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Poly-β-Hydroxybutyrate Turnover in *Azorhizobium caulinodans* Is Required for Growth and Affects *nifA* Expression

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***Azorhizobium caulinodans* is able to fix nitrogen in the free-living state and in symbiosis with the tropical legume *Sesbania rostrata*. The bacteria accumulate poly-β-hydroxybutyrate (PHB) under both conditions. The structural gene for PHB synthase, *phbC*, was inactivated by insertion of an interposon. The mutant strains obtained were devoid of PHB, impaired in their growth properties, totally devoid of nitrogenase activity ex planta (Nif⁻), and affected in nucleotide pools and induced Fix⁻ nodules devoid of bacteria. The Nif⁻ phenotype was the consequence of the lack of *nifA* transcription. Nitrogenase activity was partially restored to a *phbC* mutant by constitutive expression of the *nifA* gene. However, this constitutive *nifA* expression had no effect on the nucleotide content or on growth of the *phbC* mutant. It is suggested that PHB is required for maintaining the reducing power of the cell and therefore the bacterial growth. These observations also suggest a new control of *nifA* expression to adapt nitrogen fixation to the availability of carbon and reducing equivalents.**

Azorhizobium caulinodans, isolated from stem nodules of the tropical legume *Sesbania rostrata*, is able to grow in the free-living state at the expense of dinitrogen (11, 12). Expression of the nitrogen fixation genes (*nif* genes) and the *fixABCX* operon depends on the transcriptional activator NifA, as in other members of the class *Proteobacteria* (2, 36). Expression of *nifA*, in turn, is controlled by both the oxygen and the ammonia status of the cell, via the FixLJ, FixK, and NtrBC/NtrYX proteins and the HF-I-like protein, NrfA (18, 19, 22, 35). *A. caulinodans* can adapt its energy metabolism to a wide range of oxygen concentrations (5, 15, 21, 25, 29) and accumulate reserves of poly-β-hydroxybutyrate (PHB) both in the free-living state and during symbiosis (43).

PHB and other polyhydroxyalkanoate granules are frequently found in bacteria. These polymers serve as carbon and energy storage compounds and also as a sink for reducing equivalents (1). The most common biosynthetic pathway for PHB, first described for *Alcaligenes eutrophus* (44), starts with the formation of acetoacetyl coenzyme A (acetoacetyl-CoA) by condensation of two acetyl-CoA molecules catalyzed by a β-ketothiolase. An NADPH-dependent acetoacetyl-CoA reductase catalyzes the reduction of acetoacetyl-CoA to β-hydroxybutyryl–CoA. Finally, PHB synthase, encoded by the *phbC* gene, polymerizes the β-hydroxybutyryl residues to a polyester molecule. The PHB molecule can be depolymerized and converted back to acetyl-CoA via a PHB depolymerase and an NAD-dependent β-hydroxybutyrate dehydrogenase. The regulation of PHB synthesis and degradation is controlled at the level of β-ketothiolase and β-hydroxybutyrate dehydrogenase enzyme activities (1, 41), allowing rapid modulation of the level of reductive power in the cell. This fine-tuning of the reducing power may prevent inhibition of several enzymes of the tricarboxylic acid (TCA) cycle (13).

Whether PHB is a source of carbon and energy during nitrogen fixation and in symbiosis is unclear. Rhizobia such as *Rhizobium tropici*, *Rhizobium etli*, *Bradyrhizobium japonicum*, and *A. caulinodans* accumulate PHB in the free-living state (43, 46) and in mature nodules (16, 23, 34), whereas *Sinorhizobium meliloti* accumulates PHB in the free-living state and during the early stages of nodule development only (17). A *phbC* mutant strain of *R. etli* displays higher nitrogenase activity in symbiosis than does the wild type (9). In contrast, Bergersen et al. (4) reported that stored PHB in isolated *B. japonicum* bacteroids can support nitrogenase activity during darkness and thereby prolong the period of nitrogen fixation beyond that of a bacteroid suspension containing less PHB.

Here, we investigated the involvement of PHB accumulation in the energy metabolism in free-living *A. caulinodans* bacteria, and its role in sustaining nitrogen fixation, by studying the phenotype of *phbC* mutants.

MATERIALS AND METHODS

Bacterial strains, plasmids, growth conditions, and nitrogenase and β-galactosidase assays. The bacterial strains and plasmids are listed in Table 1 or schematized in Fig. 1. All media used for *A. caulinodans* are based on the same mineral salt solution previously described (12). Nitrogen-free minimal medium used routinely for nitrogenase assays was LSO, which contains lactate and succinate (12). LSN medium is LSO containing 20 mM ammonium sulfate as a nitrogen source. The carbon source concentrations used for growth in nitrogen-containing medium were 56 mM lactate (LN), 18 mM succinate (SN), and 24 mM pyruvate (PN). YLS is LSN medium containing 0.1% yeast extract. Nitrogenase assays of bacteria in the free-living state were performed as reported previously (10, 12). In selected experiments, the oxygen tension in the gas phase was adjusted to 1, 3, 6, or 10%. β-Galactosidase assays of bacteria under the free-living state were performed as described previously (32). *Escherichia coli* strains were grown in Luria-Bertani medium, and *R. etli* strains were grown in PY

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TABLE 1. Bacterial strains and plasmids

Strain or plasmid	Description	Reference
Strains		
ORS571	*A. caulinodans* wild-type strain	11
5795	ORS571 ethyl methanesulfonate mutant; Nif$^-$ Fix$^-$	10
57601	ORS571 *fixK* mutant; Nif$^-$ Fix$^-$	22
57592	ORS571 *fixL* mutant; Nif$^-$ Fix$^-$	19
57593	ORS571 *fixJ* mutant; Nif$^-$ Fix$^-$	19
CA430	ORS571 *phbC*::Ω-Spc^r	This work
CA439	ORS571 *phbC*::Ω-Spc^r	This work
SAM100	*R. etli phaC*::Ω-Km^r	9
Plasmids		
pΔ10.59	*R. etli phaC* gene	This laboratory
pRS1022	ORS571 *nifA* gene expressed constitutively from Km^r promoter	18
pLRSA2	ORS571 *nifA* gene expressed from its own promoter	36
pRS2002	Translational *nifH-lacZ* fusion	19
pRS2010	Transcriptional *nifA-lacZ* fusion	18
pRS2028	Transcriptional *nifA*Δ*-lacZ* fusion	20
pRS2004	Translational *fixK-lacZ* fusion	22
pRS3012	Transcriptional *fixN-lacZ* fusion	29

rich medium (6). Batch cultures of *R. etli* were grown in minimal medium complemented with 10 mM pyruvic acid as previously described (8). Antibiotics were used at the following concentrations: for *A. caulinodans*, 200 μg of carbenicillin per ml and 100 μg of kanamycin per ml; for *R. etli*, 30 μg of kanamycin per ml; for *E. coli*, 100 μg of ampicillin per ml, 25 μg of kanamycin per ml, and 30 μg of gentamicin per ml; for all, 10 μg of tetracycline per ml and 50 μg of spectinomycin per ml.

Recombinant DNA techniques and construction of plasmids and mutant strains. Plasmid isolation, DNA ligation, restriction mapping, exonuclease III treatment, transformation, genomic DNA extraction, Southern blotting, and hybridization were performed by standard methods, as described previously (3). An *E. coli* S17-1 colony containing pRS592, a plasmid of an *A. caulinodans Sau*3A genomic DNA bank inserted into pLA29-17 (18), was selected by complementation of the Nif$^-$ phenotype of the mutant strain 5795 (10). The 12-kb *Xho*I fragment from pRS592, containing *phbC*, was transferred into pVK100 (26) to yield pRS5930 (Fig. 1) and into the single *Sal*I site of pJQ200mp18 (39) to yield pSP1600. Plasmid pRSΩ6 (Fig. 1) is a derivative of pRS5930 obtained after partial digestion with *Eco*RI and ligation with a 4-kb *Eco*RI fragment carrying a Ω-Gus-Sm^r Spc^r (glucuronidase-spectinomycin resistant) interposon from pWM5 (31). The location of the interposon at the correct *Eco*RI site in the *phbC* gene was verified by restriction analysis. Similarly, after partial digestion of pSP1600 with *Eco*RI, the 2-kb *Eco*RI Ω-Spc^r interposon from pHP45Ω-Spc^r (38) was inserted into the *phbC* gene, to yield pSP1601. The 13-kb *Xho*I/*Pst*I fragment of pSP1601, containing the interposon, was then recovered as a *Pst*I fragment (with the *Pst*I site of the vector polylinker) and ligated into the *Pst*I site of pSUP202 (42) to yield pSP1605 (Fig. 1). Plasmid pSP1611 is a derivative of pSP1605 in which the tetracycline resistance gene is interrupted with the kanamycin resistance gene from pHP45Ω-Km^r (14) (Fig. 1). To construct *phbC* mutant strains, spectinomycin-resistant, kanamycin-sensitive strains were isolated from two independent experiments after introducing pSP1611 into *A. caulinodans* wild type by conjugation and subsequent recombination of the interposon in the genome. Mutants resulting from double recombination of the Ω-Sp^r interposon at the correct location in the *phbC* gene were verified by Southern hybridization of their genomic DNA with DNA fragments of pSP1600 and the interposon as probes. Plasmids pRS2010 and pRS2028 are suicide vectors carrying transcriptional *nifA-lacZ* fusions that were used for recombination of the fusions in the host genome. Plasmid pRS2028 carries the same *lacZ*-Km cartridge from pKOK5 (27) at position +268 as in pRS2010 (18) and a deletion of the *nifA* coding sequence from the nucleotides +103 to +211 (positions are in reference to the transcription start site). The details of the construction of pRS2028 are reported elsewhere (20).

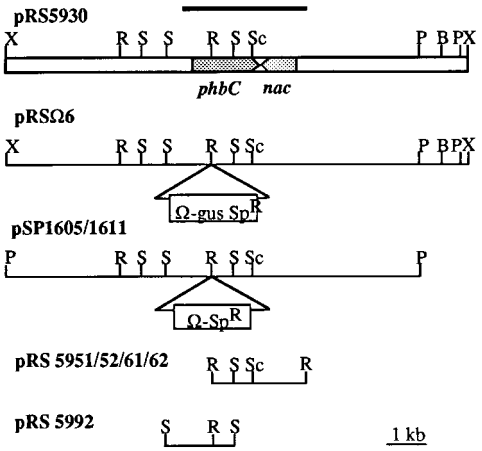

FIG. 1. Genetic and physical map of the *A. caulinodans phbC* region. The positions of *phbC* and *nac* genes are indicated by arrows. Restriction site abbreviations: B, *Bam*HI; P, *Pst*I; R, *Eco*RI; S, *Sac*I; Sc, *Sca*I; X, *Xho*I. Ω-Sp^r represents the Ω-streptomycin/spectinomycin resistance interposon. Ω-gus-Spc^r represents the glucuronidase-streptomycin/spectinomycin resistance interposon. The horizontal bar indicates the DNA fragment whose nucleotide sequence was determined.

Deletion mapping of the *phbC* region. pTZ18P2 is a derivative of pTZ18R (Pharmacia), containing two *Pac*I restriction sites introduced by ligation of appropriate oligonucleotide duplexes on either side of the polylinker. pRK415P1 is a derivative of pRK415 (24) into which a single *Pac*I restriction site has been introduced. To obtain sequential deletions of the pRS5930 12-kb *Xho*I fragment which restore nitrogen fixation to the mutant strain 5795, the fragment was first cloned into the *Sal*I site of pTZ18P2 in the two possible orientations. The resulting plasmids were digested with *Kpn*I and *Xba*I, treated with exonuclease III (Pharmacia), and religated. The deleted fragments were then recovered as *Pac*I fragments and ligated into pRK415P1. One of these deletions enabled us to isolate a 2.5-kb *Eco*RI fragment (one *Eco*RI site from the plasmid and the other from the fragment) which, once inserted into pRK415 (pRS5951 and pRS5952 [Fig. 1]), still restored nitrogen fixation of strain 5795 in the free-living state. This 2.5-kb *Eco*RI fragment was transferred into pBKS$^+$ (Pharmacia), yielding pRS5961 and pRS5962.

DNA sequencing. The nucleotide sequences of DNA inserts in pRS5961, pRS5962, and pRS5992 (Fig. 1) were determined with the Taquence kit (U.S. Biochemical). Data were compiled with the Genetics Computer Group program. The Blast and FASTA programs of the National Center for Biotechnology Information server were used for similarity searches.

Other methods and nodulation tests. PHB was assayed by the spectrophotometric method of Law and Slepecky (28). Spectral absorption was monitored between 200 and 300 nm to ensure that a single and symmetrical peak appeared at 235 nm. Free nucleotide content was assayed as described by Bravo and Mora (8). Immunodetection with antibodies against NifA was performed as previously described (33). Inoculation of *S. rostrata* and determination of the nitrogenase activity associated with roots were performed as previously described (10). Each series included six plants inoculated with the same strain to assay nitrogenase activity 4 weeks postinoculation. Noninoculated controls were included in each experiment, and no nodules were observed on control plants.

Nucleotide sequence accession number. The *phbC* gene EMBL accession no. is AJ006237.

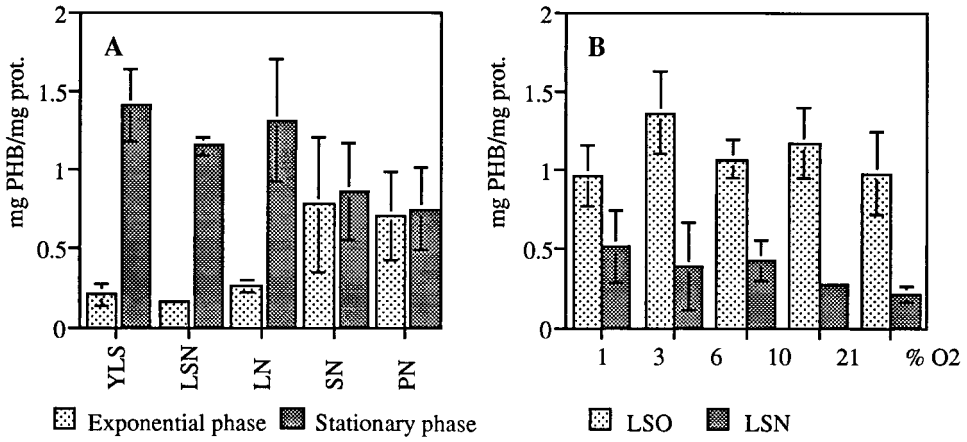

FIG. 2. PHB accumulation in the wild-type strain, expressed in milligrams of PHB per milligram of protein (prot.). (A) Aerated cultures with various carbon sources in the presence of ammonia: YLS, rich medium; LSN, lactate and succinate; LN, lactate; SN, succinate; PN, pyruvate. (B) PHB accumulation after 4 h of incubation under various oxygen tensions in the presence (LSN) or the absence (LSO) of ammonia.

RESULTS

PHB accumulation in *A. caulinodans* ORS571 wild-type bacteria. PHB accumulation was assayed in ORS571 cultures grown in minimal medium supplemented with various carbon sources and in rich medium. PHB was detected in all aerated ORS571 cultures (Fig. 2A). During the exponential phase, the PHB content was less than 0.4 mg of PHB/mg of protein when the medium contained lactate as carbon source (YLS, LSN, and LN [Fig. 2A]), whereas it increased during the stationary phase (ca. 1.4 mg of PHB/mg of protein). In contrast, there was little difference in the PHB content between the exponential and stationary phases, during growth in medium with succinate or pyruvate as sole carbon source (SN and PN [Fig. 2A]). An important increase in the PHB content was observed when the cells were transferred from exponential phase in YLS medium (0.2 mg of PHB/mg of protein [Fig. 2A]) to nitrogen-free medium after 3 h of incubation (Fig. 2B). The PHB content was not dependent on oxygen tension in the gas phase, while the addition of ammonia prevented the accumulation of PHB (Fig. 2B).

PHB accumulation in *fixK* and *nifA* mutant strains of *A. caulinodans* in LSO medium was similar to that in ORS571; thus, it does not appear to be dependent on nitrogen fixation (data not shown).

Identification of the PHB synthase structural gene. Strain 5795, isolated after ethyl methanesulfonate mutagenesis, was previously described as a regulatory Nif$^-$ mutant strain that did not synthesize several polypeptides induced under nitrogen deficiency (10). A plasmid, pRS5930 (Fig. 1), containing a 12-kb *Xho*I fragment that restored growth of strain 5795 on nitrogen-free medium was first obtained. Different deletions of the fragment were isolated to determine the smallest complementing region. This enabled us to subclone a 2.5-kb *Eco*RI fragment (pRS5951 and pRS5952 [Fig. 1]) which was sufficient to restore growth and nitrogenase activity to strain 5795. The nucleotide sequence of the 2.5-kb DNA fragment in plasmids pRS5961 and pRS5962 was found to contain two open reading frames (ORFs). The first, of 950 nucleotides (nt), encodes a polypeptide similar to transcriptional regulators of the LysR family, the highest similarity score being with the *nac* gene product of *Klebsiella aerogenes* (7). The other ORF, in the opposite orientation, extends beyond the sequenced fragment and corresponds to the 1,300 nt of the 3′ end of a gene whose deduced translation product had significant similarities to *phaC*/*phbC* gene products of other bacteria. The nucleotide sequence of the upstream region (pRS5992 [Fig. 1]) led to the identification of a complete ORF of 1,800 nt encoding a 600-amino-acid polypeptide (66.5 kDa). This ORF is preceded by an AGGA motif 6 nt upstream from the putative ATG start codon. The highest similarity scores were obtained with the predicted products of the *phbC* genes of *S. meliloti* (47) (57% identity) and *R. etli* (9) (56% identity). pRS5930 but not pRSΩ6 (which carries an interposon in the ORF) was found to restore wild-type growth on pyruvate as carbon source and to result in 50% of the maximal PHB content for the *phbC* mutant strain of *R. etli* SAM100 (9). Thus, the ORF encodes a functional equivalent of the PHB synthase and is hereafter referred to as the *phbC* gene.

Growth characteristics of the *phbC* mutant strains and PHB accumulation. *phbC* mutant strains were constructed (see Materials and Methods) and designated CA430 and CA439. The growth rates of CA430 and CA439 were found to be much lower than that of the wild type in that the mutant strains formed only tiny colonies on rich and minimal solid media after 3 days. Moreover the doubling times of strain CA430 and the wild type were 162 and 132 min, respectively, in rich liquid medium (YLS) and 384 and 192 min, respectively, in LSN medium under 1% oxygen.

The PHB content of strains CA430 and CA439 grown in lactate-containing medium was below 0.14 mg of PHB/mg of protein. Plasmid pRS5930, but not pRSΩ6, restored PHB accumulation to strain CA430 (e.g., 1.67 mg of PHB/mg of protein in LSO medium under 3% O_2 in the gas phase). Because the PHB content of strain 5795, grown in lactate, is similarly affected, this phenotype is consistent with a mutation in the *phbC* gene.

Nitrogenase activity in the free-living state and symbiotic properties of the *phbC* mutant. Like strain 5795, strains CA430 and CA439 were unable to grow on nitrogen-free solid medium. Under optimal nitrogen fixation conditions (LSO medium under 3% O_2–97% Ar), the *phbC* mutant CA430 was completely devoid of nitrogenase activity (Fig. 3A). Similarly, no activity was detected at 6 and 1% oxygen in the gas phase (data not shown). Incubation at 3% oxygen tension under nitrogen instead of argon did not result in nitrogenase activity (data not shown). Nitrogenase activity was restored to 80% of the wild-type level (59.5 ± 2.3 versus 70.2 ± 6.2 nmol of

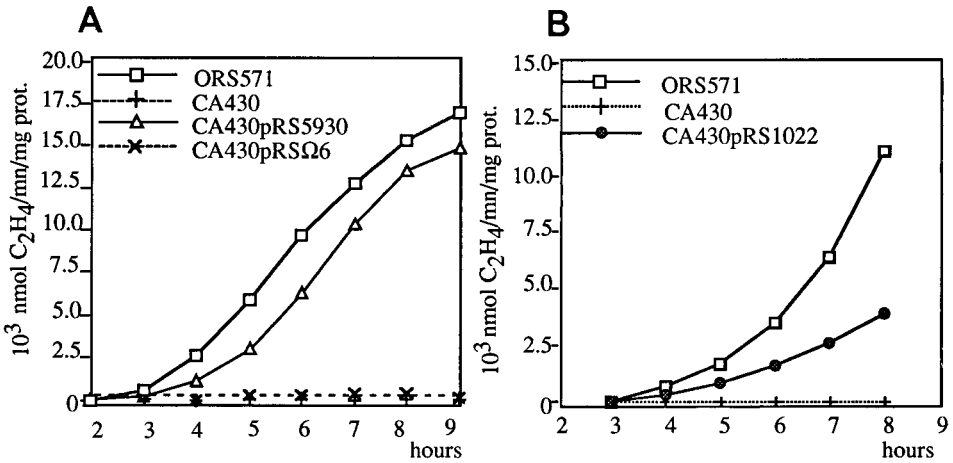

FIG. 3. (A) Kinetics of nitrogenase activity under 3% oxygen, in *A. caulinodans* ORS571 (wild type), CA430 (*phbC* mutant), CA430pRS5930, and CA430pRSΩ6. (B) Kinetics of nitrogenase activity under 3% oxygen, in *A. caulinodans* ORS571 (wild type), CA430 (*phbC* mutant), and CA430pRS1022 (*phbC* mutant with constitutively expressed *nifA*). prot., protein.

C_2H_4/min/mg of protein) by the introduction of pRS5930, but not of pRSΩ6 (Fig. 3A).

The nodulation properties and nitrogenase activity of ORS571 wild-type and *phbC* mutant strains were examined 4 weeks after inoculation of *S. rostrata* plantlets. Plants inoculated with the wild type harbored large nodules which displayed a specific nitrogenase activity of 0.20 ± 0.10 nmol of C_2H_4/min/mg of nodule (mean of three assays with six plants). Plants inoculated with strain CA430 displayed a Fix^- phenotype, and the specific nitrogenase activity was 0.02 ± 0.02 nmol of C_2H_4/min/mg of nodule (mean of three assays with six plants). The mutant strain CA430 induced essentially numerous small nodules totally devoid of nitrogenase activity which did not contain bacteria. However, we have observed the presence of a few large nitrogen-fixing nodules in addition to the small ones on some plants, which may result from *phbC* revertant strains. Plants inoculated with the mutant strain containing pRS5930 developed large nodules with specific nitrogenase activities similar to those induced by the wild type (0.14 ± 0.04 nmol of C_2H_4/min/mg of nodule).

Nucleotide content of ORS571 wild-type and *phbC* mutant strains. The absence of nitrogenase activity in strains CA430 and CA439 was surprising since *phbC* mutants of other rhizobia have been reported to be able to fix nitrogen in the free-living state and during symbiosis (9, 37). We postulated that the absence of PHB causes an imbalance in carbon metabolism, impairing the TCA cycle and, consequently, the supply of energy to nitrogenase. Therefore, the free nucleotide contents of the wild type and mutant CA430 were quantified under conditions of derepression of nitrogenase activity (LSO medium under 3% O_2–97% Ar). The NADH pool was found to be higher in the mutant than in the wild type, reflecting a lower NAD/NADH ratio in the mutant. The AMP level was much higher in the mutant than in the wild type, indicating decreased ATP availability (Table 2). The same results were observed for the *R. etli phaC* mutant strain (9). Furthermore, wild-type nucleotide contents were restored to the *A. caulinodans phbC* mutant strain by introducing pΔ10.59 (Table 2), which contains the *R. etli phaC* gene (Table 1). This indicates that, as in *R. etli*, PHB is a sink for reductive power in *A. caulinodans*.

Analysis of the regulation of *nif* gene expression in the *phbC* mutant strain CA430. A *nifH-lacZ* fusion carried by pRS2002 was not expressed in strain CA430 (data not shown), indicating that the lack of nitrogenase activity was due not to the inactivation of nitrogenase but rather to the absence of its synthesis. A chromosomal *nifA-lacZ* fusion was not expressed in CA430 (Fig. 4A), and this was correlated with the absence of NifA polypeptide synthesis, tested with antibodies against NifA (data not shown). In *A. caulinodans*, *nifA* expression is controlled by FixK, whose synthesis of which is controlled by FixLJ in response to microaerobic conditions (19, 22). In order to know if the *phbC* mutation affected expression or activity of one of these regulators, expression of *fixK* and *fixN* (a gene under the control of *fixK*) was examined. The *fixK-lacZ* fusion and the *fixN-lacZ* fusion were more strongly expressed in the *phbC* mutant than in the wild type but were not expressed in a *fixL* mutant (57592) and a *fixJ* mutant (57593), respectively (Fig. 4C and D). Thus, the defective *nifA* expression in the *phbC* mutant was not due to the inhibition of FixL, FixJ, or FixK synthesis or activities.

TABLE 2. Nucleotide contents of *A. caulinodans* wild-type and *phbC* mutant strains in nitrogen-free medium under an oxygen tension of 3%

Nucleotide(s)	Nucleotide content (nmol/mg of protein) for strain:			
	ORS571	CA430	CA430pRS1022	CA430pΔ10.59
NAD^+	1.241 ± 0.150	1.626 ± 0.103	1.680 ± 0.196	1.189 ± 0.110
NADH	0.426 ± 0.039	2.287 ± 0.149	2.538 ± 0.315	0.284 ± 0.047
NAD^+/NADH	2.946 ± 0.588	0.714 ± 0.090	0.671 ± 0.127	3.822 ± 0.337
AMP	0.016 ± 0.008	0.553 ± 0.057	0.550 ± 0.071	0.022 ± 0.017
ADP	0.535 ± 0.050	0.188 ± 0.025	0.228 ± 0.056	0.426 ± 0.109
ATP	0.291 ± 0.026	0.013 ± 0.007	0.017 ± 0.015	0.236 ± 0.114

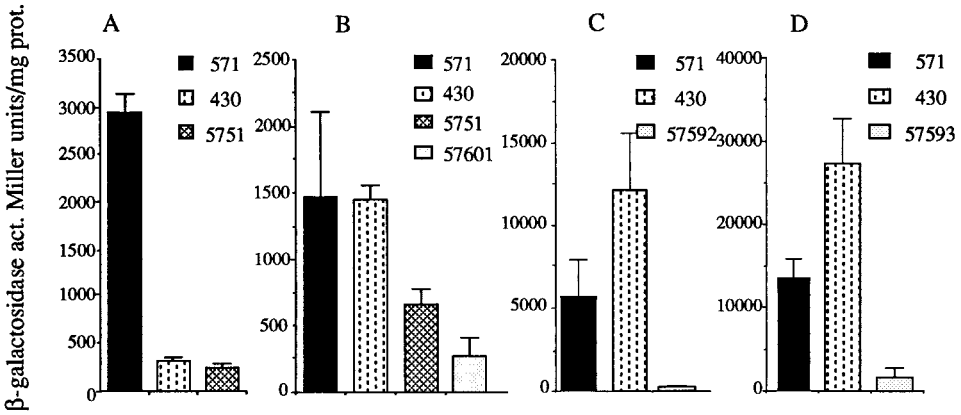

FIG. 4. Relative expression of *nif* and *fix-lacZ* fusions in the wild-type and mutant strains. (A) Chromosome-borne *nifA-lacZ*; (B) chromosome-borne *nifA*Δ*-lacZ*; (C) plasmid-borne *fixK-lacZ* (pRS2004); (D) plasmid-borne *fixN-lacZ* (pRS3012). β-Galactosidase activity (act.), expressed in Miller units per milligram of protein (prot.), was assayed in nitrogen-fixing conditions, in wild-type (571), *phbC* mutant (430), *nrfA* mutant (5751), *fixK* mutant (57601), *fixL* mutant (57592), and *fixJ* mutant (57593) strains. Data are the means of three independent determinations.

It has recently been shown that a DNA region at the beginning of the *nifA* coding sequence is involved in the regulation of *nifA* expression in response to ammonia. This region is linked to the location of NrfA control (18, 20). The expression of a chromosomal transcriptional *nifA-lacZ* fusion, carrying a deletion of this region (*nifA*Δ*-lacZ*), is abolished in a *fixK* mutant background and was found to partially escape NrfA control (20) (Fig. 4B). In contrast to the *nifA-lacZ* fusion (Fig. 4A), the *nifA*Δ*-lacZ* fusion is fully expressed in the *phbC* mutant (Fig. 4B), suggesting that this region is also involved in the control by *phbC*.

When *nifA* was expressed from a constitutive promoter (pRS1022) in CA430, 30% of the wild-type nitrogenase activity in the free-living state was restored to the mutant (Fig. 3B). This was not observed when *nifA* was expressed from its own promoter (pLRSA2 [data not shown]). However, pRS1022 did not restore PHB accumulation (data not shown), the nucleotide content (Table 2), or growth on nitrogen-free solid medium or on ammonia-containing medium.

DISCUSSION

Here, we report the characterization and nucleotide sequence of the PHB synthase gene *phbC* of *A. caulinodans*. The translation product of *A. caulinodans phbC* is similar to 18 of the *phaC*/*phbC* translation products available in the database, and as expected, the highest similarity was with the alpha subgroup of the *Proteobacteria*. The *phbC* gene of *A. caulinodans* is most probably monocistronic, as in *R. etli* (9) and *S. meliloti* (47).

The cloning of an *A. caulinodans* genomic DNA region, complementing the nitrogen fixation defect of strain 5795, led to the isolation of a DNA fragment containing a complete *nac*-like gene and the distal two-thirds of *phbC*. However, a DNA fragment containing the complete *nac*-like gene and not *phbC* did not complement the Nif$^-$ phenotype of strain 5795. Furthermore, the inactivation of the *nac*-like gene did not lead to a Nif$^-$ phenotype (data not shown). As it was subsequently found that strain 5795 did not accumulate PHB, it was assumed to instead carry a mutation in the *phbC* gene. Why the Nif$^-$ phenotype of strain 5795 was restored by a fragment that contains only the distal two-thirds of *phbC* remains unclear.

Accumulation of PHB in bacteria in the presence of an excess of the carbon source differs markedly from one species to another and depends on environmental conditions. The main factor that triggers PHB accumulation in members of the family *Azotobacteriaceae* is oxygen limitation (1). In other bacteria, for example, *Azospirillum brasilense*, the C/N ratio determines the synthesis of the polymer (45). PHB breakdown in *A. caulinodans* is inhibited in oxygen-limiting conditions and stimulated by an excess of ammonia (43). We observed an increase in the PHB content during the stationary phase when lactate was the carbon source. In the exponential phase of growth, the synthesis of the polymer was rapid and inversely correlated with nitrogen availability at all oxygen tensions tested. Thus, PHB synthesis in *A. caulinodans* appears to be controlled mainly by physiological nitrogen.

Inactivation of the *phbC* gene by insertion of an interposon led to a decrease of growth of the strain. Analysis of CA430 showed that PHB accumulation is required for optimal growth in all conditions tested. Interestingly, its growth rate in a medium containing lactate was low (in particular, at 1% oxygen tension). However, the amount of PHB in wild-type cells under these conditions is low during the exponential phase. This suggests that the absence of PHB synthesis, and hence the absence of PHB turnover, is deleterious for the bacteria, even in conditions where there is no substantial accumulation of the polymer. In addition, the content of reduced nucleotide NADH, under conditions of nitrogen fixation, was higher in the mutant strain than in the wild type. This reflects a decrease in TCA cycle efficiency, which could explain the poor growth of the *phbC* mutant strain. A *phbC* mutant strain of *R. etli* also displays an increased amount of reduced NADH cofactors and a reduced ability to oxidize various substrates (9). In the *Azotobacteriaceae*, PHB has been suggested to be a redox regulator (1, 41). Thus, PHB metabolism appears essential for maintaining a pool of NADH preventing inhibition of TCA cycle enzymes. This model of TCA cycle regulation proposed for *R. etli* and *Azotobacter beijerinckii* is likely to apply to *A. caulinodans*.

The Nif$^-$ phenotype of the *phbC* mutant of *A. caulinodans* in the free-living state was due to an impaired *nifA* expression, whereas the Fix$^-$ phenotype results mainly from the lack of bacteroids. When *nifA* was transcribed from a constitutive promoter, neither the PHB accumulation nor the nucleotide content was restored to the mutant strain. This explains why the nitrogenase activity can only be partially restored in the free-living state, while the growth is strongly impaired in the presence or absence of ammonia.

The nodulation capability of the mutant appeared unaffected, but most of the nodules induced by the CA430 strain were small and did not contain bacteroids. The Fix^+ nodules observed among the Fix^- nodules on roots of plants inoculated with the mutant strain may result from revertants. The Fix^- phenotype of CA430 contrasts with the Fix^+ phenotype in symbiosis of *R. etli* and *S. meliloti* (9, 37, 47) *phbC* mutants. This shows that PHB turnover in *A. caulinodans* is essential for growth both in the free-living state and during symbiosis. In addition, PHB turnover is required for nitrogen fixation, as its absence inhibits *nifA* transcription.

The inhibition of NifA synthesis was one of the most intriguing properties of the *phbC* mutant of *A. caulinodans*. It was suggested that the alteration in the redox state and/or in the pool of ATP observed in the *phbC* mutant is responsible for the alteration of the activity of one of the regulatory proteins involved in *nifA* transcription. However, inactivation of NtrBC and NtrYX can be ruled out since *nifA* transcription in *ntr* mutants is only partly affected (35). FixK, the other regulatory protein that controls *nifA* expression, is active, as shown by the expression of a *fixN-lacZ* fusion in CA430. Interestingly, the *nifA* deletion, which allows escape from NrfA control, also allowed escape from *phbC* mutation. This suggests a mode of control of gene expression independent of the promoter region as reported in the case of NrfA. It is unknown whether the *phbC* mutation acts on NrfA or on another unidentified factor. NrfA, in addition to a role in *nifA* translation, may be involved in *nifA* transcription via an effect on the chromosome structure (20). In *A. caulinodans*, DNA supercoiling of *nifA* promoter, mediated by DNA gyrase, is necessary for *nifA* transcription (40). In vitro and in vivo studies of DNA gyrase of *E. coli* show that its activity depends on the ATP/ADP ratio (30, 48). Thus, the low ATP/ADP ratio in the *phbC* mutant of *A. caulinodans* may inhibit the DNA gyrase, thereby specifically affecting the DNA supercoiling of the *nifA* promoter, and consequently inhibit *nifA* transcription. To date, the control of *nifA* expression by NrfA and in response to PHB metabolism is unique to *A. caulinodans*, whereas other rhizobia impaired in PHB synthesis are Nif^+.

In conclusion, the PHB turnover reflects the potential of the cell to adapt anabolism under nitrogen fixation conditions to the availability in carbon and reducing equivalents. Besides a well-established metabolic role of PHB as a redox regulator to maintain optimal cell growth, we propose another mechanism of control through the expression of key regulatory genes that could explain the severe growth defect of the *A. caulinodans phbC* mutant strain.

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