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Poly(3-hydroxybutyrate) hyperproduction by a global nitrogen regulator NtrB mutant strain of *Paracoccus denitrificans* PD1222

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One sentence summary: Overproduction of short-length polyhydroxyalkanoates by the *P. denitrificans* NtrB mutant grown with nitrate has been shown by fluorescence and transmission electron microscopy. This hyperaccumulation of polyhydroxyalkanoates has been associated with increased phaC gene expression, determined by qRT-PCR, and an elevated intracellular accumulation of acetyl-CoA measured by fluorescence.

Editor: Tim Daniell

ABSTRACT

Paracoccus denitrificans PD1222 accumulates short-length polyhydroxyalkanoates, poly(3-hydroxybutyrate), under nitrogen-deficient conditions. Polyhydroxybutyrate metabolism requires the 3-ketoacyl-CoA thiolase PhaA, the acetoacetyl-CoA dehydrogenase/reductase PhaB and the synthase PhaC for polymerization. Additionally, *P. denitrificans* PD1222 grows aerobically with nitrate as sole nitrogen source. Nitrate assimilation is controlled negatively by ammonium through the two-component NtrBC system. NtrB is a sensor kinase that autophosphorylates a histidine residue under low-nitrogen concentrations and, in turn, transfers a phosphoryl group to an aspartate residue of the response regulator NtrC protein, which acts as a transcriptional activator of the *P. denitrificans* PD1222 *nasABGHC* genes. The *P. denitrificans* PD1222 NtrB mutant was unable to use nitrate efficiently as nitrogen source when compared to the wild-type strain, and it also overproduced poly(3-hydroxybutyrate). Acetyl-CoA concentration in the *P. denitrificans* PD1222 NtrB mutant strain was higher than in the wild-type strain. The expression of the *phaC* gene was also increased in the NtrB mutant when compared to the wild-type strain. These results suggest that accumulation of poly(3-hydroxybutyrate) in the NtrB mutant strain of PD1222 responds to the high levels of acetyl-CoA that accumulate in the cytoplasm as consequence of its inability to efficiently use nitrate as nitrogen source.

Keywords: acetyl-CoA; nitrogen regulator NtrB; nitrate assimilation; *Paracoccus*; PhaC synthase; polyhydroxyalkanoate

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INTRODUCTION

Prokaryotic microbes may accumulate cytoplasmic biopolymers such as polyhydroxyalkanoates (PHAs) that act as carbon and energy storage material in response to nutrient imbalance, e.g. under conditions where excess carbon sources are available (García *et al.* 1999; Madison and Huisman 1999; Prieto *et al.* 2007). PHAs are biodegradable thermoplastics consisting of 3-hydroxycarboxylic acid units that show a great potential as replacement for petroleum-based plastics. PHAs are of significant interest in biotechnology because they are environmental eco-friendly, non-toxic, biocompatible and constitute a source for a commercially useful pool of chiral monomers (Muhammadi, Muhammad and Shafqat 2015). Design of efficient and economic biological processes has become a challenge and key steps such as polyhydroxyalknoate production processes with pure or mixed cultures, from raw microbial process development to downstream processing, are currently optimized (Kourmentza *et al.* 2017). It is worth mention that PHA production based on raw materials requires the use of suitable microbial strains, non-hazardous methods for PHA recovery and reutilization of waste streams from the PHA production process (Koller *et al.* 2017). There are two distinct groups: the short-chain-length polyhydroxyalkanoates (scl-PHAs) composed of units from three to five carbon atoms and the medium-chain-length polyhydroxyalkanoates (mcl-PHAs) that contain units with six to fourteen carbons (Madison and Huisman 1999; Prieto *et al.* 2007; Chen 2009). The PHA biosynthetic pathway includes the formation of hydroxyacyl-CoA (HA-CoA) and its polymerization through the PHA synthase-catalyzed reaction (Liebergesell, Rahalkar and Steinbüchel 2000; Rehm 2007). Type I, III and IV synthases often use scl-HA-CoA for PHA polymerization (Huisman *et al.* 1991; Matsusaki *et al.* 1998; Schubert, Steinbüchel and Schlegel 1998), whereas type II enzymes utilize mcl-HA-CoA as substrate (McCool and Cannon 2001; Ashby, Solaiman and Foglia 2002; Hang *et al.* 2002; Satoh *et al.* 2002; Chen *et al.* 2004). In addition to PHA synthases involved in PHA polymerization, phasins are proteins with a regulatory and structural role that are usually associated with PHA granules (Jendrossek and Handrick 2002; Pötter and Steinbüchel 2005; Chen 2009; Jendrossek 2009; Galán *et al.* 2011).

Most of the bacterial strains that produce PHAs are Gram negative, but several genera of Gram-positive bacteria are also able to produce these polymers (Muhammadi, Muhammad and Shafqat 2015). Among Gram-negative bacterial strains, one group including *Cupriavidus necator* (Vandamme and Coenye 2004), *Azospirillum brasilense* (Sun *et al.* 2000) and *Paracoccus denitrificans* produces scl-PHAs, while pseudomonads mainly produce mcl-PHA. A few strains of *Pseudomonas* synthesize a mixture of scl- and mcl-PHAs, such as *Pseudomonas* sp. 61-3, *Pseudomonas oleovorans* B-778, *Pseudo. stutzeri* 1317 and *Pseudo. pseudoalcaligenes* YS1 and CECT5344 (Matsusaki *et al.* 1998; Ashby, Solaiman and Foglia 2002; Hang *et al.* 2002; Chen *et al.* 2004; Manso *et al.* 2015). This capability may require two separate biosynthetic pathways, for scl-PHA, mainly poly(3-hydroxybutyrate) and mcl-PHA production, with different PHA synthases (Chanasit *et al.* 2016). The poly(3-hydroxybutyrate) biosynthetic pathway consists of three enzymatic reactions, the condensation of two acetyl-coenzyme A (acetyl-CoA) molecules into acetoacetyl-CoA by the 3-ketoacylCoA thiolase PhaA, the NADPH-dependent reduction of acetoacetyl-CoA to (R)-3-hydroxybutyryl-CoA by the (R)-specific acetoacetyl-CoA dehydrogenase/reductase PhaB and the (R)-3-hydroxybutyryl-CoA monomer polymerization into poly(3-hydroxybutyrate) by the poly(3-hydroxybutyrate) polymerase PhaC (Muhammadi, Muhammad and Shafqat 2015). In addition, poly(3-hydroxybutyrate) can be synthesized *de novo* from fatty acid biosynthesis and β-oxidation pathways, with sugars and fatty acids as initial substrates (Satoh *et al.* 2002; Chanasit *et al.* 2016). In the case of mcl-PHAs synthesis, two pathways are possible. The first occurs when the intermediates from the fatty acid *de novo* biosynthesis are used for PHA biosynthesis through a transacylase. The second pathway takes place when the carbon source is oxidized through the fatty acid β-oxidation pathway, whereby the (R)-specific enoyl-CoA hydratase catalyzes the oxidation of enoyl-CoA to (R)-3-hydroxyacyl-CoA, which is used as substrate for the mcl-PHA synthase PhaC (Fukui, Shiomi and Doi 1998; Hoffmann *et al.* 2002).

Paracoccus denitrificans PD1222 can grow with nitrate either aerobically as the sole nitrogen source (nitrate assimilation) or under anaerobic conditions (denitrification) by using it as both nitrogen source and electron acceptor (Richardson *et al.* 2001). The *P. denitrificans* nitrate assimilation system (NAS) is encoded by the *nasABGHC* gene cluster that includes the structural genes for nitrate and nitrite transport as well as the catalytic subunits of the nitrate and nitrite reductases (Gates *et al.* 2011; Luque-Almagro *et al.* 2011). Ammonium repression of the *P. denitrificans nasABGHC* genes is dependent on the Ntr system. Mutational analysis of the *P. denitrificans ntrBCYX* genes shows that while *ntrBC* genes are required for nitrate assimilation, *ntrYX* genes can only partially restore growth on nitrate in the absence of *ntrBC* genes (Luque-Almagro *et al.* 2017).

In this work, the capacity of a *P. denitrificans* PD1222 NtrB mutant to overproduce poly(3-hydroxybutyrate) has been investigated through transmission electron microscopy, poly(3-hydroxybutyrate) yield recovery, *phaC* gene expression analysis and determination of acetyl-CoA levels. These results demonstrated that the NtrB mutant overproduces poly(3-hydroxybutyrate) when growing with nitrate as the sole nitrogen source in response to an increased concentration of cytoplasmic acetyl-CoA.

MATERIALS AND METHODS

Bacterial strains and growth conditions

Paracoccus denitrificans PD1222 wild-type, and NtrB and NtrY mutant strains were routinely cultured under aerobic conditions at 30°C in a defined mineral salt medium (Harms *et al.* 1985) with shaking at 225 rpm. Ten millimolar potassium nitrate and/or 10 mM ammonium chloride were used as nitrogen source(s) and 30 mM sodium succinate was the sole carbon source in media adjusted to pH 7.2. In all cases, an aerobic overnight culture, prepared from a frozen stock in mineral salt medium supplemented with 10 mM ammonium chloride was centrifuged and used as inoculum. Spectinomycin was used as antibiotic at 25 μg mL^{-1} concentration in the cultures of the wild-type strain, NtrB and NtrY mutants, because the wild-type (parental) strain was a spontaneous mutant resistant to this antibiotic. The NtrB mutant was also cultured with 25 μg mL^{-1} kanamycin, because it harbors a kanamycin-resistant cassette inserted into the *ntrB* gene, and the NtrY mutant was also cultured with 50 μg mL^{-1} streptomycin, because a streptomycin-resistant cassette was inserted into the *ntrY* gene (Luque-Almagro *et al.* 2017).

Analytical determinations

Cell growth was routinely measured by estimating protein concentration in whole cells (Shakir *et al.* 1994) because PHAs display strong absorbance at 600 nm. PHAs were detected by fluorescence using 1 mL aliquots of each culture and cells were

stained with the Nile red reagent. PHAs fluoresced maximally at excitation wavelength between 520 and 550 nm, with emission wavelength between 600 and 620 nm (Zuriani *et al.* 2013). Nitrate concentration was determined spectrophotometrically as described previously (Cawse 1967).

Transmission electron microscopy

Cells were grown aerobically with nitrate as the sole nitrogen source. Aliquots were stained by Nile red to identify the highest fluorescence intensities, and at that point they were harvested, washed twice in M9 minimal medium and fixed for 24 h in 2% (w/v) glutaraldehyde in the same solution. Cells were suspended in 1% (w/v) OsO_4 for 1 h, gradually dehydrated in acetone 30, 50, 70, 90 and 100% (v/v), 30 min each, and finally treated with propylene oxide (two changes, 10 min each). Afterwards, cells were embedded sequentially into 2:1, 1:1, 1:2 propylene oxide-resin. Ultrathin sections (thickness 50 nm) were cut with a Leica Ultracut R ultramicrotome (Leica Inc, Buffalo, USA) using a diatome diamond knife. The sections were picked up with 200 mesh cupper grids coated with a layer of uranyl acetate for 2 min and lead citrate for 3 min and subsequently observed in a Jeol JEM-1400 (Tokyo, Japan) transmission electron microscope with an accelerating voltaje of 80 kV (Manso *et al.* 2015). These analyses were carried out by using the microscopy facilities at the Central Service for Research Support (SCAI), University of Córdoba (Spain).

Poly(3-hydroxybutyrate) polymers extraction

Paracoccus denitrificans strains (350-mL cultures) were grown aerobically with nitrate as the sole nitrogen source, and the cells were harvested after reaching the highest fluorescence intensities when stained with Nile red. Half of the cultures were used to determinate cell dry weight and the other half was used to isolate scl-PHA. For this purpose, cell pellets were resuspended in 10 mL chloroform and broken by cavitation with ultrasound (5 pulses for 20 s at 70 W). After centrifugation for 5 min at 6000× *g*, chloroform soluble phase was recovered from the bottom and it was added, slowly and with agitation, to a clean tube containing five volumes of precooled methanol. Chloroform (additional 10 mL) was used to resuspend the insoluble phase, and a second extraction step was carried out by incubation for 30 min at 65°C. This final mixture was placed on ice for 30 min. Finally, the precipitated scl-PHA was recovered by centrifugation at 8000× *g* for 30 min. The pellet was air dried and resuspended in 500 μL of chloroform. Finally, scl-PHA was dried at 65°C overnight and recovered to estimate scl-PHA dry weight. This method was a modified protocol previously described (Manso *et al.* 2015).

Determination of acetyl-CoA concentration

Paracoccus denitrifcans wild-type strain and NtrB mutant were cultured aerobically with nitrate, ammonium or nitrate plus ammonium as nitrogen source(s) and harvested by centrifugation when displayed the highest fluorescence intensities (staining with Nile red). Cells were broken by cavitation (3 pulses of 5 s at 90 W) and centrifuged at 20 000× *g*. Supernatants were deproteinized by using 10% trichloroacetic acid. After 5 min incubation on ice, samples were centrifuged at 20 000× *g* and supernatants were recovered for further analysis. Acetyl-CoA was measured from deproteinized total extracts by using the Acetyl-Coenzyme A Assay Kit (Sigma) according to the instructions provided by the manufacturer. The concentration of acetyl-CoA was estimated by using calibration plots previously elaborated with a stock solution of acetyl-CoA.

Quantification of *P. denitrificans phaC* synthase gene expression

Paracoccus denitrificans cells were washed in a buffer containing 25 mM Tris-HCl (pH 8.0) with 1% glucose and 10 mM EDTA (ethylenediaminetetraacetic acid). RNA isolations were performed using the Qiagen RNA extraction kit (RNeasy midi kit). DNase incubation was carried out in the column with RNase-free DNase set (Qiagen) and an additional post-column treatment was required with DNase I (Ambion). The concentration and purity of the RNA samples were measured by using an ND1000 spectrophotometer (Nanodrop Technologies). Synthesis of total cDNA was achieved in a 20 μL final volume containing 500 ng RNA, 0.7 mM dNTPs, 200 U SuperScript II Reverse Transcriptase (Invitrogen) and 3.75 mM random hexamers (Applied Biosystems). Samples were initially heated at 65°C for 5 min and then incubated at 42°C for 50 min, followed by incubation at 70°C for 15 min. The cDNA was purified using Favorprep Gel/PCR purification kit (Favorgen) and the concentration was measured using a Nanodrop. The iQ5 Multicolour Real-Time PCR Detection System (Bio-Rad) was used in a 25 μL reaction (final volume), containing 2 μL of diluted cDNA (12.5, 2.5 and 0.5 ng), 0.2 μM of each primer; PhaCR: 5′-TCTTCTGCAATGGCGAGGACGAGAC-3′ and PhaCF: 5′-CGAGCACCTGCCAGGCGACC-3′ and 12.5 μL of iQ SYBR Green Supermix (Bio-Rad). Target cDNAs and reference samples were amplified three times in separate PCR reactions. Samples were initially denatured by heating at 95°C for 3 min, followed by 40 cycles of amplification (95°C, 30 s; test annealing temperature, 60°C, 30 s; elongation and signal acquisition, 72°C, 30 s). For relative quantification of the fluorescence values, a calibration curve was made using dilution series from 80 to 0.008 ng of *P. denitrificans* PD1222 genomic DNA sample. Data were normalized using *dnaN* as housekeeping gene with *dnaN*-1F': 5′-CATGTCGTGGGTCAGCATAC-3′ and *dnaN*-1R': 5′-CTCGCGACCATGCATATAGA-3′ primers.

Statistics

Statistical analyses were performed using SPSS v 21.0.0.0. Student's *t*-test (two-tailed) was applied for experiments involving pairwise comparisons.

RESULTS

PHA gene cluster arrangements

The *P. denitrificans* PD1222 genome contains two gene clusters involved in short-length PHA metabolism (Fig. 1). The synthase *phaC* gene is located together with the depolymerase *phaZ* gene, the phasin *phaP* gene and the regulatory *phaR* gene, whereas the 3-ketoacylCoA thiolase *phaA* and the acetoacetyl-CoA dehydrogenase/reductase *phaB* genes are present at a different *locus* (Maehara *et al.* 2002). In other α-proteobacteria, such as *A. brasilense*, and in the β-proteobacterium *C. necator*, the *phaC* gene is clustered together with the *phaA*, *phaB* and *phaR* genes. In *Pseudo. oleovorans*, the PHA gene cluster includes two synthase-encoding genes, whereas in *P. pseudoalcaligenes* CECT5244 there are three synthase genes, the *phaC* gene involved in scl-PHA synthesis, and the *phaC1* and *phaC2* genes required for mcl-PHA synthesis, which are located in two different clusters (Fig. 1). The

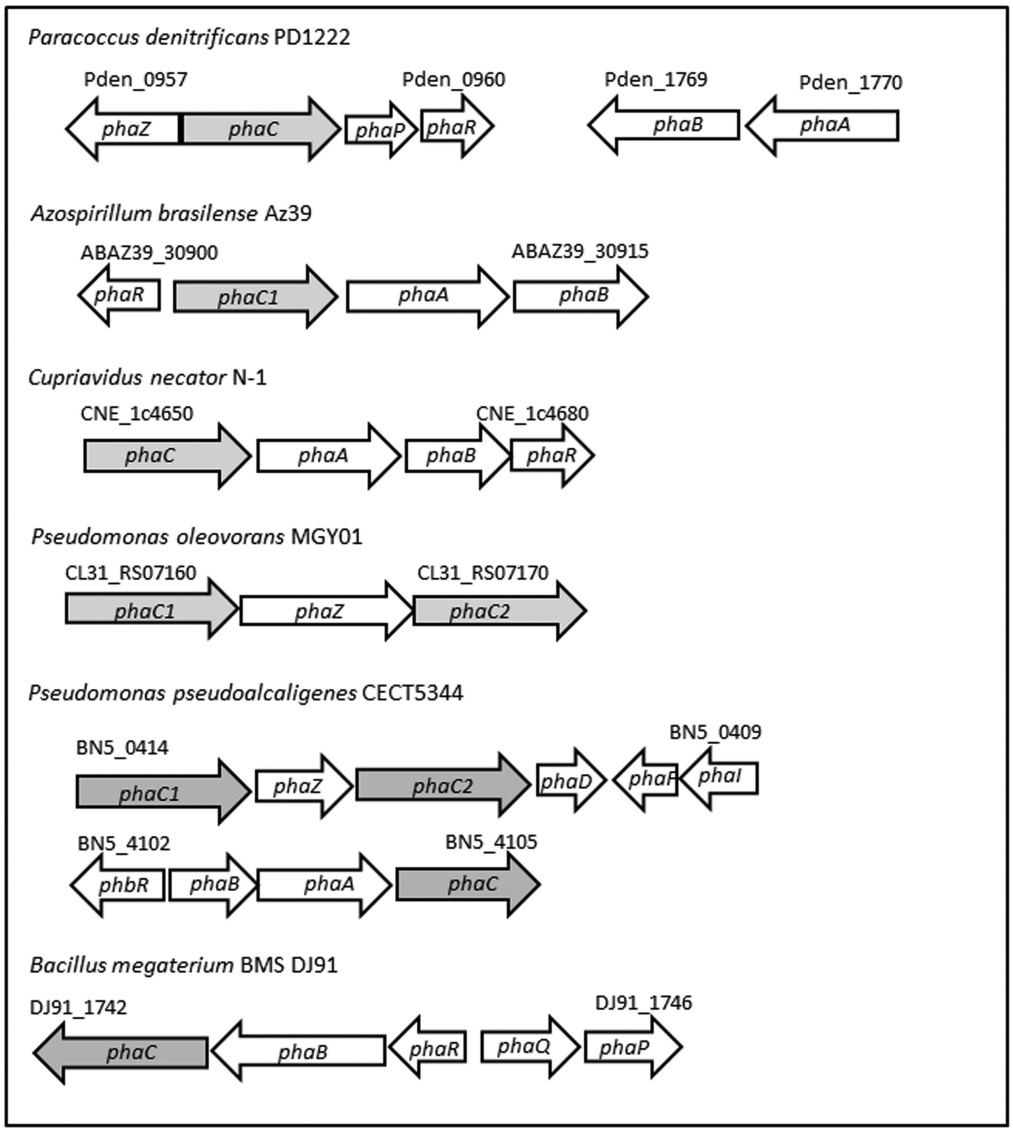

Figure 1. PHA gene cluster arrangements in different bacterial strains. The *P. denitrificans* PD1222 *pha* gene cluster includes the depolymerase *phaZ* (Pden_0957), the synthase *phaC* (Pden_0958), the phasin *phaP* (Pden_0959) and the regulatory *phaR* (Pden_0960) genes. The acetoacetyl-CoA dehydrogenase/reductase *phaB* (Pden_1769) and the β-ketoacyl-CoA thiolase *phaA* (Pden_1770) genes are arranged in a different cluster.

P. denitrificans PD1222 PhaC (ABL69069) enzyme belongs to the class I of synthases for scl-PHA production, and shows homology (62% identity) with other class I synthases present in other α-proteobacteria such as *Rhodobacter capsulatus* (WP_023918062). However, *P. denitrificans* PD1222 PhaC showed low similarity to *A. brasilense* PhaC (AIB16267.1) and only shared 37% identity with the *C. necator* PhaC synthase (AEI76811.1). In the Gram-positive *Bacillus megaterium*, the *pha* gene cluster contains an additional gene, *phaQ*, which codes for a new class of poly(3-hydroxybutyrate)-responsive repressor that regulates other *pha* genes (Lee *et al.* 2004).

Analysis of poly(3-hydroxybutyrate) production in the *P. denitrificans* PD1222 wild-type and NtrB and NtrY mutants

Two *P. denitrificans* mutant strains defective in the *ntrB* and *ntrY* genes have been previously generated (Luque-Almagro *et al.* 2017). The wild-type strain of *P. denitrificans* PD1222 grew well with nitrate as the sole nitrogen source, and almost consumed all nitrate supplied after 18 h growth. The NtrY mutant also consumed all nitrate, and only showed a slightly slower growth rate when compared to the wild-type strain (Fig. 2). By contrast, the NtrB mutant of *P. denitrificans* showed reduced growth rate on nitrate with a lag phase substantially larger than that observed for the wild-type strain. When the scl-PHA production was analyzed by using the Nile red staining method (Zuriani *et al.* 2013), fluorescence was detected in all strains, but the maximal intensity level was lower in both wild-type and NtrY mutant strains than in the *P. denitrificans* PD1222 NtrB mutant (Fig. 2). The fluorescence intensity in the NtrB mutant was only increased with nitrate as the sole nitrogen source, while with ammonium or ammonium plus nitrate as nitrogen source(s) the NtrB mutant presented a low level of fluorescence similar to that shown by the wild-type and NtrY mutant strains (not shown). When cultures of *P. denitrificans* wild-type, NtrB and NtrY mutants reached their maximal fluorescence intensities, cells were subjected to transmission electron microscopy (Fig. 3). The scl-PHA gran-

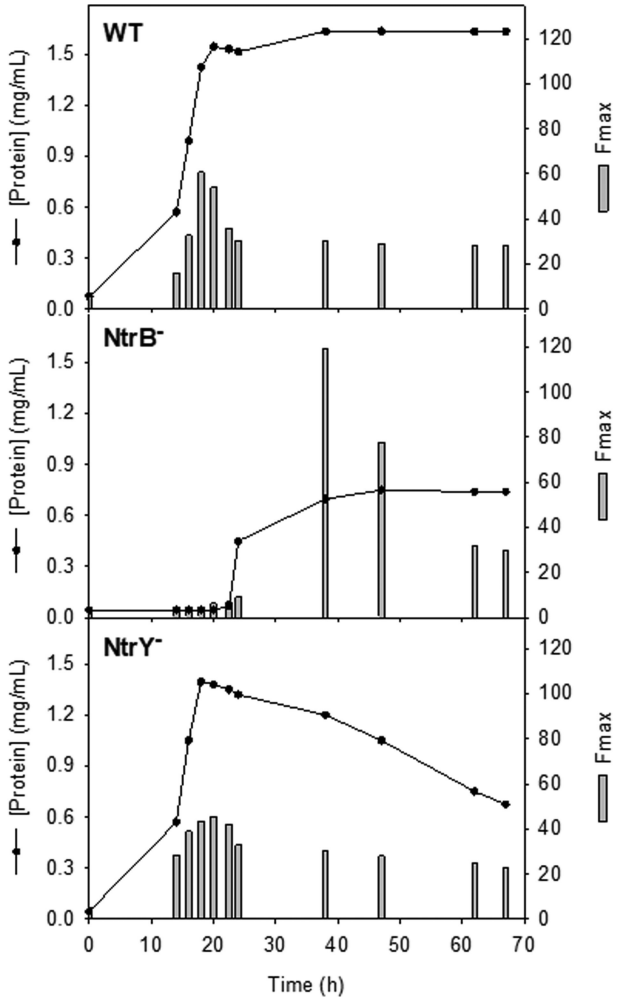

Figure 2. PHA production by the *P. denitrificans* PD1222 wild-type strain and the NtrB and NtrY mutant strains. Cells were cultured in media with nitrate as the sole nitrogen source and bacterial growth (measured as protein concentration) and PHA accumulation (maximal fluorescence emission, Fmax, after Nile red staining) were determined along the growth curves.

ules were extracted from the *P. denitrificans* PD1222 wild-type and NtrB mutant strains, and whereas the *P. denitrificans* wild-type strain accumulated about 17% of its dry weight as poly(3-hydroxybutyrate), the NtrB mutant hyperaccumulated poly(3-hydroxybutyrate) where the polymer represented about 93% of the cell dry weight (Table 1).

Analysis of *phaC* gene expression and acetyl-CoA levels in *P. denitrificans* PD1222 wild-type strain and NtrB mutant

Paracoccus denitrificans PD1222 wild-type strain and NtrB mutant were grown with nitrate, nitrate plus ammonium or ammonium as nitrogen source(s) until they reached their highest level of fluorescence when stained with Nile red. Cells were harvested by centrifugation, total RNA was extracted, and cDNA was synthetized to perform quantitative analysis of *phaC* gene expression by quantitative real-time polymerase chain reaction. In the wild-type strain, maximal expression of the *phaC* gene was observed in cells grown with nitrate plus ammonium, but in the NtrB mutant, the *phaC* gene was more highly expressed when nitrate was used as the sole nitrogen source (Fig. 4).

Acetyl-CoA concentration was also determined in both *P. denitrificans* wild-type and NtrB mutant cells grown with nitrate, nitrate plus ammonium or ammonium as nitrogen source(s). Cells were stained with Nile red to evaluate the highest level of fluorescence intensity, and at that point they were harvested by centrifugation and broken by cavitation. After centrifugation, supernatants corresponding to the soluble cell fraction were used to determine acetyl-CoA concentration. The wild-type strain and the NtrB mutant showed a similar acetyl-CoA content either in the presence of ammonium as sole nitrogen source or with ammonium plus nitrate as nitrogen sources. However, when nitrate was the sole nitrogen source, the NtrB mutant strain accumulated a higher concentration of acetyl-CoA than the wild-type strain (Fig. 5).

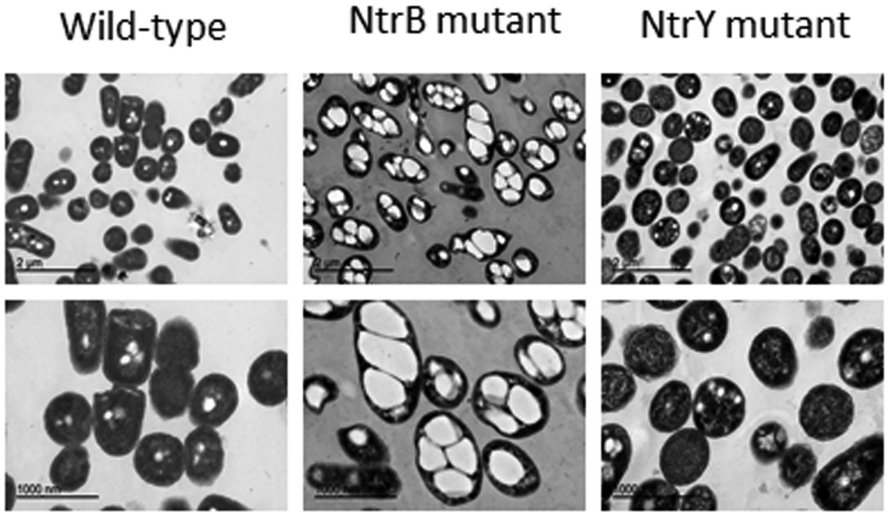

Figure 3. Transmission electron microscopy images from *P. denitrificans* PD1222 wild-type strain and NtrB and NtrY mutants. Cells were cultured with nitrate as the sole nitrogen source, harvested by centrifugation when they reached their maximal fluorescence intensities (stained with Nile red) and prepared as indicated in Materials and methods.

Table 1. Polyhydroxyalkanoate (scl-PHA) extraction of *P. denitrificans* wild-type and NtrB mutant strains.

Strain	A_{600}	[Protein] (mg/ml)	Cells dry weight (mg)*	PHB dry weight (mg)*	% cell dry weight
Wild-type	0.73 ± 0.03	0.75 ± 0.02	10 ± 0.23	1.66 ± 0.30	16.66 ± 3.01
NtrB	0.83 ± 0.02	0.66 ± 0.01	10 ± 0.17	9.26 ± 0.81	92.66 ± 8.12

*xThree independent replicates (for each bacterial strain) were statistically analyzed with a significative difference within samples $P < 0.01$.

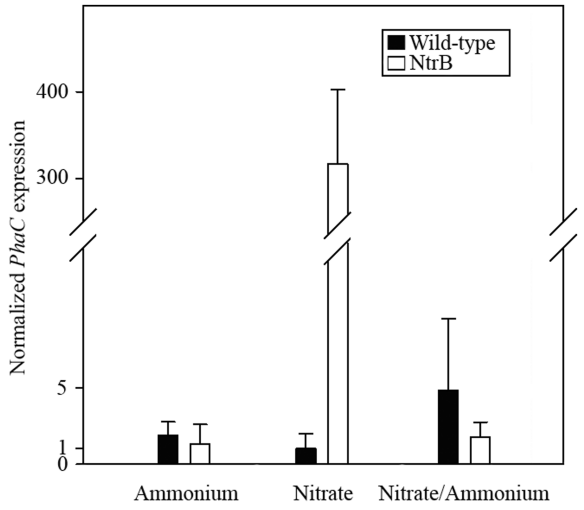

Figure 4. Quantification of the *P. denitrificans* PD1222 *phaC* gene expression in the wild-type strain and the NtrB mutant of *P. denitrificans* PD1222. Strains were cultured with nitrate, ammonium or nitrate plus ammonium as nitrogen source(s), and *phaC* gene expression was analyzed as indicated in Materials and methods. Data were normalized relative to expression of the housekeeping gene *dnaN*.

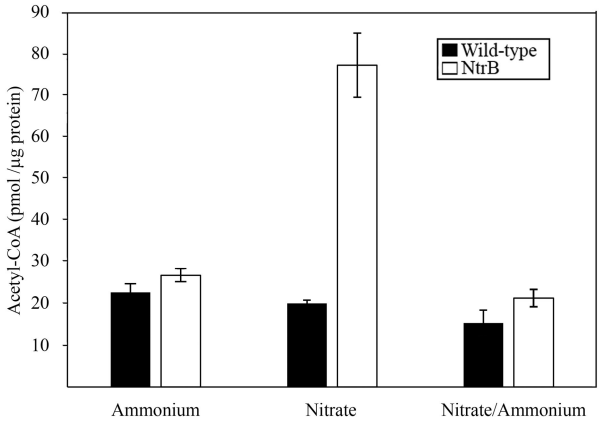

Figure 5. Determination of intracellular acetyl-CoA concentration in *P. denitrificans* PD1222 wild-type and NtrB mutant strains. Cells were cultured with nitrate, ammonium or nitrate plus ammonium as nitrogen source(s), harvested by centrifugation and broken by cavitation. Acetyl-CoA was determined in the cytoplasmic fraction as specified in Materials and methods.

DISCUSSION

Paracoccus denitrificans PD1222 is a soil denitrifying bacterium that can also grow aerobically with nitrate as the sole nitrogen source. Under these culture conditions, the *nasABGHC* genes for nitrate assimilation are induced. Nitrate assimilation is a widespread metabolic process among proteobacteria that usually is controlled at the transcriptional level by nitrate induction and by ammonium repression (Luque-Almagro *et al.* 2011). The NAS is positively regulated by nitrate/nitrite through the NasTS regulatory proteins, where NasT is a transcriptional antiterminator and NasS a nitrate/nitrite sensor. Recently, it has been demonstrated that nitrate not only activates transcription of the *nasABGHC* genes of *P. denitrificans* PD1222, but also exerts a positive control at the level of translation (Luque-Almagro *et al.* 2017). Expression of the *P. denitrificans nasABGHC* genes is also activated under low-nitrogen conditions (in the absence of ammonium) mainly through the global nitrogen regulatory NtrBC system. NtrB is a sensor kinase that autophosphorylates a key histidine residue under low-nitrogen conditions and it transfers a phosphoryl group to the NtrC response regulator protein on a specific aspartate residue, which in turn activates transcription of different genes required for nitrogen metabolism (Luque-Almagro *et al.* 2017). *Paracoccus denitrificans* PD1222 presents an additional two-component regulatory system, NtrYX, which seems not to play an important role in regulation of nitrate assimilation (Luque-Almagro *et al.* 2017). *Paracoccus denitrificans* PD1222 wild-type cells accumulate poly(3-hydroxybutyrate) under aerobic-nitrate culture conditions because nitrate is a poor nitrogen source when compared to ammonium or organic nitrogen. However, the NtrB mutant strain hyperaccumulated poly(3-hydroxybutyrate) under this condition, but this characteristic was not shared by the NtrY mutant (Figs 2 and 3).

Many prokaryotic microbes accumulate PHAs, mainly as poly(3-hydroxybutyrate), but only a few of them can be used for industrial production purposes. As suitable candidate for PHA production, the microorganism should display fast growth with cheap carbon sources, and high conversion efficiency of substrate into product for high PHA accumulation in the cells, which also need to be easily broken before PHA isolation (Chen 2002). These polymers have attracted commercial interest because, in addition to be completely biodegradable, they are non-toxic, biocompatible and, also, sources for commercially useful pool of chiral monomers (Muhammadi, Muhammad and Shafqat 2015). Two gene clusters involved in PHA metabolism have been described in *P. denitrifcans* PD1222 (Fig. 1). Although polyhydroxyalknoates production has been extensively studied in Gram-negative bacteria, the PHA isolated from the Gram-positive *B. megaterium* and *B. subtilis* have been reported to display several advantages such as the lack of the toxic lipo-polysaccharides and the expression of self-lysing genes on completion of PHA biosynthetic process that enable these bacteria to release these compounds and thus compete as potential candidate for commercial production of PHAs (McCool and Cannon 2001; Singh, Patel and Kalia 2009).

The reduction of the production costs and the utilization of industrial wastes via their bioconversion into bioplastics are currently in the spotlight to carry out PHA production at the industrial scale. In this sense, pseudomonads are considered one of the best source for PHAs production because they can accumulate high amounts in the cell (more than 90% of cell dry weight), they can use a wide variety of industrial wastes as carbon source and some of strains can co-produce scl- and mcl-PHA (Tortajada, Ferreira da Silva and Prieto 2013; Kourmentza *et al.* 2015; Manso *et al.* 2015; Chanasit *et al.* 2016). However, high levels of poly(3-hydroxybutyrate) production by

P. denitrificans DSMZ 413 has been reported in cells cultured in batch reactor with glycerol as carbon source and yeast extract as nitrogen source, with a poly(3-hydroxybutyrate) content of 72% of cell dry weight (Kalaiyezhini and Ramachandran 2015). In this sense, the NtrB mutant of *P. denitrificans* is a suitable candidate to produce scl-PHAs because this strain can accumulate poly(3-hydroxybutyrate) up to 93% of the cell dry weight (Fig. 3 and Table 1).

It has been previously described that *P. denitrifcans* accumulates scl-PHAs, mainly poly(3-hydroxybutyrate), under nitrogen-deficient conditions (Kojima *et al.* 2004). *Paracoccus denitrifcans* PD1222 *pha* genes were induced at the transcriptional level by low-nitrogen conditions when cells were cultured with nitrate as the sole nitrogen source (Fig. 4). Also, the hyperaccumulation of scl-PHA in the NtrB mutant could be related to a nitrate-dependent increase of the PHA synthase *phaC* gene expression (Fig. 4). Curiously, it has been proposed that *A. brasilense ntrB* and *ntrC* genes are involved in poly(3-hydroxybutyrate) synthesis in the presence of ammonium as nitrogen source (Sun *et al.* 2000). In the case of scl-PHA production in the *P. denitrifcans* NtrB mutant, hyperaccumulation was only observed with nitrate as the sole nitrogen source. Furthermore, induction of *P. denitrificans pha* genes was directly related with an increase in the cytoplasmic acetyl-CoA concentration (Fig. 5). Therefore, under nitrogen-sufficient conditions (i.e. with ammonium as nitrogen source) acetyl-CoA is used in the tricarboxylic acid cycle for energy supply and formation of amino acids. By contrast, under low-nitrogen conditions (i.e. with nitrate as nitrogen source) the energy requirement decreases, and as a consequence acetyl-CoA concentration increases, allowing to be diverted to scl-PHAs synthesis (Kojima *et al.* 2004). This hypothesis was supported by the increased concentration of acetyl-CoA detected in the *P. denitrificans* NtrB mutant (Fig. 5), a strain that is unable to efficiently assimilate nitrate when compared with the wild-type strain.

In summary, we have demonstrated that a *P. denitrificans* NtrB mutant hyperaccumulates scl-PHAs (poly-3-hydroxybutyrate) becoming a potential bacterial strain to overproduce these biodegradable polymers. In cells grown with nitrate, the acetyl-CoA content increases and the PHA synthase *phaC* gene is induced for poly(3-hydroxybutyrate) accumulation.

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