APPLIED AND ENVIRONMENTAL MICROBIOLOGY, Jan. 2000, p. 113–117 Vol. 66, No. 1
0099-2240/00/$04.00+0

The *ntrB* and *ntrC* Genes Are Involved in the Regulation of Poly-3-Hydroxybutyrate Biosynthesis by Ammonia in *Azospirillum brasilense* Sp7

JUN SUN,[1] XUAN PENG,[2] JAN VAN IMPE,[2] AND JOS VANDERLEYDEN[1]*

F. A. Janssens Laboratory of Genetics[1] and Bioprocess Technology and Control,[2] K.U. Leuven, B-3001 Heverlee, Belgium

Received 6 July 1999/Accepted 10 October 1999

Azospirillum brasilense **Sp7 and its *ntrA* (*rpoN*), *ntrBC*, and *ntrC* mutants have been evaluated for their capabilities of poly-3-hydroxybutyrate (PHB) accumulation in media with high and low ammonia concentrations. It was observed that the *ntrBC* and *ntrC* mutants can produce PHB in both low- and high-C/N-ratio media, while no significant PHB production was observed for the wild type or the *ntrA* mutant in low-C/N-ratio media. Further investigation by fermentation analysis indicated that the *ntrBC* and *ntrC* mutants were able to grow and accumulate PHB simultaneously in the presence of a high concentration of ammonia in the medium, while little PHB was produced in the wild type and *ntrA* (*rpoN*) mutant during active growth phase. These results provide the first genetic evidence that the *ntrB* and *ntrC* genes are involved in the regulation of PHB synthesis by ammonia in *A. brasilense* Sp7.**

Poly-3-hydroxybutyrate (PHB), a thermoplastic produced by numerous microorganisms as an energy and/or carbon storage material under conditions of nutrient imbalance, has attracted attention for its biodegradability and biocompatibility (2). However, a major limitation in the commercialization of PHB in a wide range of applications is its high production cost (5, 6). Much effort has been devoted to lowering the production cost by developing more efficient fermentation and recovery processes (15); selecting new potential microorganisms, including genetically engineered bacteria (7, 26); metabolic engineering of PHB biosynthetic pathways in higher organisms, such as *Saccharomyces cerevisiae* (14), insects (33), and plants (34); using alternative cheaper carbon sources (3, 22); and investigating the precise control mechanisms involved in PHB biosynthesis (25, 27).

Intensive studies on the metabolic pathways for PHB biosynthesis and molecular analyses of PHB biosynthesis genes in various bacteria have been conducted in order to understand the mechanisms of PHB biosynthesis and subsequently to construct genetically engineered microorganisms or even plants for more efficient production of PHB. In *Ralstonia eutropha* (formerly known as *Alcaligenes eutrophus*), acetyl coenzyme A (acetyl-CoA) is converted to PHB in the following three steps: (i) formation of acetoacetyl-CoA, (ii) stereoselective reduction of acetoacetyl-CoA to D-(−)-3-hydroxybutyryl-CoA, and (iii) ligation of D-(−)-3-hydroxybutyryl to the growing chain of PHB. Since the first *phb* gene was isolated from *Zoogloea ramigera* (24), more than 30 different PHB biosynthesis genes have been cloned from various bacteria (15). Some genes involved in the formation of the PHB granule have also been recently characterized (25).

For most PHB-producing bacteria, only little PHB accumulation can be observed during the active growth phase of cells, so a long growth phase is essential for high-density cell cultivation (2). Nutrient limitation is needed for initiation of PHB accumulation, and generally ammonia is considered the critical control factor decoupling the growth of cells and PHB production. However, some bacteria, such as *Azotobacter vinelandii* strain UWD (obtained by chemical mutagenesis [23]), *Alcaligenes latus* (8), and *Pseudomonas putida* KT2442 (9), are able to accumulate large amounts of PHB or polyhydroxyalkanoate (PHA) during exponential growth. The inactivation of inhibition of ammonia of the accumulation of PHB has industrial potential for improvement of process control and productivity (16).

Azospirillum, a genus of free-living nitrogen-fixing bacteria, has been studied intensively in the past decades for its physiological and genetic properties. Some of the species, such as *Azospirillum brasilense* and *Azospirillum lipoferum*, are noted for their capabilities of accumulation of intracellular PHB with a relatively high content (up to 88% of the dry biomass) under unbalanced nutrient conditions such as oxygen limitation and a high C/N ratio (12, 28).

In this study, the regulation of PHB production by ammonia was investigated for *A. brasilense* Sp7 and its *ntrA* (*rpoN*), *ntrBC*, and *ntrC* mutants. The significant differences in PHB production by the *ntrBC* and *ntrC* mutants versus the *ntrA* (*rpoN*) mutant and wild type during the exponential growth phase in the presence of a high concentration of ammonia in the medium demonstrate the involvement of the *ntrB* and *ntrC* genes in the regulation of PHB biosynthesis by ammonia in *A. brasilense* Sp7.

MATERIALS AND METHODS

Bacterial strains and growth conditions. The bacterial strains used in this study are listed in Table 1. All the strains were routinely grown in MMAB medium (31) at 30°C. Kanamycin (25 μg/ml) was added to the medium when required. Because the *ntrB* and *ntrC* genes are organized in one operon, the *ntrB* mutant, constructed by polar mutation, is an *ntrBC* mutant (18).

Culture conditions for PHB production. For test tube cultures of bacteria, 5-ml aliquots of MMAB medium in 20-ml test tubes were each inoculated with 1 loop of bacteria from a fresh plate or 0.15 ml of preculture from another test tube and then incubated at 30°C for 24 h while shaken at 200 rpm. The batch fermentation was performed in a 2-liter O_2-stat fermentor as described previously (20). The concentration of dissolved oxygen (DO_2) was controlled at a constant level by varying the air flow into the fermentor according to the measured DO_2 value so that the air flow rate could be used as an indicator for the oxygen uptake rate (20).

* Corresponding author. Mailing address: F.A. Janssens Laboratory of Genetics, K.U. Leuven, Kardinaal Mercierlaan 92, B-3001 Heverlee, Belgium. Phone: 0032-16-329679. Fax: 0032-16-321966. E-mail: jozef.vanderleyden@agr.kuleuven.ac.be.

TABLE 1. *A. brasilense* strains used in this study

Strain	Relevant characteristic(s)	Reference
Sp7	Wild type (ATCC 29145)	23
FAJ301	*rpoN*::*kan* Km^r; *A. brasilense* Sp7 *ntrA* (*rpoN*) mutant	21
7194	*ntrB*::Tn*5*-194 Km^r; *A. brasilense* Sp7 *ntrB* mutant	18
7148	*ntrC*::Tn*5*-148 Km^r; *A. brasilense* Sp7 *ntrC* mutant	18

Analytical procedures. Cell growth was monitored by measuring the optical density at 600 nm with a Perkin-Elmer Lambda 2 UV–visible-spectrum spectrophotometer. Biomass concentration, defined as cell dry weight per milliliter of culture broth, was determined by weighing dry cells with a microbalance (Mettler, Zurich, Switzerland) as described previously (32). L-Malate and ammonia concentrations in the culture broth were determined with test kits from Boehringer Mannheim (Mannheim, Germany). The PHB concentration was determined with a gas chromatograph (HP6890 Plus; Hewlett-Packard, Wilmington, Del.) equipped with an automatic sampler (HP7683; Hewlett-Packard) and a J&W DB-WAX capillary column (0.53 mm by 15 m, 1-μm film thickness) by using benzoic acid as the internal standard (4). Non-PHB biomass was obtained by subtracting the amount of PHB from the biomass, while the PHB content was defined as the ratio of PHB to cell dry weight, expressed as a percentage. All the data in this paper are average values of at least two replicates.

RESULTS

A. brasilense Sp7 and its *ntrA* (*rpoN*), *ntrBC*, and *ntrC* mutants were grown in MMAB medium with different initial C/N ratios obtained by varying the concentration of malate or NH_4Cl. After incubation at 30°C for 24 h, the biomass and PHB concentrations were measured and compared. The results are shown in Fig. 1.

PHB production by *A. brasilense* Sp7 and its *ntrA* (*rpoN*) mutant increased with the C/N ratio of the medium, and no PHB could be detected in low-C/N-ratio media because at least half of the initial amount of ammonia was still present in the media when malate was depleted (data not shown). In contrast with the wild type and *ntrA* (*rpoN*) mutant, the *ntrBC* and *ntrC* mutants were able to synthesize PHB even in low-C/N-ratio media.

The above results can be interpreted in two ways: (i) PHB biosynthesis coincides with the growth of cells for the *ntrBC* or *ntrC* mutant, or (ii) initiation of PHB accumulation occurs at much higher ammonia concentrations in the *ntrBC* or *ntrC* mutant. In order to elucidate the involvement of the *ntrB* and *ntrC* genes in the regulation of PHB production by ammonia in *A. brasilense* Sp7, the wild type and mutant strains were grown in a bioreactor, allowing more precise monitoring and control of culture conditions. MMAB medium was supplemented with 10 g of malate and 1.35 g of NH_4Cl per liter (initial C/N ratio = 10), and the DO_2 concentration was set at 30%, which was reported to be optimal for PHB accumulation in *A. brasilense* (28). No nitrogen fixation can occur under these culture conditions since the nitrogen fixation process is repressed by the high DO_2 concentration and the presence of combined nitrogen. Therefore, the influence of diazotrophic growth can be excluded (9, 10). The results are shown in Fig. 2.

It can be observed that *A. brasilense* Sp7 and its *ntrA* (*rpoN*) mutant produced only small amounts of PHB in the active growth phase, while no additional PHB accumulated during the stationary phase (Fig. 2A and B). The respiration of cells (indicated by the air flow rate) increased drastically at the end of the exponential growth phase, which is consistent with the results of a previous study (20). Nevertheless, PHB production was not triggered during the stationary phase, since about 20 mM NH_4Cl was still present in the medium. For the wild type, the PHB concentration reached its maximum and cell growth entered the stationary phase at 10 h of fermentation even though there was about 4 g of malate per liter left in the medium (Fig. 2A). However, the *ntrBC* and *ntrC* mutants not only produced a larger amount of PHB during the growth phase than the wild type but also continued to synthesize PHB in the stationary phase despite a high concentration of ammonia in the medium (Fig. 2C and D). Eventually about 40 and 22% PHB content accumulated in the *ntrBC* and *ntrC* mutants, respectively. However, a relatively long lag phase was observed for the *ntrBC* and *ntrC* mutants when the DO_2 concentration was higher than 30%, and the respiration of the mutants was much lower than that of the wild type, as can be deduced from

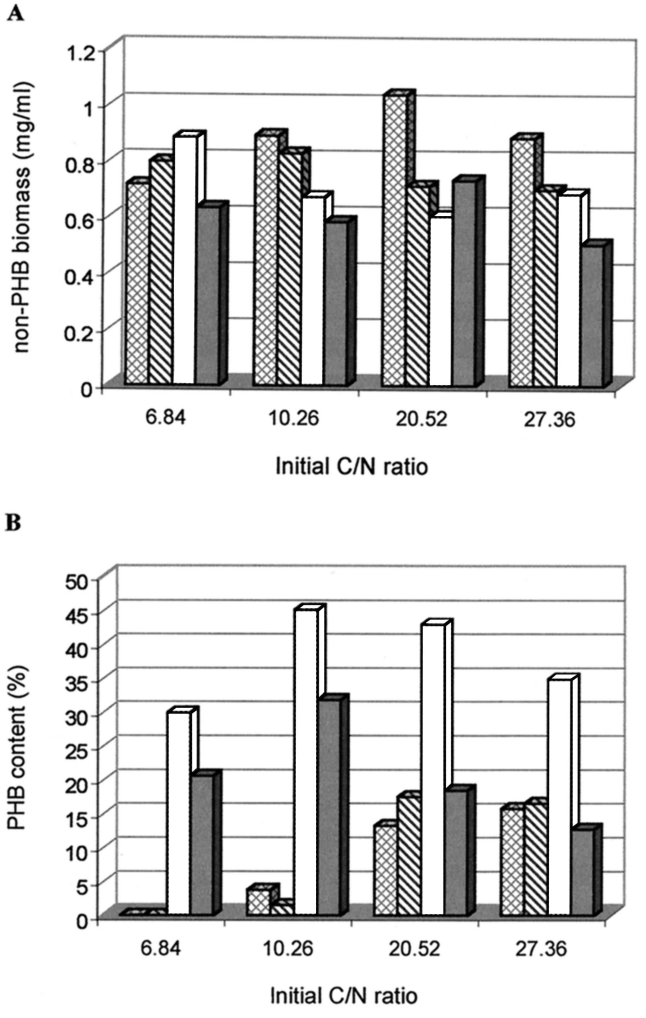

FIG. 1. Comparison of non-PHB biomass production and PHB content of *A. brasilense* Sp7 (▨) and its *ntrA* (▧), *ntrBC* (⊡), and *ntrC* (■) mutants in MMAB medium with different initial C/N ratios. The concentration of malate was 15 g/liter, while the concentrations of NH_4Cl were 3, 2, 1, and 0.75 g/liter, corresponding to the different initial C/N ratios.

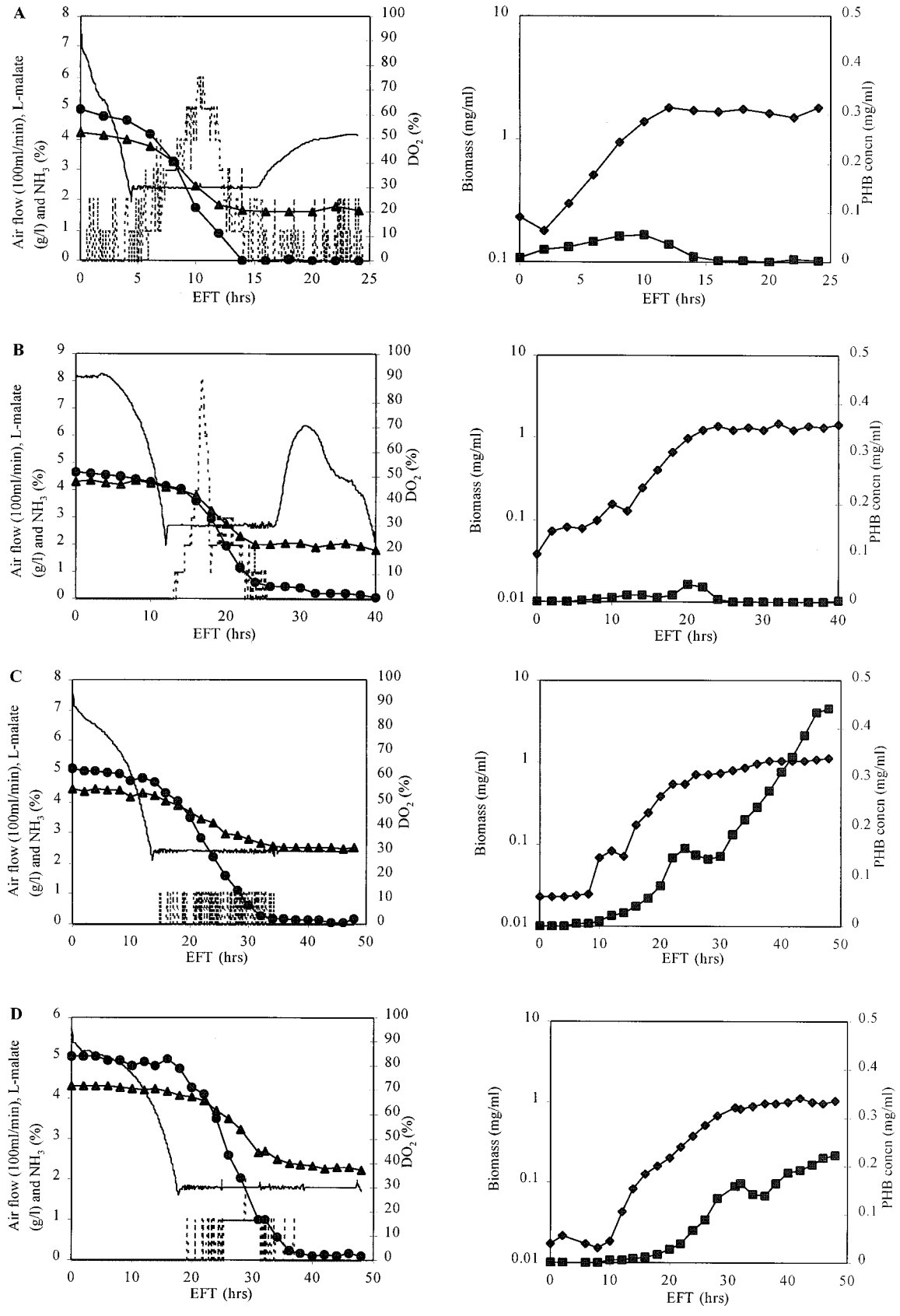

FIG. 2. Time course of fermentation of *A. brasilense* Sp7 (A) and its *ntrA* (*rpoN*) (B), *ntrBC* (C), and *ntrC* (D) mutants. EFT, elapsed fermentation time. Symbols: ——, DO_2; – – –, air flow; ●, L-malate concentration; ▲, ammonia concentration; ◆, biomass concentration; ■, PHB concentration.

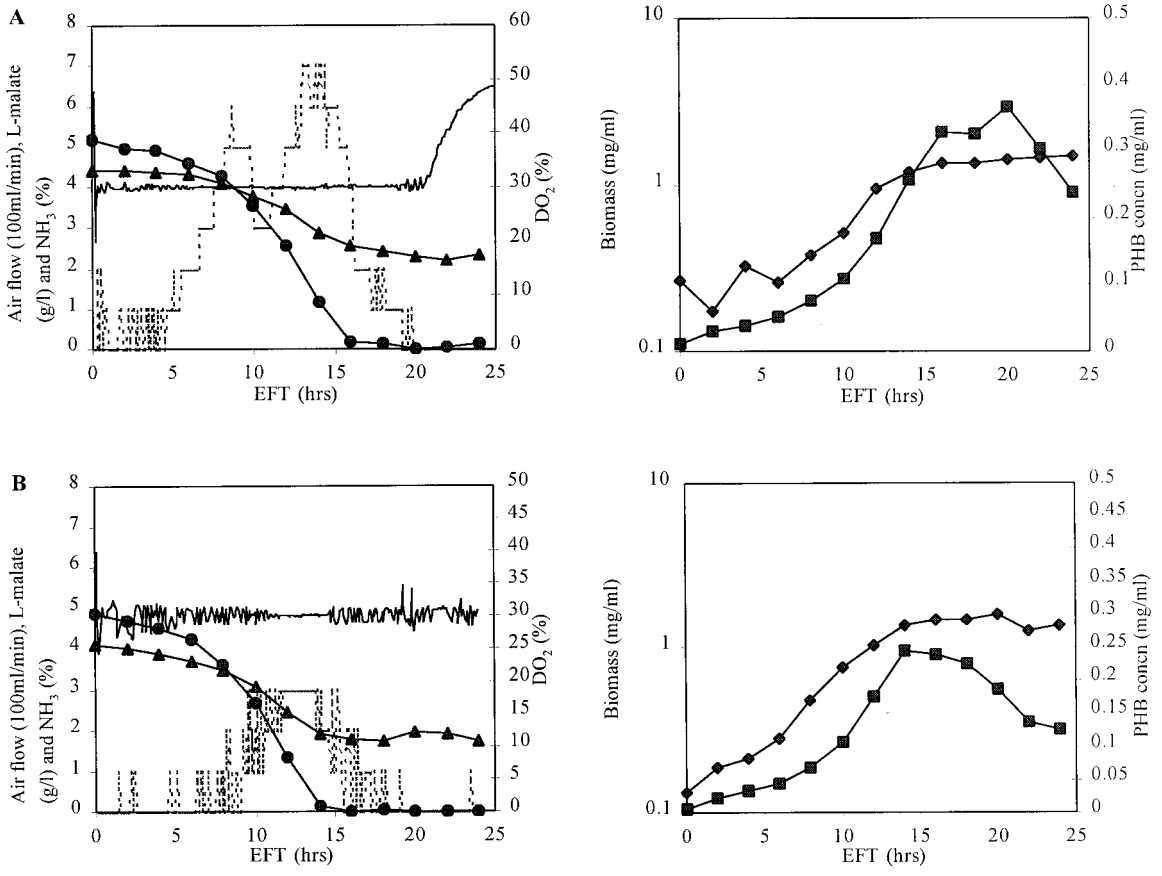

FIG. 3. Time course of fermentation of the *A. brasilense* Sp7 *ntrBC* (A) and *ntrC* (B) mutants with control of the DO_2 concentration from the beginning of fermentation. The symbols and abbreviation are the same as for Fig. 2.

the air flow. This implies that a high DO_2 value might inhibit the growth of the *ntrBC* and *ntrC* mutants. The active growth phases are similar for the wild type and the *ntrBC* and *ntrC* mutants.

In order to demonstrate and exclude the inhibition influence of a high DO_2 concentration on the growth of the *ntrBC* and *ntrC* mutants, the DO_2 concentration was kept at 30% from the beginning of fermentation by sparging N_2 into the fermentor. The results are shown in Fig. 3. It can be observed that the growth properties of the *ntrBC* and *ntrC* mutants were similar to that of the wild type, while their PHB production coincided with the active growth. However, the PHB concentration decreased during the stationary phase because the malate was exhausted and PHB was likely used as the alternative carbon source for growth maintenance.

DISCUSSION

The regulatory genes *ntrB* and *ntrC*, encoding the two-component sensor-activator regulatory system NtrB-NtrC (13), have been previously characterized in *A. brasilense* (18). The results of studies on the phenotype of the *ntrBC* and *ntrC* mutants indicate that NtrB and NtrC are not strictly required for nitrogen fixation in *Azospirillum*, although the nitrogenase activity of the *ntrC* mutant was partially reduced. No significant difference of ammonia uptake rate has been observed for the *ntrBC* mutant and the wild type of *A. brasilense* (30). However, NtrC has been shown to be involved in nitrate utilization as a nitrogen source in *A. brasilense*, and the *ntrBC* mutant displayed nitrogenase activity which was partially resistant to ammonia inactivation (17). The regulation of the *amtB* gene, encoding an ammonia transporter, by the Ntr system has been recently demonstrated (30). In *P. putida* KT2442, which can synthesize PHA during exponential growth when grown on fatty acids, a two-component system homologous to the sensor kinase-response regulator couple LemA-GacA was recently found to be involved in the regulation of PHA synthesis (19). However, no study on the relationship between NtrBC and PHB production has been reported so far.

In this study, some intriguing phenomena from the fermentation data for the *ntrBC* and *ntrC* mutants have been observed. Firstly, the respiration of the *ntrBC* and *ntrC* mutants diminished greatly compared to that of the wild type, indicating that the *ntrBC* genes might be involved in the regulation of genes encoding respiratory enzymes. Secondly, the long lag phase of the *ntrBC* and *ntrC* mutants (Fig. 2C and D) implies that the *ntrBC* genes are probably also involved in the regulation of the tolerance of high oxygen concentrations by *A. brasilense*. Thirdly, the results for PHB production by the *ntrBC* and *ntrC* mutants indicate explicitly the involvement of the *ntrB* and *ntrC* genes in the regulation of PHB production by ammonia in *A. brasilense*. The *ntrBC* and *ntrC* mutants can produce PHB continuously, whether in the active growth phase or stationary phase or whether or not a high concentration of ammonia is present in the medium. Nevertheless, a transition of PHB production from exponential growth phase to station-

ary phase can still be observed in the fermentation time courses of the *ntrBC* and *ntrC* mutants. Therefore, it can be reasonably concluded that inactivation of the *ntrB* and *ntrC* genes not only couples the PHB production and the active growth of cells but also eliminates the inhibition effect of ammonia on PHB biosynthesis by *A. brasilense* Sp7.

The coupling of PHB production and cell growth has application potential for significant improvement of productivity and facilitation of process control. This study provides evidence of the involvement of the *ntrB* and *ntrC* genes in the regulation of PHB production and therefore supports further investigation of this relationship. It will be of interest to identify the target gene(s) of the NtrB-NtrC two-component system.

ACKNOWLEDGMENTS

J.S. is a recipient of a doctoral scholarship from the Research Council, K.U. Leuven. X.P. is a recipient of a scholarship from K.U. Leuven. This study was supported in part by Project OT/95/20 of the K.U. Leuven Research Council.

We thank A. Van Dommelen for her advice, and we also acknowledge C. Elmerich for the gift of the *ntrBC* and *ntrC* mutants of *A. brasilense* Sp7.

REFERENCES

1. **Albrecht, S. L., and Y. Okon.** 1980. Cultures of *Azospirillum*. Methods Enzymol. **69:**740–749.
2. **Anderson, A. J., and E. A. Dawes.** 1990. Occurrence, metabolism, metabolic role, and industrial uses of bacterial polyhydroxyalkanoates. Microbiol. Rev. **54:**450–472.
3. **Bourque, P. Y., Y. Pomerleau, and D. Groleau.** 1995. High-cell-density production of poly-β-hydroxybutyrate (PHB) from methanol by *Methylobacterium extorquens*: production of high-molecular-mass PHB. Appl. Microbiol. Biotechnol. **44:**367–376.
4. **Braunegg, G., B. Sonnleitner, and R. M. Lafferty.** 1978. A rapid gas chromatographic method for the determination of poly-β-hydroxybutyric acid in microbial biomass. Eur. J. Appl. Microbiol. Biotechnol. **6:**29–37.
5. **Byrom, D.** 1987. Polymer synthesis by microorganisms: technology and economics. Trends Biotechnol. **5:**246–250.
6. **Choi, J., and S. Y. Lee.** 1997. Process analysis and economic evaluation for poly(3-hydroxybutyrate) production by fermentation. Bioprocess Eng. **17:** 335–342.
7. **Choi, J., S. Y. Lee, and K. Han.** 1998. Cloning of the *Alcaligenes latus* polyhydroxyalkanoate biosynthesis genes and uses of these genes for enhanced production of poly(3-hydroxybutyrate) in *Escherichia coli*. Appl. Microbiol. Biotechnol. **64:**4897–4903.
8. **Hänggi, U. J.** 1990. Pilot scale production of PHB with *Alcaligenes latus*, p. 65–70. *In* E. A. Dawes (ed.), Novel biodegradable microbial polymers. Kluwer Academic Publishers, Dordrecht, The Netherlands.
9. **Hartmann, A., and R. H. Burris.** 1987. Regulation of nitrogenase activity by oxygen in *Azospirillum brasilense* and *Azospirillum lipoferum*. J. Bacteriol. **169:**944–948.
10. **Hartmann, A., H. Fu, and R. H. Burris.** 1986. Regulation of nitrogenase activity by ammonium chloride in *Azospirillum* spp. J. Bacteriol. **165:**864–870.
11. **Huisman, G. W., E. Wonink, G. de Koning, H. Preusting, and B. Witholt.** 1992. Synthesis of poly(3-hydroxyalkanoates) by mutant and recombinant *Pseudomonas* strains. Appl. Microbiol. Biotechnol. **38:**1–5.
12. **Itzigsohn, R., O. Yarden, and Y. Okon.** 1995. Polyhydroxyalkanoate analysis in *Azospirillum brasilense*. Can. J. Microbiol. **41:**73–76.
13. **Keener, J., and S. Kustu.** 1988. Protein kinase and phosphoprotein phosphatase activities of nitrogen regulatory proteins NTRB and NTRC of enteric bacteria: role of the conserved amino-terminal domain of NTRC. Proc. Natl. Acad. Sci. USA **85:**4976–4980.
14. **Leaf, T. A., M. S. Peterson, S. K. Stoup, D. Somers, and F. Srienc.** 1996. *Saccharomyces cerevisiae* expressing bacterial polyhydroxybutyrate synthase produces poly-3-hydroxybutyrate. Microbiology **142:**1169–1180.
15. **Lee, S. Y.** 1996. Bacterial polyhydroxyalkanoates. Biotechnol. Bioeng. **49:**1–14.
16. **Lee, S. Y., and H. N. Chang.** 1995. Production of poly(hydroxyalkanoic acid). Adv. Biochem. Eng. Biotechnol. **52:**27–58.
17. **Liang, Y. Y.** 1992. Etude du rôle des gènes *nifA* at *ntrBC* dans la régulation de l'experssion des gènes *nif* chez *Azospirillum brasilense* Sp7. Ph.D. thesis. Institut Pasteur, Paris, France.
18. **Liang, Y. Y., F. Arsène, and C. Elmerich.** 1993. Characterization of the *ntrBC* genes of *Azospirillum brasilense* Sp7: their involvement in the regulation of nitrogenase synthesis and activity. Mol. Gen. Genet. **240:**188–196.
19. **Madison, L. L., and G. W. Huisman.** 1999. Metabolic engineering of poly(3-hydroxyalkanoates): from DNA to plastic. Microbiol. Mol. Biol. Rev. **63:**21–53.
20. **Marchal, K., J. Sun, V. Keijers, H. Haaker, and J. Vanderleyden.** 1998. A cytochrome cbb_3 (cytochrome *c*) terminal oxidase in *Azospirillum brasilense* Sp7 supports microaerobic growth. J. Bacteriol. **180:**5689–5696.
21. **Milcamps, A., A. Van Dommelen, J. Stigter, J. Vanderleyden, and F. de Bruijn.** 1996. The *Azospirillum brasilense rpoN* gene is involved in nitrogen fixation, nitrate assimilation, ammonium uptake and flagellar biosynthesis. Can. J. Microbiol. **42:**467–478.
22. **Page, W. J., and A. Cornish.** 1993. Growth of *Azotobacter vinelandii* UWD in fish peptone medium and simplified extraction of poly-β-hydroxybutyrate. Appl. Environ. Microbiol. **59:**4236–4244.
23. **Page, W. J., and O. Knosp.** 1989. Hyperproduction of poly-β-hydroxybutyrate during exponential growth of *Azotobacter vinelandii* UWD. Appl. Environ. Microbiol. **55:**1334–1339.
24. **Peoples, O. P., S. Masamune, C. T. Walsh, and A. J. Sinskey.** 1987. Biosynthetic thiolase from *Zoogloea ramigera*. III. Isolation and characterization of the structural gene. J. Biol. Chem. **262:**97–102.
25. **Prieto, M. A., B. Bühler, K. Jung, B. Witholt, and B. Kessler.** 1999. PhaF, a polyhydroxyalkanoate-granule-associated protein of *Pseudomonas oleovorans* GPo1 involved in the regulatory expression system for *pha* genes. J. Bacteriol. **181:**858–868.
26. **Renner, G., G. Haage, and G. Braunegg.** 1996. Production of short-side-chain polyhydroxyalkanoates by various bacteria from the rRNA superfamily III. Appl. Microbiol. Biotechnol. **46:**268–272.
27. **Slater, S., K. L. Houmiel, M. Tran, T. A. Mitsky, N. B. Taylor, S. R. Padgette, and K. J. Gruys.** 1998. Multiple β-ketothiolases mediate poly(β-hydroxyalkanoate) copolymer synthesis in *Ralstonia eutropha*. J. Bacteriol. **180:**1979–1987.
28. **Tal, S., and Y. Okon.** 1985. Production of the reserve material poly-β-hydroxybutyrate and its function in *Azospirillum brasilense* Cd. Can. J. Microbiol. **31:**608–613.
29. **Tarrand, J. J., N. R. Krieg, and J. Dobereiner.** 1978. A taxonomic study of the *Spirillum lipoferum* group, with the description of a new genus, *Azospirillum* gen. nov. and two species, *Azospirillum lipoferum* (Beijerinck) comb. nov. and *Azospirillum brasilense* sp. nov. Can. J. Microbiol. **24:**967–980.
30. **Van Dommelen, A., V. Keijers, J. Vanderleyden, and M. de Zamaroczy.** 1998. (Methyl)ammonium transport in the nitrogen-fixing bacterium *Azospirillum brasilense*. J. Bacteriol. **180:**2652–2659.
31. **Vanstockem, M., K. Michiels, J. Vanderleyden, and A. P. Van Gool.** 1987. Transposon mutagenesis of *Azospirillum brasilense* and *Azospirillum lipoferum*: physical analysis of Tn*5* and Tn*5*-Mob insertion mutants. Appl. Environ. Microbiol. **53:**410–415.
32. **Wang, F., and S. Y. Lee.** 1997. Production of poly(3-hydroxybutyrate) by fed-batch culture of filamentation-suppressed recombinant *Escherichia coli*. Appl. Environ. Microbiol. **63:**4765–4769.
33. **Williams, M. D., J. A. Rahn, and D. H. Sherman.** 1996. Production of a polyhydroxyalkanoate biopolymer in insect cells with a modified eukaryotic fatty acid synthase. Appl. Environ. Microbiol. **62:**2540–2546.
34. **Williams, S. F., and O. P. Peoples.** 1996. Biodegradable plastics from plants. Chemtech **26:**38–44.