altschul et. al., 1997). Sequence alignments were done with the Clustal W program (Thompson et al., 1994) and edited using GeneDoc (K.B. Nicholas and J.B. Nicholas Jr., GeneDoc [http://www.cris.com/~Ketchup/genedoc.shtml]).

Oligonucleotide primers were synthesized (General Biotechnology, Rehovot, Israel) by the phosphoramidate method, using a Pharmacia 4-Primer Gene Assembler, according to the Codon Usage Database compiled from the codon usage tabulated from GenBank (Nakamura et al., 1998). Based on known *bdhA* sequences, two primers were designed to amplify the putative *bdhA* fragment from total DNA of *A. brasilense* Sp7: *bdhA* - R, 5'-CCAGCCGCCGTCCAT-3', and *bdhA* - F, 5'-TGGGGCCGCATCATCAACATC-3'. The primers anneal with *bdhA* of *Sinorhizobium meliloti* and *Rhodobacter* in positions 394 to 765 and 391 to 765, respectively. PCR was performed with an automated PCR thermoblock (Mastercycler gradient; Eppendorf, Netheler, Hamburg).

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

Strain or plasmid	Relevant characteristic(s)[a]	Reference or source
Strains		
Azospirillum spp.		
A. brasilense Sp7 (ATCC 29145)	Wild- type strain	Okon et al., 1977
A. brasilense *glnBglnZ* mutant	*glnB*::kan/*glnZ*::Ω $km^r Sp^r Sm^r$, *A. brasilense* Sp7 *glnBglnZ* double mutant	De Zamaroczy et al., 1996
A. brasilense ntrB mutant	*ntrB*::Tn5-194 Km^r; *A. brasilense* Sp7 *ntrB* mutant	Liang et al., 1993
A. brasilense ntrC mutant	*ntrC*::Tn5-148 Km^r; *A. brasilense* Sp7 *ntrC* mutant	Liang et al., 1993
Escherichia coli		
DH5α	*hsdR17 endA1 thi-1 relA1 recA1 supE44 ΔlacU169(φ80lacZΔM15)*	Sambrock et al., 1989
HB101	*F^-hsdS20 ($r^-_B m^-_B$) recA13 ara-14 proA2 lacY1 galK2 rpsL20 xyl-5 supE44*	Sambrock et al., 1989
S17.1	*pro thi endA recA hsdR* with RP4-2-Tc::*Mu*-Km::Tn7 integrated in chromosome, Sm^r	Simon et al., 1983
Plasmids		
pUC18	Ap^r, *Col*E1 replicon, *lacZ*, cloning vector	Yanisch-Perron et al., 1985
pLAFR3	Tc^r, pLAFR1 derivative containing *HaeII* fragment of pUC8	Staskavicz et al., 1987
pFAJ1701	Ap^r, Tc^r, MCS, *trfA*, *gusA*, *oriV* (Inc Pα)oriT, T:*trpA*	Dombrecht et al., 2000
pUC 7.6	Ap^r, pUC18, containing 10 Kb *SalI* *A. brasilense* Sp7 *bdhA*, *acsA2* fragment	This study
pP13S2	Ap^r, pUC18, containing 2 Kb *SalI* *A. brasilense* Sp7 *phbA* fragment	Kadouri et al., 2002
pP2EP5	Ap^r, pUC19, containing 5 Kb *Eco*RI-*Pst*I *A. brasilense* Sp7 *phbC* fragment	Kadouri et al., 2002
pUC control	Ap^r, pUC18, containing 400 bp *Bam*HI-*Xba*I *A. brasilense* Sp7 promotorless control fragment	This study
pGUS *phbA*	Ap^r, Tc^r, pFAJ1701 containing 532 bp *Bam*HI-*Xba*I *A. brasilense* Sp7 promotor area of *phbA* fragment	This study

Table 1. Continued.

Strain or plasmid	Relevant characteristic(s)[a]	Reference or source
pGUS *phbC*	Ap^r, Tc^r, pFAJ1701, containing 300 bp *Bam*HI-*Xba*I *A. brasilense* Sp7 promotor area of *phbC* fragment	This study
pGUS *acsA2*	Ap^r, Tc^r, pFAJ1701, containing 638 bp *Bam*HI-*Xba*I *A. brasilense* Sp7 promotor area of *acsA2* fragment	This study
pGUS control	Ap^r, Tc^r, pFAJ1701, containing 400 bp *Bam*HI-*Xba*I *A. brasilense* Sp7 promotorless area, control fragment	This study

[a]Abbreviations: Tc, tetracycline; Ap, ampicillin; Km, kanamycine.

Construction of A. brasilense phbA-, phbC- and acsA2-GUS fusions ntrB, ntrC and glnBglnZ mutants

*Bam*HI-*Xba*I fragments of 535, 324 and 648 bp, including the putative promoter regions (357 bp-*phbA*, 300 bp-*phbC* and 372 bp-*acsA2* upstream to the respective putative start codons) were cloned into pFAJ1701 (Dombrecht et al., 2000) yielding pGUS *phbA*, pGUS *phbC* and pGUS *acsA2*, respectively, bearing translational fusions with the promoter regions with the GUS gene. The resulting constructs were transformed into *E. coli* S17.1 and further mobilized by biparental mating into *A. brasilense* Sp7 and its mutant derivatives for *ntrB*, *ntrC* and *glnBglnZ*. *Azospirillum* transconjugants were selected on a minimal medium supplemented with the appropriate antibiotics. A transconjugant of *A. brasilense* Sp7 containing a 400-bp *Bam*HI-*Xba*I promotorless fragment was created as described above. This construct which is used as a control in GUS expression experiments was then transformed into *E. coli* S17.1 and mobilized into *A. brasilense* Sp7.

β-glucuronidase assays

In order to investigate if the differences in PHB accumulation observed in cultures grown under high and low C:N ratios correspond to changes in the gene expression, *phbA*, *phbC*, *acsA2-gusA* fusions were assayed in *A. brasilense* Sp7 in a wild type and in *ntrB*, *ntrC* and *glnBglnZ* mutant backgrounds. Overnight, 48 h cultures and a 48 h culture washed in saline and further incubated for 48 h (starvation culture) of *A. brasilense* transconjugants were centrifuged at 4,500

rpm for 8 min and resuspended in 0.085% saline solution. One ml of the suspension was used to inoculate tubes containing 4 ml of a high or low C:N ratio medium, which were then incubated for 4 hours at 37°C. β-glucuronidase activity was assayed spectrophotometrically using the GUS extraction buffer and the substrate p-nitrophenyl-β-D-glucuronide (PNPG) (Jefferson, 1987; Jefferson et al., 1986). β-glucuronidase activity is expressed as Miller units (Miller, 1972).

GUS expression in wheat seedlings. Wheat seeds (*Triticum aestivum* cv. Atir, (Hazera, Haifa) were surface sterilized by consecutive immersions in 10% commercial bleach for 30 sec and in 70% ethanol for 30 sec. The seeds were rinsed five times with sterile distilled water. The treated seeds were then germinated in the dark, at 25°C on sterile Petri dishes containing moist Whatman paper. Overnight cultures of the *A. brasilense* Sp7 transconjugants (approximately 10^8 bacteria per ml), were used to inoculate three day old seedlings. After 4 hr incubation on a rotary shaker (170 rpm) at 30°C, the seedlings were moved to a growth chamber for further incubation.

One day and two days post inoculation, seedlings were stained for 24 hr, at 37°C in 0.1 M phosphate buffer (pH=7.0) containing 0.5 mg/ml X-Gluc, 0.33 mg/ml $K_3(Fe(CN)_6$ and 0.42 mg/ml $K_4(Fe(CN)_6)$.

Nucleotide sequence accession number

The *A. brasilense* DNA sequences encoding for acetoacetyl-CoA synthetase (accession number AF447493), β-hydroxybutyrate dehydrogenase (AF355575), for a permease ABC transporter (AF508178, partial sequence) and for glutathione s-transferase (AF508179, partial sequence) were deposited in GenBank.

3. Results

Cloning and sequence analysis of *A. brasilense* Sp7. Two primers were designed based on *bdhA* sequences of *S. meliloti*. Using total DNA of *A. brasilense* Sp7 as the template in PCR, a 371-bp product, homologous to known *bdhA* genes, was obtained. Following sequencing, specific oligonucleotide primers (*bdhA* 580-F 5-GTGCAGAAGCAGATCCGAC-3, *bdhA* 726-R 5-ATCGGAGCACAGGAAAAC-3) were synthesized and used for PCR screening of an *E. coli* HB101 cosmid library containing partially *EcoR1*-restricted total DNA of *A. brasilense* Sp7 in PLAFR3. A 20 kb clone containing a DNA fragment including a region containing *bdhA* was isolated. This fragment was subcloned in pUC18, resulting in a 7.6 kb subclone. About 4.5 kb of the above subclone was sequenced by primer walking, and four open reading frames (ORFs) were

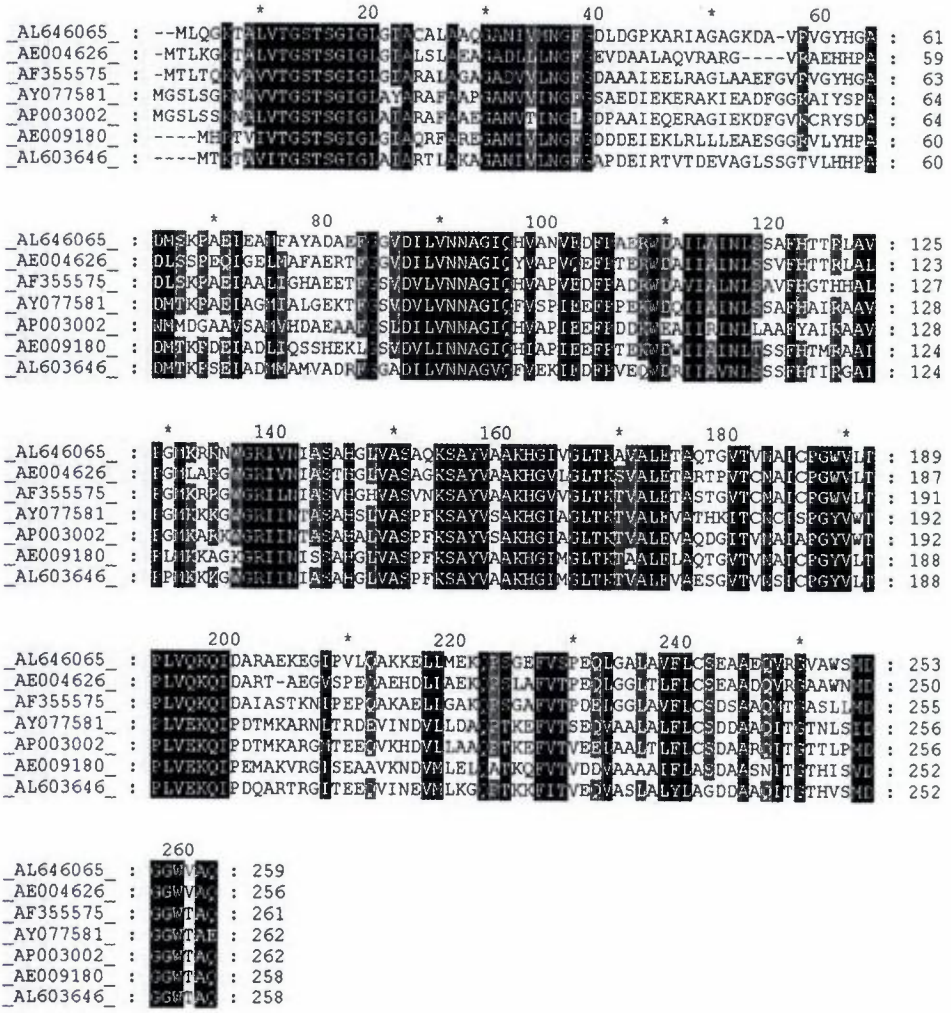

Figure 1. Multiple alignment of the deduced amino acid sequence of *bdhA* from *A. brasilense* (accession no. AF355575) with corresponding sequences of *bdhA* from *R. solanacearum* (accession no. AL646065), *P. aeruginosa* (accession no. AE004626), *B. japonicum* (accession no. AY077581), *M. loti* (accession no. AP003002), *A. tumefaciens* (accession no. AE009180), and *S. meliloti* (accession no. AL603646). Three levels of similarity are shown according to the default settings of GeneDoc.

detected. Homology analysis of the deduced amino acid sequence of the first ORF exhibited similarity with a permease ABC transporter protein. The second ORF showed high similarity with the AcsA2 proteins of *Mesorhizobium*

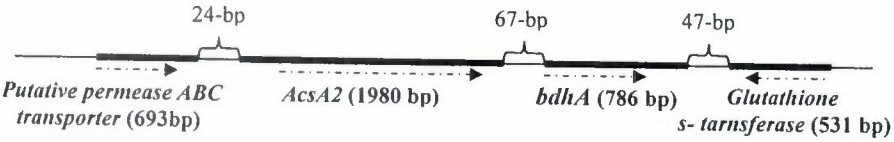

Figure 2. A physical map of a *A. brasilense* Sp7 putative permease ABC transporter (ORF 1), *acsA2*, *bdhA* and a putative glutathione s-transferase genes (ORF 4). Both ORF 1 and 4 are partial sequences. Arrows indicate the location and direction of transcription of the genes.

loti (GenBank accession no. AP003009; 62% identity, 76% similarity), *S. meliloti* (GenBank accession no. AL591784; 61% identity, 72% similarity), and *Agrobacterium tumefaciens* C58 (GenBank accession no. AE009343; 52% identity, 66% similarity). The third ORF showed high similarity with the *bdhA* proteins of *Ralstonia solanacearum* (GenBank accession no. AL646065; 68% identity, 78% similarity), *Pseudomonas aeruginosa* (GenBank accession no. AE004626; 67% identity, 79% similarity), *Bradyrhizobium japonicum* (GenBank accession no. AY077581; 57% identity, 73% similarity), *M. loti* (GenBank accession no. AP003002; 57% identity, 72% similarity), *A. tumefaciens* str. C58 (GenBank accession no. AE009180; 58% identity, 74% similarity), and *S. meliloti* (GenBank accession no. AL603646; 55% identity, 71% similarity). A multiple alignment of the deduced amino acid sequence of the *bdhA* from *A. brasilense* strain Sp7 with deduced amino acid sequences from homologous *bdhA* genes from other bacteria is shown in Fig. 1. A fourth ORF, which was not completely sequenced, showed high similarity with a glutathione s- transferase. Molecular analysis revealed that there is apparently only one copy of the *bdhA* gene in *A. brasilense* Sp7. First attempts to knock out the *bdhA* gene were unsuccessful.

A physical map of the putative permease ABC transporter (ORF 1), the *acsA2*, the *bdhA* and the partial glutathione s-transferase (ORF 4), was established (Fig. 2). The partial probable glutathione s-transferase (ORF 4) of *A. brasilense* is transcribed in the opposite direction to the other three genes. The putative permease ABC transporter (ORF 1) gene is 693-bp long, followed by the 1980-bp long *acsA2* and the 786-bp long *bdhA* genes.

Analysis of β-glucuronidase activity in A. brasilense phbA, phbC and acsA2

A small increase in *phbA*, *phbC* and *acsA2* promoter-driven GUS gene expression was detected in the exponential phase and in starved wild type cells of *A. brasilense* Sp7 cells under high C:N ratio conditions, as compared to low C:N conditions. A similar pattern was observed in the *glnBglnZ*, *ntrB* and *ntrC*

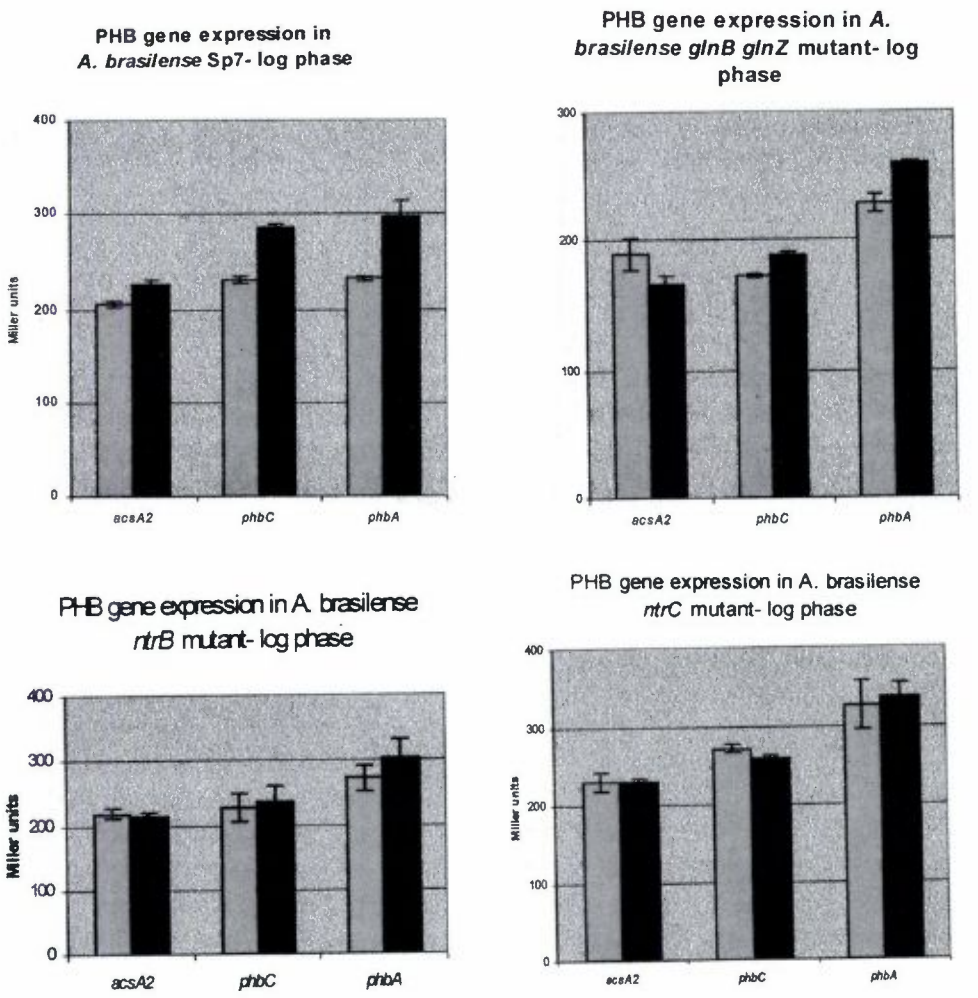

Figure 3. *phbA*, *phbC* and *acsA2* gene expression during exponential phase and under high (dark)or low (light) C:N ratio in *A. brasilense* Sp7 and in its mutants derivatives disrupted in the *glnBglnZ*, *ntrB* and *ntrC* genes. Each value represents the mean of two replicates from one experiment. Each experiment was performed four times.

background mutants but not in *acsA2-gus* fusions, the expression of which was slightly reduced in the log phase (Figs. 3 and 4). No such differences were observed in the stationary phase of in both the wild type and the mutant backgrounds (Fig. 5). No β-glucuronidase activity was seen in the negative control of *A. brasilense* Sp7 control-*gusA* fusion.

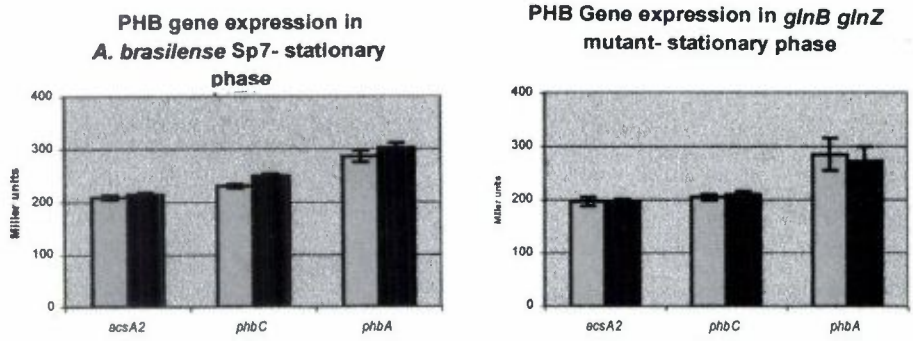

Figure 4. *phbA*, *phbC* and *acsA2* gene expression stationary phase after 48 h of growth and under high (dark) or low (light) C:N ratio in *A. brasilense* Sp7 and in its mutants derivatives disrupted in the *glnBglnZ* genes. Each value represents the mean of two replicates from one experiment. Each experiment was performed four times.

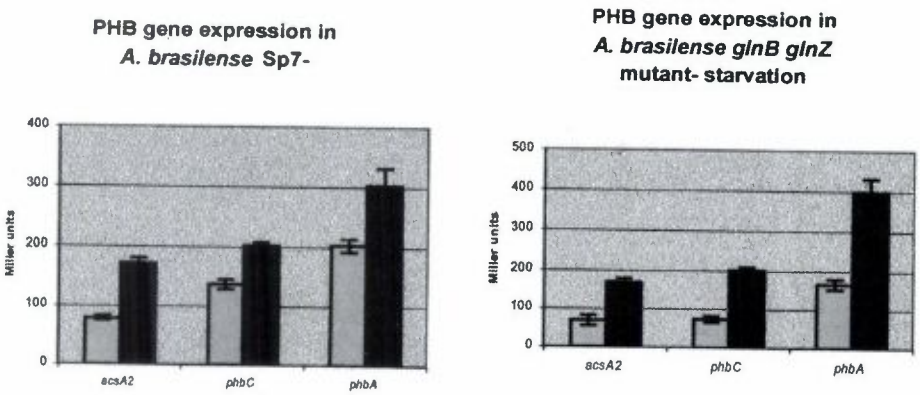

Figure 5. *phbA*, *phbC* and *acsA2* gene expression after starvation for 48 h under high (dark) or low (light) C:N ratio in *A. brasilense* Sp7 and in its mutants derivatives disrupted in the *glnBglnZ*, *ntrB* and *ntrC* genes. Each value represents the mean of two replicates from one experiment. Each experiment was performed four times.

Analysis of A. brasilense phbA, phbC and acsA2 expression on wheat roots

A. brasilense Sp7 carrying *phbA/phbC/acsA2-gusA* fusions were inoculated onto wheat seedlings and analyzed one day and two days post inoculation. After staining, root-colonizing bacteria were detected as blue zones on the root surface. Control, not inoculated roots remained white, confirming the lack of endogenous β-glucouronidase activity in wheat roots. A weak color was

observed one day post inoculation at sites of lateral root emergence and at root tips (data not shown). At two days post inoculation, a stronger expression of the GUS genes was observed, as detected by a stronger color. This was also seen in the elongation zone. Similar results were observed for all three constructs.

4. Discussion

In this study, two of the genes involved in PHB degradation were cloned and characterized. A PCR-based gene isolation strategy yielded a sequence exhibiting a high degree of similarity with D-β-hydroxybutyrate dehydrogenases from other bacteria at the amino acid level. Additional sequencing upstream and downstream of this gene (*bdhA*) yielded *acsA2*, another PHB degrading gene, coding for acetoacetyl CoA synthetase. A putative permease ABC transporter gene and a gene encoding glutathione s-transferase were also identified.

Although an acetoacetyl CoA succinate transferase (Tal et al., 1990b) may also exist, our findings suggest that the activation of the acetoacetate into acetoacetyl-CoA, may also proceed directly through the acetoacetyl-CoA synthetase pathway, as described for *Zoogloea ramigera* (Babel et al., 2001) and *Sinorhizobium meliloti* (Cai et al., 2000; Aneja et al., 2002).

In comparison to *A. brasilense acsA2 bdhA* cluster, in *Sinorhizobium meliloti* the enzyme 3-hydroxybutyrate dehydrogenase encoded by the gene *bdhA*, is organized in a *xdhA2*, *xdhB2* (xanthine dehydrogenase/oxidase) operon (Aneja and Charles, 1999). PHB depolymerase, encoded by *phbZ* which controls the breaking down of the polymer into its monomeric constituents is found in *A. brasilense* in another region of the chromosome (D. Kadouri, unpublished results).

The main factors influencing PHB accumulation in *Azospirillum* are partial oxygen pressure, and the ratio of carbon and nitrogen in the medium. PHB is formed at the end of the exponential phase and it is consumed during the stationary phase (Tal and Okon, 1985), as it can function as the sole carbon and energy source under starvation conditions (Tal and Okon, 1985; Anderson and Dawes, 1990). In order to examine the role of nutritional status and a possible interconnection of PHB and nitrogen metabolisms at the gene level, expression of *phbA-*, *phbC-* and *acsA2-gus* fusions was examined under high or low C:N ratio in the exponential and stationary phases, and under starvation conditions, in wild type and *ntrB*, *ntrC* and *glnBglnZ* mutant backgrounds.

Only relatively minor differences in gene expression levels were detected with the three *gus* constructs in all growth phases, under high and low C:N in the wild type and in the different mutant backgrounds. Although statistically significant, there is no regulatory meaning to these differences. Up or down

regulation of gene expression are expected to yield hundreds of percent differences in expression levels, as in *nifH-gus* fusions (Vande Broek et al., 1993). Therefore, it can be concluded that the expression of the *phbA*, *phbC* and *acsA2* genes is constitutive. Likewise, no relationship between growth phase and expression of *bdhA* to the growth phase associated expression of *S. meliloti bdhA* was reported by Aneja et al. (1999). Increases in PHB cell content under high C:N ratio can result from the regulation of the activity of a pool of existing enzymes. Although not energy efficient, this strategy allows the cells to react immediately to changes in the environment to proceed with the synthesis of PHB: a higher enzymatic activity was found in PHB rich cells formed under high C:N ratio (Tal et al., 1990b), and in *Ralstonia eutropha* a β-ketothiolase encoded by a *phaA* gene is inhibited by coenzyme A (Steinbuchel and Hein, 2001). The degradation of PHB may also be regulated at the protein level: in *A. brasilense* Sp7, the activity of the *bdhA* product encoding for a β-hydroxybutyrate dehydrogenase is dependent on the energetic state of the cell and on products of TCA cycle, such as NAD^+, and the enzyme is inhibited by NADH (Tal et al., 1990a). Babel et al. (2001) suggested that succinyl-CoA transferase and acetoacetyl-CoA synthetase, which catalyze the activation of acetoacetate to acetoacetyl-CoA, might be inhibited by ATP. Previous studies had shown that in *A. brasilense*, PHB synthesis is linked to the nitrogen level sensing pathways as mutants in *glnBglnZ*, *ntrB* or *ntrC* exhibit alterations in growth phase and C:N ratio regulation of PHB synthesis (Sun et al., 2000, 2002). Based on these and our results, it is suggested that still to be uncovered genes encode for functions linking the nitrogen status of the cell to the activity of enzymes involved in PHB synthesis and degradation.

A qualitative *in planta* expression analysis of *phbA*, *phbC* and *acsA2* was performed with the promoter regions of these genes fused to the GUS gene. With all three constructs, gene expression was detected, indicating that these genes are expressed during colonization. The pattern of colonization coincided with that previously observed with *nifH-gus* fusions (Vande Broek et al., 1993), i.e. the lateral roots in the emergence zone, the root tips and the root elongation zone were stained, with a stronger color obtained two days post inoculation, indicating apparently differences in bacterial cell proliferation that affect the population size rather than changes in gene expression.

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