Involvement of the Reserve Material Poly-β-Hydroxybutyrate in 
*Azospirillum brasilense* Stress Endurance and Root Colonization

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When grown under suboptimal conditions, rhizobacteria of the genus *Azospirillum* produce high levels of poly-β-hydroxybutyrate (PHB). *Azospirillum brasilense* strain Sp7 and a *phbC* (PHB synthase) mutant strain in which PHB production is impaired were evaluated for metabolic versatility, for the ability to endure various stress conditions, for survival in soil inoculants, and for the potential to promote plant growth. The carbon source utilization data were similar for the wild-type and mutant strains, but the generation time of the wild-type strain was shorter than that of the mutant strain with all carbon sources tested. The ability of the wild type to endure UV irradiation, heat, osmotic pressure, osmotic shock, and desiccation and to grow in the presence of hydrogen peroxide was greater than that of the mutant strain. The motility and cell aggregation of the mutant strain were greater than the motility and cell aggregation of the wild type. However, the wild type exhibited greater chemotactic responses towards attractants than the mutant strain exhibited. The wild-type strain exhibited better survival than the mutant strain in carrier materials used for soil inoculants, but no difference in the ability to promote plant growth was detected between the strains. In soil, the two strains colonized roots to the same extent. It appears that synthesis and utilization of PHB as a carbon and energy source by *A. brasilense* under stress conditions favor establishment of this bacterium and its survival in competitive environments. However, in *A. brasilense*, PHB production does not seem to provide an advantage in root colonization under the conditions tested.

A wide variety of microorganisms are known to produce intracellular energy and carbon storage compounds that generally are described as poly-β-hydroxybutyrate (PHB). It has been found that in most cases the polymers are polyhydroxyalkanoates (PHAs) comprising copolymers that contain different alkyl groups at the β position (41). The gram-negative nitrogen-fixing rhizobacterium *Azospirillum brasilense* lives in close association with plant roots, where it has beneficial effects on plant growth and the yields of many crops of agronomic importance (25, 26). Enzymes involved in the synthesis, accumulation, and degradation of PHAs in *A. brasilense* have been examined in detail (35–37). It has been shown that in contrast to other bacterial species, *A. brasilense* does not produce copolymers of hydroxyalkanoates; rather, it produces only homopolymers of PHB (15).

Although mutants unable to synthesize PHAs have been isolated and genetically engineered in several bacterial species (4, 13, 14, 27, 28, 33, 42), most PHA-negative mutants have been examined to determine their effects on symbiosis and on cellular metabolism (4, 27, 42). The ecological role played by PHA in bacteria under stress conditions has not been generally investigated. Understanding the role played by PHAs as intracellular storage polymers is of fundamental importance in microbiology. The accumulation, degradation, and utilization of PHAs by bacteria under stress conditions constitute a mechanism that may favor the establishment, proliferation, survival, and competition of the organisms, especially in competitive environments in which carbon and energy sources are limiting, such as those encountered in soil (24).

In previous work we isolated and sequenced the genes involved in PHB biosynthesis in *A. brasilense* strain Sp7. A *phbC* (PHB synthase) mutant strain in which PHB production was impaired was obtained and characterized (18). The ability of the wild-type strain to endure starvation conditions was greater than that of the mutant strain. However, extracellular polysaccharide production was greater in the *phbC* mutant strain than in the wild type (18).

To gain insight into the possible influence of PHB in the free-living state and to increase our understanding of the role played by PHB in survival and proliferation of *Azospirillum* spp., we conducted experiments in which *A. brasilense* strain Sp7 and the mutant strain were evaluated for metabolic versatility and for the ability to endure various environmental stress conditions. Additionally, we assessed the ability of the mutant strain to survive in soil inoculants and its ability to promote plant growth.

**MATERIALS AND METHODS**

*A. brasilense* strains and growth conditions. Wild-type strain Sp7 (38) and strain Sp7 *phbC::Km*, a Tn5 mutant of *Sp7* defective in PHB production (18), were maintained on nutrient agar (Difco) slants. To induce PHB accumulation, bacteria were grown in a medium with a high carbon-to-nitrogen (C/N) ratio (2). Kanamycin was added at a concentration of 25 μg/ml for the Tn5 mutant strain. Bacterial cultures were incubated on a rotary shaker (170 rpm) at 30°C.

Metabolic versatility. Metabolic versatility experiments were performed by using BIOLOG GN plates (Biolog, Hayward, Calif.). *A. brasilense* cultures were successively streaked three times on tryptic soy agar plates (Difco). Twenty-four-
hour colonies were suspended in saline (9 g/liter of NaCl) to a final optical density at 540 nm of 0.1. Suspended bacteria were used for plate inoculation and incubated for 24 h at 30°C. Utilization or the lack of utilization of each carbon source was verified visually and with an MR-5000 microplate reader (Dynatech, Brussel, Belgium) at 590 nm.

Ability to grow on different carbon sources. The carbon sources used were malic acid, succinate, lactose, pyruvate, l- (+) -arabinose, d- (+) -galactose, l- (+) -fructose, and L-glutamic acid, each at a final concentration of 0.037 M. To limit the extent of aggregation during growth, a low-C/N-ratio medium was used, which contained 0.963 g of NH₄Cl per liter (2). All other components were the same as those in the high-C/N-ratio medium. Fifty milliliters of medium in a 125-ml flask was inoculated with 1 ml of an overnight culture of A. brasilense at an optical density at 540 nm of 0.1. Growth was measured with a Genesis 5 spectrophotometer (Spectronic-Unicam, Rochester, N.Y.).

Stress endurance. In all experiments described below, 2-ml aliquots of over-night cultures of A. brasilense were used to inoculate flasks containing 25 ml of high-C/N-ratio medium and grown for 24 h. The number of viable cells (CFU per milliliter) was determined by dilution plating before each experiment and at the end of each experiment. For each experiment the same initial number of cells (wild-type strain Sp7 and mutant strain) was used (between 5 x 10⁶ and 7 x 10⁷ cells/ml). In heat resistance experiments, 10 ml of cells was resuspended in a 1.5-liter tube and incubated in a water bath at 50°C for 70 min. Bacterial viability was determined every 10 min. Resistance to UV irradiation was tested by placing 20 ml of cells in a 90-mm plastic petri dish and exposing them to short-wave UV irradiation (254 nm) from a VL-6LC UV lamp (Vilber-Lourmat, Cedex, France) for 60 s. Bacterial viability was determined every 5 to 10 s. Survival of cells under desiccation conditions was tested by placing 100 µl of bacteria on coverslips (18 by 18 mm). The coverslips were air dried in a Gelman class-100 laminar flow apparatus (Gelman, Milan, Italy) with a constant airflow of 0.45 m/s for 60 min. The cells were resuspended in 10 ml of potassium phosphate buffer (0.06 M, pH 6.8) and plated. The sensitivity of both strains to osmotic pressure was evaluated by adding 25-ml portions of 2 and 4 M glucose solutions to 25-ml portions of bacterial suspensions, which yielded final glucose concentrations of 1 and 2 M, respectively. The bacteria were incubated at 30°C for 24 h. Sensitivity to osmotic shock was estimated by adding 25 ml of a Tris-glyceral solution (0.05 M Tris, 4 M glyceral; pH 7.6) to 25 ml of a cell suspension and incubating the preparation for 30 min at 30°C. The cells were then centrifuged (4,000 x g, 10 min) and resuspended in 50 ml of distilled sterilized H₂O₂. Bacterial viability was determined as described above.

Sensitivity of A. brasilense to hydrogen peroxide and antibiotics. One-hundred-microliter portions of cells were evenly spread on high-C/N-ratio solid medium in 90-mm petri dishes. Aliquots (25 µl) of a 1.5% solution of H₂O₂ were placed on 13-mm Whatman discs and left to air dry for 15 min. A single disc was placed in each dish. The dishes were incubated at 30°C for 24 h. The size of the halo around each disc was considered a measure of the sensitivity of the strain. Likewise, 6-mm Whatman discs containing the following antibiotics were placed on individuals plates: gentamicin (25 µg/ml), chloramphenicol (50 µg/ml), ampicillin (100 µg/ml), tetracycline (10 µg/ml), sulfafurazol (100 µg/ml), nalidixic acid (50 mg/ml), and gentian violet (1%, wt/vol). The sensitivity of each strain was determined every 10 min. Resistance to UV irradiation was tested by placing 100 µl of bacteria on coverslips (18 by 18 mm). The coverslips were air dried in a Gelman class-100 laminar flow apparatus (Gelman, Milan, Italy) with a constant airflow of 0.45 m/s for 60 min. The cells were resuspended in 10 ml of potassium phosphate buffer (0.06 M, pH 6.8) and plated. The sensitivity of both strains to osmotic pressure was evaluated by adding 25-ml portions of 2 and 4 M glucose solutions to 25-ml portions of bacterial suspensions, which yielded final glucose concentrations of 1 and 2 M, respectively. The bacteria were incubated at 30°C for 24 h. Sensitivity to osmotic shock was estimated by adding 25 ml of a Tris-glyceral solution (0.05 M Tris, 4 M glyceral; pH 7.6) to 25 ml of a cell suspension and incubating the preparation for 30 min at 30°C. The cells were then centrifuged (4,000 x g, 10 min) and resuspended in 50 ml of distilled sterilized H₂O₂. Bacterial viability was determined as described above.

Results

Surface-sterilized seeds of wheat (cv. Atir) were placed in pots containing sterile, washed sianic sand. Three days after germination 1-ml portions of overnight cultures of wild-type strain S7 or the phbC mutant strain (3 x 10⁶ cell/ml) were used to inoculate the soil of each plant. Potassium phosphate buffer (0.06 M, pH 6.8) was applied as a control. The plants were grown in a greenhouse at 25°C with a 15-h light period and were watered every 3 days with 5 ml of tap water. Wheat and corn were grown for 14 and 33 days after inoculation, respectively. The experiments were repeated twice, with eight replicates for each treatment.

Root colonization by wild-type and PHB-defective mutant strains. Surface-sterilized seeds of wheat (cv. Atir) were placed in pots containing sterile, washed sianic sand. Three days after germination 1-ml portions of overnight cultures of wild-type strain S7 or the phbC mutant strain (3 x 10⁶ cell/ml) were used to inoculate the soil of each plant. Potassium phosphate buffer (0.06 M, pH 6.8) was applied as a control. The plants were grown in a greenhouse at 25°C with a 15-h light period and were watered every 3 days with 5 ml of tap water. Wheat and corn were grown for 14 and 33 days after inoculation, respectively. The experiments were repeated twice, with eight replicates for each treatment.

IAA production. A. brasilense cells were grown for 24 h (log phase) or 48 h (stationary growth phase) in a high-C/N-ratio medium. Tryptophan (0.1 mM) was added in order to enhance indoleacetic acid (IAA) production by the bacteria (29). Production of IAA in culture supernatant was assayed by the Pilet-Chollet method, as described by Dobbeleare et al. (7). This method was shown to be more sensitive and more specific than the Salkowski-based colorimetric technique (10). For the reaction, 1 ml of reagent R1, consisting of 12 g of FeCl₃ per liter in 7.9 M H₂SO₄, was added to 1 ml of a sample supernatant, mixed well, and left in the dark for 30 min at room temperature. Absorbance was measured at 530 nm.

Statistics. All experiments were performed at least three times (unless indicated otherwise) and were highly reproducible. Therefore, data from one replicate of each experiment are presented below. Data were subjected to t test analysis or one-way analysis of variance followed by a post hoc test, as indicated below for each experiment.

Results

Metabolic versatility and ability to grow on different carbon sources. No apparent differences in utilization of an array of carbon sources in BIOLOG GN plates by A. brasilense wild-type strain S7 and by a phbC-deficient mutant strain were detected. However, formation of a more intense color was observed in the wells of the plates inoculated with the wild-type
In batch cultures with the carbon sources tested, the generation times of the wild-type strain were always shorter than those of the PHB-deficient mutant. Under these conditions the generation times of the wild type and the mutant were as follows: with malic acid, 6.37 and 11.24 h, respectively; with succinate, 5.14 and 6.37 h, respectively; with pyruvate, 3.01 and 3.52 h, respectively; with d-(-) fructose, 5.85 and 7.81 h, respectively; and with L-glutamic acid, 9.26 and 11.7 h, respectively.

**Stress endurance.** Exposure of both wild-type and mutant strains to stress conditions revealed that the wild-type strain was more resistant than the PHB-deficient mutant counterpart, as measured by viable counting.

When bacteria were incubated at 50°C in a water bath, the *A. brasilense* phbC mutant died rapidly. After 70 min of exposure, only 0.8% of the initial number of bacteria remained alive, compared to 36.3% for wild-type strain Sp7 (Fig. 1A). Irradiated Sp7 wild-type cells exhibited greater tolerance to UV than mutant cells exhibited; 19.7 and 2% of the wild-type and mutant cells remained viable after 60 s of exposure, respectively (Fig. 1B).

*A. brasilense* wild-type strain Sp7 had a higher survival rate following a glycerol-induced osmotic shock. Also, the viability of the mutant strain was especially affected in solutions with a high concentration of glucose. Mutant cells were also very sensitive to desiccation, and their survival rate was more than sixfold less than the survival rate of the wild-type strain (Table 1).

When grown in the presence of hydrogen peroxide, the mutant strain exhibited considerably greater sensitivity to this oxidizing agent than the wild-type strain exhibited; the halos around the impregnated discs were 6.8 cm and about 4.8 cm for the mutant and the wild type, respectively (Fig. 2). No significant differences in sensitivity to the antibiotics tested were detected between wild-type strain Sp7 and the mutant strain (data not shown).

Mutation of the PHB synthase gene also resulted in phenotypic alteration of the aggregation properties of *A. brasilense*, and the extent of aggregation of the mutant strain was threefold greater than that of the wild-type strain after 48 h of growth in a high-C/N-ratio medium (Table 2).

Analysis of cell motility by phase-contrast microscopy revealed that the mutant strain swam significantly faster than its wild-type counterpart when both organisms were grown in a high-C/N-ratio medium (Table 2). In chemotaxis experiments with fructose, sodium malate (data not shown), and seeds of sweet corn as attractants, a turbid band composed of bacterial cells was apparent 24 h after inoculation with the wild-type strain, perhaps indicating that there was more efficient growth (data not shown). Glycogen, inulin, D-arabitol, 3-methyl glucose, α-methyl-D-glucoside, β-methyl-D-glucoside, D-psicose, stachyose, acetic acid, β-hydroxybutyric acid, α-ketovaleric acid, D-malic acid, L-malic acid, lactamide, methyl pyruvate, propionic acid, succinamic acid, L-alanine, L-glutamic acid, glycyl-L-glutamic acid, glycerol, and fructose 6-phosphate were utilized by both strains.

**TABLE 1. Resistance of *A. brasilense* wild-type strain Sp7 and a phbC mutant to osmotic pressure and desiccation**

| Strain          | Osmotic pressure | Osmotic shock | Desiccation |
|-----------------|------------------|---------------|-------------|
|                 | Glucose (1 M)         | Glucose (2 M) |             |
| Wild-type Sp7   | (2.5 ± 0.4) × 10⁸ A | 10 ± 0.54) × 10⁶ A | (7.8 ± 0.6) × 10⁶ A | (2.85 ± 1.2) × 10⁶ A |
| phbC mutant     | (1.5 ± 0.1) × 10⁸ A | 14 ± 0.8) × 10⁵ B | (1.8 ± 1.2) × 10⁵ B | (4 ± 2.7) × 10⁵ B |

* a The initial number of cells for each treatment was 6 × 10⁹ cells. The experiment was performed three times, and similar results were obtained each time. The values are the means ± standard errors for three replicates per treatment. Within a column different letters after values indicate that there is a significant difference at a P value of 0.05, as determined by a t test.
strain. No band was apparent after inoculation of the mutant, demonstrating that it exhibited reduced chemotaxis towards the attractants compared to the chemotaxis of the wild-type strain (Fig. 3).

**Survival in inoculant carriers.** Seven days after inoculation and after incubation under suboptimal conditions, survival of the wild-type and mutant strains varied considerably when the results obtained with the different carriers were compared; peat sustained the largest populations of inoculated bacteria, and perlite sustained the smallest. In all cases, the wild-type strain survived better than the mutant survived, but the differences between the carriers were very large; while the mutation in the \( \text{phbC} \) gene resulted in a 30% reduction in survival in peat, it resulted in 3-fold and almost 30-fold reductions in survival in sand and perlite, respectively (Table 3).

**Phytostimulatory effects of wild-type strain Sp7 and the \( \text{phbC} \) mutant strain.** The plant growth promotion results after plants were inoculated with \( A. \text{brasiliense} \) were similar with the wild-type and mutant strains, both under sterile conditions in petri dishes and in soil in the greenhouse. The root length was significantly increased after 72 h for sterile plants that were inoculated in vitro (6.3 ± 0.5, 5.3 ± 0.5, and 3.1 ± 0.4 cm for the wild-type and mutant strains and the control, respectively). No significant differences in root length, root dry weight, leaf length, and leaf dry weight were observed for plants inoculated with the two strains and grown in the greenhouse, while growth promotion effects were still detectable (Table 4).

**Root colonization by the wild-type strain and the PHB-defective mutant strain.** No significant differences in root colonization were observed when plants inoculated with \( A. \text{brasiliense} \) were similar with the wild-type and mutant strains, both under sterile conditions in petri dishes and in soil in the greenhouse. The root length was significantly increased after 72 h for sterile plants that were inoculated in vitro (6.3 ± 0.5, 5.3 ± 0.5, and 3.1 ± 0.4 cm for the wild-type and mutant strains and the control, respectively). No significant differences in root length, root dry weight, leaf length, and leaf dry weight were observed for plants inoculated with the two strains and grown in the greenhouse, while growth promotion effects were still detectable (Table 4).

**IAA production.** When tryptophan was added to the growth medium, after 48 h a higher level of IAA was detected in the \( \text{phbC} \) mutant than in the wild-type strain (8.3 and 5.1 mg of IAA/g [dry weight] of bacteria, respectively). No significant differences (\( P = 0.05 \)) in the amounts of IAA produced were detected after 24 h of growth (6.5 mg of IAA/g [dry weight] of bacteria). No IAA production was detected by the method used when tryptophan was not added to the growth medium.

**DISCUSSION**

In this study, we conducted a series of experiments aimed at assessing the significance of the reserve material PHB for the survival of \( A. \text{brasiliense} \) subjected to various physical and chemical stresses under controlled conditions and in inoculant carriers and on the ability of the bacterium to promote plant growth.

\( A. \text{brasiliense} \) is very versatile in terms of its ability to utilize organic compounds as carbon and energy sources (11). Although there was no apparent difference in the carbon sources utilized by wild-type strain Sp7 and the \( \text{phbC} \) mutant strain.
(18), it was evident that the wild type utilized carbon sources faster and to a greater extent, as reflected by shorter generation times. Similar findings were reported by Cevallos et al. (4), who observed that the mutation in the phbC gene severely impaired the ability of Rhizobium etli to grow in minimal media supplemented with glucose or pyruvate. Although it has been suggested that carbon is not a limiting factor for microorganisms in the rhizosphere (5), the ability of soil inhabitants to grow quickly on available compounds in the rhizosphere probably affects their capacity for root colonization (17). It has been hypothesized that an important factor influencing rhizosphere competence is the growth rate of the bacteria. A correlation between rhizosphere competence and growth rate was shown previously for Pseudomonas fluorescens (34). As the C/N ratio in the rhizosphere favors PHB accumulation in Azospirillum (24) and the phbC mutant used in this study exhibited decreased growth rates on the carbon sources tested, we assume that accumulation and utilization of PHB may enhance the competitiveness of A. brasilense in the environment.

The ability of A. brasilense to tolerate various stresses was significantly affected by the lack of PHB accumulation, which resulted in lower stress endurance, in accordance with the findings of Tal and Okon (35). Also, a wild-type strain of Pseudomonas oleovorans survived ethanol and heat challenges better than a PHA depolymerase-deficient strain (31). Early work on Ralstonia eutropha suggested that PHA utilization is associated with respiration and oxidative phosphorylation (12). In a recent study performed by Ruiz et al. (31), an increase in guanosine tetraphosphate (ppGpp) levels appeared to occur concomitantly with PHA degradation. This phenomenon was observed only in wild-type P. oleovorans and not in a PHA depolymerase-deficient strain unable to degrade the polymer. There is strong evidence that ppGpp induces expression of the rpoS gene (9). This gene encodes a transcription factor that activates the expression of genes involved in protection against damaging agents, such as ethanol, H$_2$O$_2$, high temperature, or high salt concentration (30). It may well be that the same cascade of events is involved in A. brasilense PHB-containing cells exposed to stress. In order to further associate the significant role of a functioning PHB cycle with stress endurance, we isolated and sequenced an intercellular PHB depolymerase (phbZ) gene from A. brasilense strain Sp7 (an essential gene in PHB degradation), and an A. brasilense phbZ mutant was obtained (Kadouri, unpublished data). Although in this mutant PHB is produced at high levels, it cannot be utilized by the bacteria. This mutant exhibited the same phenotypic features as the phbC mutant. The ability of the phbZ mutant to endure starvation conditions, UV irradiation, heat, and osmotic shock and its ability to grow in the presence of hydrogen peroxide were significantly lower than those of the wild type. Thus, as the two mutants are altered similarly, the phenotype of the phbC mutant can be attributed to a defective PHB cycle.

It was previously observed that the motility exhibited by the mutant strain was greater than that of the wild type (18). Here we show that the mutant swims two to three times faster than the wild-type strain. However, under the conditions tested (carbon-free medium) the increased motility of the phbC mutant did not result in an elevated chemotactic response when the organism was placed in the presence of attractants, probably because of the absence of PHB as an intracellular energy storage compound that could be utilized by the bacteria for motility towards an attractant during starvation or because of pleiotropic effects of the mutation on the chemotactic response.

An increase in root binding in short-time root adhesion assays attributed to elevated production of exopolysaccharide and capsular polysaccharides was observed in the A. brasilense phbC mutant strain compared to the root binding of the wild type (18). Differences in the exopolysaccharide and capsular polysaccharide contents between wild-type and mutant strains can also explain the ability to aggregate displayed by the latter strain in this study. Azospirilla are known for their capacity to aggregate and flocculate under diverse stress conditions, and

| Strain | Bacterial viability (%) |
|--------|-------------------------|
|        | Sianic sand | Peat | Perlite |
| Wild-type Sp7 | 66 ± 5.5 A | 87 ± 5.3 A | 17.2 ± 3.1 A |
| phbC mutant | 21 ± 3.4 B | 63 ± 7.3 B | 0.6 ± 0.3 B |

*The values are the percentages of viable cells present after treatment. The initial number of cells for each treatment was 3 x 10^6 cells. The values are means ± standard errors for three replicates from one representative experiment. Each experiment was carried out three to five times, and similar results were obtained each time. Within a column different letters after values indicate that there is a significant difference at a P value of 0.05, as determined by a t test.
previous studies have shown that extracellular polysaccharides are involved in cell aggregation (1, 3, 21, 32). Although the mutant strain’s ability to adhere to roots is greater than that of the wild type, no difference in root colonization was observed between the two strains. In A. brasilense, two different modes of attachment to roots are known. One involves an adhesion process in which binding to the root is relatively weak and reaches a maximal level within 2 h. The second step, beginning after 8 h of incubation and reaching a maximal level after 16 h, is root colonization (22). Scanning electron micrographs of roots inoculated with Azospirillum have revealed bacterial cells embedded in a fibrillar matrix on root surface. It is not yet clear whether this matrix consists of bacterial polysaccharide or root mucigel (39).

Dobbelaere et al. (7) showed that IAA produced by A. brasilense is important for stimulating root development. The pHbC mutant strain produced more IAA than the wild type produced in tryptophan-amended medium, and tryptophan can be detected in the rhizosphere (16).

In this study, root colonization and growth promotion effects were similar in plants inoculated with the pHbC mutant and plants inoculated with the wild-type strain, and similar levels of colonization may explain similar levels of growth promotion. Although more polysaccharides and IAA were produced in the mutant strain, no differences in colonization or growth promotion were seen. At this stage, we do not know if increased production occurs in the rhizosphere as well. On the other hand, stresses expected in the rhizosphere (hydric stress, osmotic changes) were apparently minimized under the conditions in which the plant experiments were performed (optimal temperature for plant growth, high soil moisture), thus reducing the relevance of PHB production for survival. Additionally, the relatively high concentration of bacteria used for inoculation may also have limited competition with other microorganisms within the soil, thus allowing better establishment of both strains. It remains to be determined whether under inoculation and growth conditions found in the field the impaired stress resistance and reduced growth rate of the pHbC mutant are translated into reduced colonization and growth promotion, although this strain has some beneficial features compared with the wild type.

The fact that PHB-accumulating cells exhibit increased stress endurance is of great importance for commercial bacterioinoculants. Stress endurance varies according to the inoculant preparation methods and the storage conditions. The survival of A. brasilense in carriers was significantly reduced after a 6-month storage period (8), probably due to stresses that developed during storage under suboptimal conditions, such as a lack of moisture, heat stress, and a limited available carbon source. The role of PHB as an intracellular energy and carbon storage compound which can enhance survival during starvation periods has been examined in various bacteria. For Bacillus megaterium and R. eutropha the survival of wild-type strains was greater than that of PHB-negative mutants (19).

Starvation experiments with A. brasilense showed that there was a clear decrease in the ability of a pHbC mutant to survive compared to the ability of wild-type strain Sp7 to survive (18). In field experiments carried out in Mexico with maize and wheat, better and more consistent results were obtained when plant inoculants prepared with PHB-rich Azospirillum cells were used (6, 8). In our work we found that when inoculants were stored under suboptimal conditions, there was a clear decrease in the ability of the pHbC mutant to survive compared to the ability of wild-type strain Sp7 to survive. It can be concluded that production of PHB is critically important for improving the shelf life, efficiency, and reliability of commercial inoculants.

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