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Involvement of *glnB*, *glnZ*, and *glnD* Genes in the Regulation of Poly-3-Hydroxybutyrate Biosynthesis by Ammonia in *Azospirillum brasilense* Sp7

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The role of three key nitrogen regulatory genes, *glnB* (encoding the P_{II} protein), *glnZ* (encoding the P_z protein), and *glnD* (encoding the GlnD protein), in regulation of poly-3-hydroxybutyrate (PHB) biosynthesis by ammonia in *Azospirillum brasilense* Sp7 was investigated. It was observed that *glnB glnZ* and *glnD* mutants produce substantially higher amounts of PHB than the wild type produces during the active growth phase. *glnB* and *glnZ* mutants have PHB production phenotypes similar to that of the wild type. Our results indicate that the P_{II}-P_z system is apparently involved in nitrogen-dependent regulation of PHB biosynthesis in *A. brasilense* Sp7.

A wide variety of bacteria can produce a thermoplastic poly-3-hydroxybutyrate (PHB) as an energy and carbon storage compound under unbalanced nutrient conditions (1, 11). Because of the biodegradability and biocompatibility of PHB and because PHB has a wide range of applications, the biochemistry of PHB (16, 17, 18, 19, 21), its uses in genetic and metabolic engineering (9, 15, 20, 22, 26, 35), and its application in tissue engineering (23, 24, 25, 36) and material engineering (13) have been intensively studied.

In most PHB-producing bacteria, production of only a little PHB is observed during the active growth phase of cells. Therefore, a long time is needed for bacteria to reach a high-density non-PHB cell biomass before accumulation of large amounts of PHB can occur (1, 11). Nutrient limitation is necessary to trigger PHB accumulation, and generally ammonia is used as the critical control factor for uncoupling the growth of cells and PHB production. However, some bacteria, such as *Azotobacter vinelandii* UWD (14), *Alcaligenes latus* (4, 34), and *Pseudomonas putida* KT2442 (7), are able to accumulate a large amount of PHB or polyhydroxyalkanoates during exponential growth, but the genetic reasons for this are not well defined.

The PHB-producing abilities of *Azospirillum*, a genus of free-living nitrogen-fixing bacteria, have been studied previously. Some species, such as *Azospirillum brasilense* and *Azospirillum lipoferum*, can accumulate high levels of PHB (up to 88% of the cell dry biomass) under unbalanced nutrient conditions, such as oxygen limitation or a high C/N ratio (8, 29). However, it was observed in a previous study that *ntrB* and *ntrC* mutants of *A. brasilense* Sp7 can grow and produce PHB simultaneously even when a large amount of ammonia is present in the medium, indicating that *ntrB* and *ntrC* are involved in regulation of PHB biosynthesis by ammonia in *A. brasilense* Sp7 (28). Because inactivation of inhibition of PHB production by ammonia has industrial potential for improving process control and productivity (10), further studies were performed to investigate the involvement of other nitrogen regulatory genes in controlling PHB production in *A. brasilense* Sp7.

The P_{II} and P_z proteins, encoded by the *glnB* and *glnZ* genes, respectively, have similar structures but perform different functions (3). These two proteins are involved in sensing the intracellular nitrogen status (2). They occur in two forms, the native form when there is excess nitrogen and the uridylylated form under nitrogen-limiting conditions (2). Uridylylation and deuridylation of the P_{II} and P_z proteins are catalyzed by another nitrogen-sensing protein, GlnD, which functions as uridylyltransferase and uridylyl-removing enzyme and is encoded by the *glnD* gene (31, 32).

In this study, *glnB*, *glnZ*, *glnB glnZ*, and *glnD* mutants were examined to determine PHB production under low-C/N-ratio conditions. The bacterial stains used in this study are listed in Table 1. All of the strains were routinely grown in MMAB medium (33) at 30°C. Kanamycin (25 μg/ml), tetracycline (10 μg/ml), spectinomycin (50 μg/ml), and streptomycin (100 μg/ml) were added to the medium when required. Batch fermentation was performed in a 2-liter pH-stat and O_2-stat fermentor as described previously (12). The concentration of dissolved

TABLE 1. Bacterial strains used in this study

Strain	Relevant characteristic(s)	Reference
Sp7 (= ATCC 29145)	Wild type	30
7628	*glnB*::*kan* Km^r, *A. brasilense* Sp7 *glnB* mutant	3
7611	*glnZ*::Ω Sp^r Sm^r, *A. brasilense* Sp7 *glnZ* mutant	2
2812	*glnB*::*kan*/*glnZ*::Ω Km^r Sp^r Sm^r, *A. brasilense* Sp7 *glnB glnZ* double mutant	2
FAJ311	*glnD*::Tn5-B30 Tc^r, *A. brasilense* Sp7 *glnD* mutant	32

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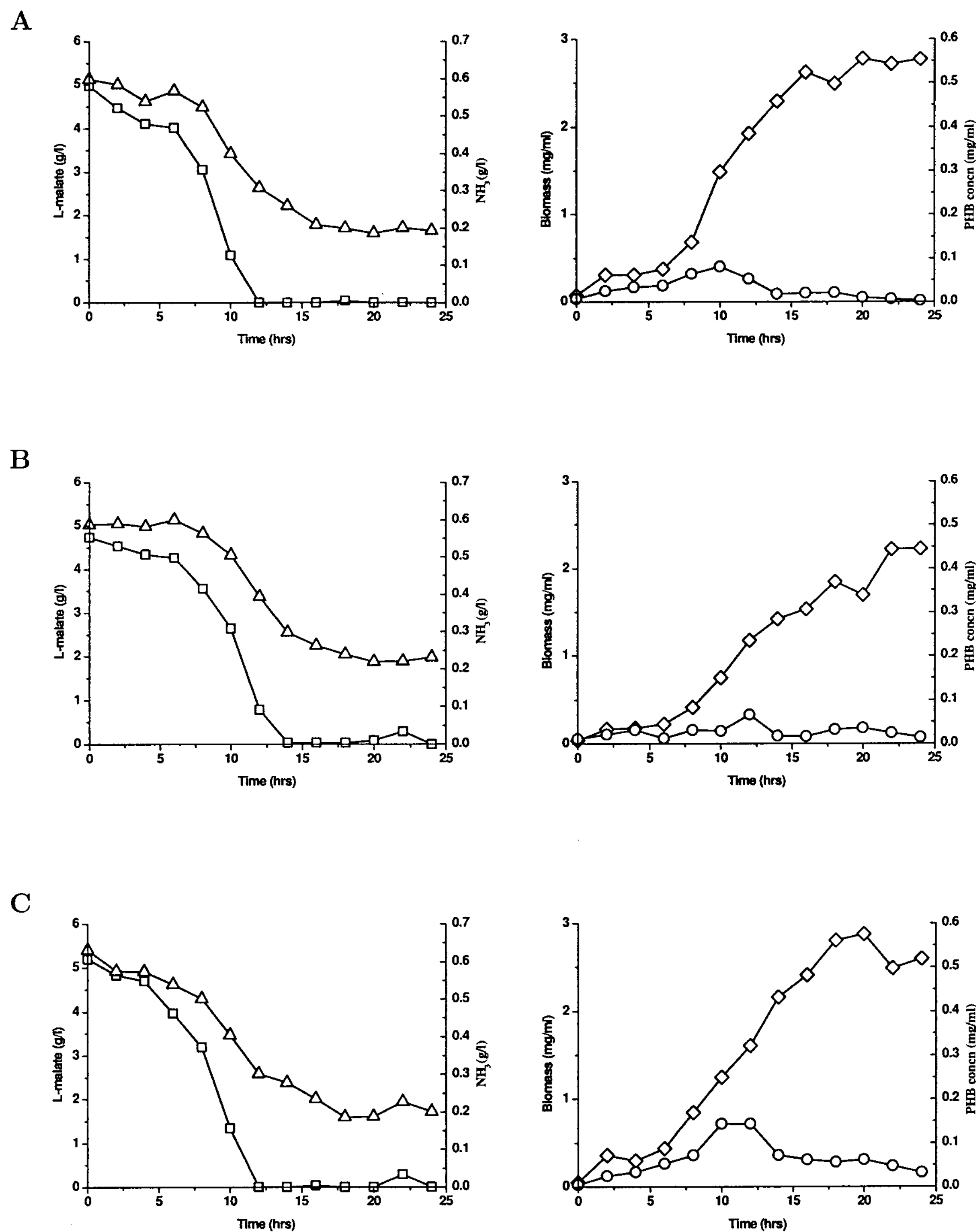

FIG. 1. Time course of fermentation in *A. brasilense* Sp7 (A) and its *glnB* (B), *glnZ* (C), *glnB glnZ* (D), and *glnD* (E) mutants. Symbols: □, L-malate concentration; △, ammonia concentration; ◇, biomass concentration; ○, PHB concentration. The data in each panel are the data from one of the reproducible independent experiments performed. All the data are averages based on at least two replicates.

oxygen (DO_2) was kept constant by varying the airflow into the fermentor based on the measured DO_2 level, so the airflow rate could be used as an indicator of the oxygen uptake rate (12).

All of the analytical procedures used to determine cell growth, biomass, and PHB concentration were performed as described previously (28). All the data below are averages based on at least two replicates.

To determine the involvement of *glnB*, *glnZ*, and *glnD* in regulation of PHB production by ammonia in *A. brasilense* Sp7,

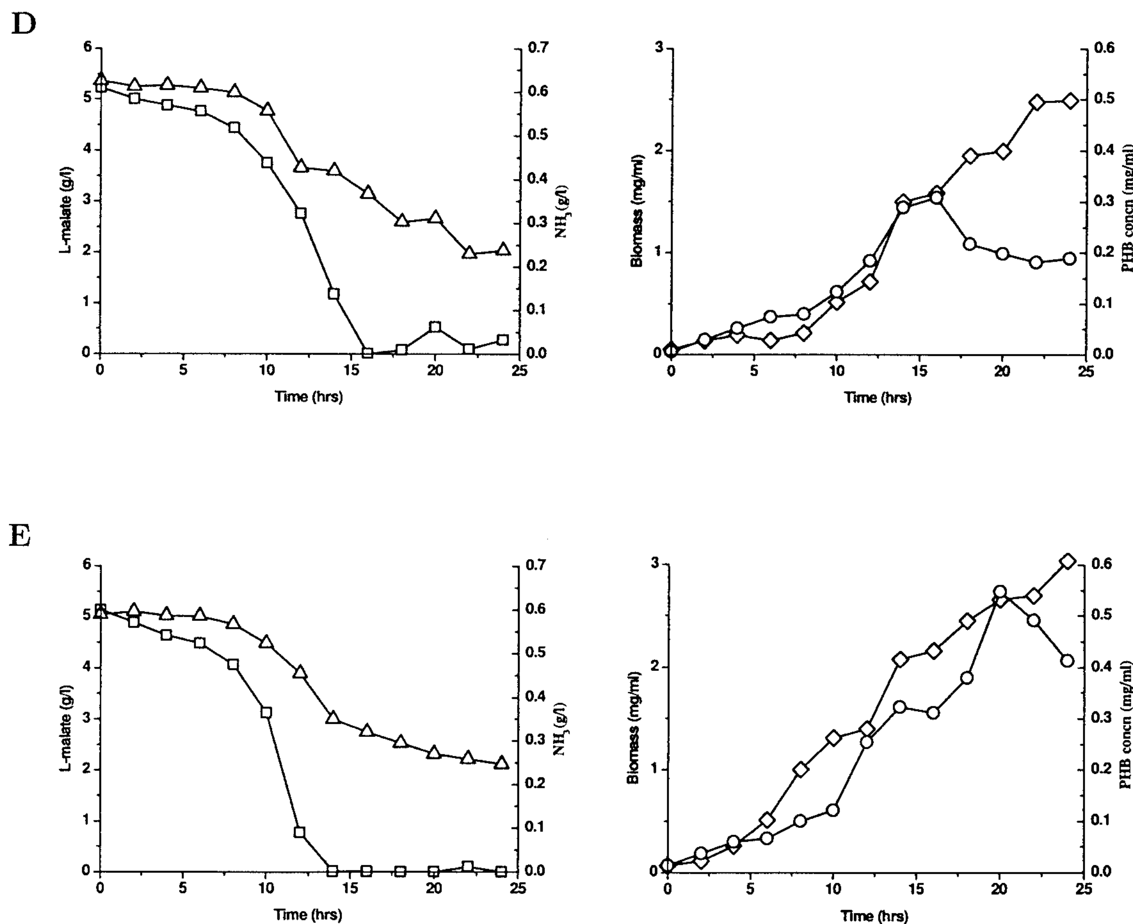

FIG. 1—*Continued.*

the wild-type and mutant strains were grown in a fermentor in order to precisely monitor and control the culture conditions. MMAB medium was supplemented with 10 g of malate per liter and 2 g of NH_4Cl per liter (initial C/N ratio, 6.8), and the DO_2 concentration was kept at 30%, which has been reported to be the optimal value for PHB production in *A. brasilense* (29). Under these culture conditions, no nitrogen fixation can occur because the nitrogen fixation process is repressed by the presence of a high concentration of combined nitrogen and a high DO_2 concentration. Therefore, the possibility that diazotrophic growth has any effect can be excluded (5, 6). The results obtained are shown in Fig. 1.

We observed that *A. brasilense* wild-type strain Sp7 can produce only small amounts of PHB during the active growth phase, which is consistent with a previous report (28). Cell growth enters the stationary phase because of depletion of the carbon source (malate). The PHB concentration decreases during the stationary phase because PHB is probably used as the alternative carbon source for growth maintenance after the supply of malate is exhausted (Fig. 1A). The *glnB* mutant has a PHB production and cell growth phenotype similar to that of the wild type (Fig. 1B). The *glnZ* mutant can produce slightly more PHB during the growth phase than the wild type and *glnB* mutant can produce, but the amount of PHB is still small (Fig. 1C). However, the *glnB glnZ* double mutant produces a significantly larger amount of PHB during the active growth phase than the wild type produces (Fig. 1D). The *glnD* mutant can accumulate even more PHB (up to 40% of the cell dry weight) during the growth phase than the *glnB glnZ* double mutant can accumulate (Fig. 1E). PHB accumulation is obviously associated with cell growth in the *glnB glnZ* and *glnD* mutants under nitrogen-excess conditions. Additionally, all four mutants have cell growth and respiration phenotypes (data not shown) similar to those of the wild type under the conditions used in this study, if the effects of other growth-limiting factors caused by mutations on PHB production are excluded. Furthermore, a mathematical modelling analysis has been done to confirm the significant differences in PHB production regulated by ammonia for the wild type and the four mutants (data not shown) (27).

It has been reported that even though the P_{II} and P_z proteins have similar structures and are similarly modified (uridylylated) in response to nitrogen limitation in cells, they are involved differently in nitrogen-dependent regulation of various physiological functions (2). However, the results of an analysis of PHB production in *glnB*, *glnZ*, and *glnB glnZ* mutants suggest that the P_{II} and P_z proteins can complement each other for control of PHB accumulation in *A. brasilense* Sp7. Surprisingly, the *glnD* mutant can also produce a large amount of PHB associated with cell growth and is not sensitive to the inhibitory

effect of ammonia on PHB accumulation. Under nitrogen-excess conditions, such as those used in this study, the P_{II} and P_z proteins are retained in their native, nonuridylylated forms (2), which are also the only forms present in a *glnD* mutant regardless of the intracellular nitrogen status (31, 32). The high level of PHB production in the *glnD* mutant might imply that in *A. brasilense* Sp7 the *glnD* gene controls regulation of PHB biosynthesis by ammonia not via the P_{II} and P_z nitrogen-sensing system but via another unknown target gene(s).

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