Applied Microbiology and Biotechnology (2018) 102:2693–2707
https://doi.org/10.1007/s00253-018-8806-y

BIOTECHNOLOGICALLY RELEVANT ENZYMES AND PROTEINS

Inactivation of an intracellular poly-3-hydroxybutyrate depolymerase of *Azotobacter vinelandii* allows to obtain a polymer of uniform high molecular mass

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Received: 21 August 2017 / Revised: 16 January 2018 / Accepted: 18 January 2018 / Published online: 12 February 2018

Abstract

A novel poly-3-hydroxybutyrate depolymerase was identified in *Azotobacter vinelandii*. This enzyme, now designated PhbZ1, is associated to the poly-3-hydroxybutyrate (PHB) granules and when expressed in *Escherichia coli*, it showed in vitro PHB depolymerizing activity on native or artificial PHB granules, but not on crystalline PHB. Native PHB (nPHB) granules isolated from a PhbZ1 mutant had a diminished endogenous in vitro hydrolysis of the polyester, when compared to the granules of the wild-type strain. This in vitro degradation was also tested in the presence of free coenzyme A. Thiolytic degradation of the polymer was observed in the nPHB granules of the wild type, resulting in the formation of 3-hydroxybutyryl-CoA, but was absent in the granules of the mutant. It was previously reported that cultures of *A. vinelandii* OP grown in a bioreactor showed a decrease in the weight average molecular weight (Mw) of the PHB after 20 h of culture, with an increase in the fraction of polymers of lower molecular weight. This decrease was correlated with an increase in the PHB depolymerase activity during the culture. Here, we show that in the *phbZ1* mutant, neither the decrease in the Mw nor the appearance of a low molecular weight polymers occurred. In addition, a higher PHB accumulation was observed in the cultures of the *phbZ1* mutant. These results suggest that PhbZ1 has a role in the degradation of PHB in cultures in bioreactors and its inactivation allows the production of a polymer of a uniform high molecular weight.

Keywords Polyhydroxybutyrate · Depolymerase · Bioplastic · Molecular mass

Introduction

Poly-3-hydroxybutyrate (PHB) is a polymer produced by many *Eubacteria* and *Archaea* for storage of carbon and energy. This polymer is intracellularly accumulated in the form of inclusion bodies named PHB granules or carbonosomes (Jendrossek and Pfeiffer 2014). PHB exhibits thermoplastic properties and is also biodegradable and biocompatible, which make it a promising industrial material that has been studied extensively for its application in the production of biodegradable plastics and other products (Chen 2009). Based on its properties of biocompatibility, new attractive applications for PHB have been proposed in the medical and pharmaceutical fields (Nigmatullin et al. 2015; Wu et al. 2009).

The mechanical properties of the polymer are very important for its applications. It is well known that the PHB homopolymer is a plastic with poor properties of stiffness and brittleness (De Koning and Lemstra 1993; Kusaka et al. 1999); however, for several polymers, a high molecular weight is one of the most important factors contributing to have high elastic modulus and high strength (Aoyagi et al. 2003). In the case of PHB, it has been shown that those polymers reaching an ultra-

Electronic supplementary material The online version of this article (https://doi.org/10.1007/s00253-018-8806-y) contains supplementary material, which is available to authorized users.

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high molecular weight (3–11 × 10^6 Da) yield improved, more ductile, and flexible materials, with high tensile strength and high elongation to break, without affecting biodegradability (Aoyagi et al. 2003; Kusaka et al. 1999).

The molecular weight of PHB can be affected by culture parameters, like medium composition, pH, temperature, and aeration conditions (Agus et al. 2010; Asenjo et al. 1995; Bocanegra et al. 2013; Myshkina et al. 2008; Peña et al. 2014; Shimizu et al. 1993). Other factors, such as the relative expression level or activity of the biosynthetic enzymes (Hiroe et al. 2012; Sim et al. 1997), the type of PHB synthase present (Agus et al. 2006; Hiroe et al. 2012), and mutations on this enzyme (Zheng et al. 2006), can also influence the molecular weight of the polymer produced.

PHB and other polyhydroxyalkanoates (PHA) are catabolized through intracellular or extracellular depolymerases, depending on its location. Intracellular PHA depolymerases hydrolyze native PHA granules that contain the polymer in an amorphous state, whereas extracellular PHA depolymerases degrade extracellular granules released from accumulating cells after death, which contain denatured and partially crystalline polymer (Jendrossek and Handrick 2002). The effect of inactivating the PHA depolymerases of *Ralstonia eutropha* on the molecular weight of the PHA produced was recently reported. Inactivation of depolymerase PhaZ6 (PhaZd1) allowed to produce an ultra-high molecular weight PHA (PHBHHx), with an average molecular weight approximately double of that produced by the original strain (Arikawa et al. 2016), showing that PHA depolymerase activity also has a significant influence on the molecular weight of the PHA accumulated.

Azotobacter spp. are able to synthesize a PHB of high molecular weight (Chen and Page 1994; Myshkina et al. 2008, Peña et al. 2014). In these species, the molecular weight of PHB can also be influenced by the medium composition and aeration conditions (Chen and Page 1994; Myshkina et al. 2008; Quagliano and Miyazaki 1997). In bioreactor cultures, *A. vinelandii* is able to produce a PHB of an ultra-high molecular weight of 4800 kDa during the exponential growth phase; however, during the stationary phase, the mean molecular weight decreases to 3600 kDa, coincident with an increased PHB depolymerase activity (Millán et al. 2016).

In *A. vinelandii*, the genetics of PHB biosynthesis and the regulatory elements involved have been widely studied (Castañeda et al. 2000; Hernández-Eligio et al. 2011, 2012; Muriel-Millán et al. 2014; Noguez et al. 2008; Peralta-Gil et al. 2002); however, the process of PHB mobilization is known only at the biochemical level (Manchak and Page 1994). In the genome of this bacterium, there are seven genes putatively coding for PHB depolymerases, but evidence for the role of their products is missing. In the present report, we characterized one of these PHB depolymerases. We demonstrated that it has hydrolytic activity on amorphous but not on crystalline PHB and it additionally has a thiolytic activity on the polymer in the presence of free coenzyme A. We also studied the effect of inactivating this gene on the molecular weight of the polymer produced in a bioreactor and on the amount of PHB produced.

Materials and methods

Bacterial strains, media, and growth conditions

Bacterial strains and plasmids used in this study are listed in Table 1. The *A. vinelandii* strains PhbZ1, PhbZ1-C, PhbZ1-OP, and phbZ1-phbC were deposited in a microbial collection (Colección Nacional de Cepas Microbianas y Cultivos Celulares, CINVESTAV) with codes CDBB-B-2007, CDBB-B-2008, CDBB-B-2009, and CDBB-B-2010, respectively. Inocula were grown on BS medium (Kennedy et al. 1986), washed with 10 mM $MgSO_4$, and transferred to the indicated medium. For PHB production, *A. vinelandii* strains were grown on peptone-yeast (PY) medium supplemented with 2% sucrose as carbon source (PYS; in g l^{-1}, sucrose, 20; yeast extract, 3.0 and peptone, 5.0) (Peña et al. 2014). Liquid cultures were carried out in 250-ml flasks containing 50 ml of medium at 250 rpm and 30 °C. *Escherichia coli* strains were grown at 37 °C on Luria-Bertani medium and M9 medium with glucose 10 g l^{-1}. *A. vinelandii* transformation was carried out as described in Bali et al. (1992). When needed, antibiotics were added at the following concentrations (μg ml^{-1}): for *A. vinelandii* rifampicin, 10; nalidixic acid, 30; kanamycin, 1; and gentamicin, 0.25 and for *E. coli* ampicillin, 100; kanamycin, 30; and spectinomycin, 100.

DNA manipulations, molecular, and microbiological procedures

DNA manipulations were performed according to standard protocols, as described (Sambrook and Russell 2001). Restriction enzymes were obtained from Thermo Scientific and used according to the manufacturer's instructions. DNA sequences were determined by the dideoxy chain termination method (Sanger et al. 1977), using a Perkin-Elmer/Applied Biosystems DNA sequencer. The oligonucleotides used in this work were synthesized at the oligonucleotide synthesis facility at the Instituto de Biotecnología/UNAM.

In order to generate specific DNA fragments suitable for cloning, PCR amplifications were carried out using Taq DNA polymerase (Thermo Scientific). The amplification protocol consisted of 1 cycle at 95 °C for 3 min and 30 cycles at 95 °C for 1 min and 1 min at variable temperatures, depending on the primer alignment temperature, 1 to 3 min at 72 °C, and a final extension for 15 min. The PCR products were purified

Table 1 Bacterial strains and plasmids

Strain or plasmid	Description	Reference or source
A. vinelandii OP	Wild type	Bush and Wilson 1959 ATCC® 13705™
A. vinelandii PhbZ1	UW136 with the *phbZ1*::Gm gene inactivation	This study CDBB-B-2007
A. vinelandii PhbZ1-C	$PhbZ_1$ with plasmid pJG03910 cointegrated in the chromosome	This study CDBB-B-2008
A. vinelandii PhbZ1-OP	OP with the *phbZ1*::Gm gene inactivation	This study CDBB-B-2009
A. vinelandii phbC-phbZ1	PhbZ1 with the *phbC*::Sp gene inactivation	This study CDBB-B-2010
A. vinelandii CS18	UW136 with the *phbC*::Sp gene inactivation	Segura et al. 2003
A. vinelandii UW136	Natural rifampin-resistant mutant of OP, nonmucoid	Bishop and Brill 1977
E. coli BL21DE3	F- *ompT hsdS*B (rB-mB-) *gal dcm rne131* (DE3)	Invitrogene
E. coli DH5α	*supE44 ΔlacU169 hsdR17 recA1 endA1 gyrA96 thi-1 relA1*	Invitrogene
E. coli MG1655	*E. coli* K-12 wild type	ATCC 700926
pBSL98	Source of the gentamycin resistance cassette	Alexeyev et al. 1995
pCS18	Plasmid carrying a *phbC*::Sp mutation	Segura et al. 2003
pET24a+	Expression vector with the selectable marker kanamycinan, C-terminal His*Tag sequence	Novagen
pJET1.2/blunt	Cloning vector	Thermo Fisher Scientific
pJG03910	pET24a + with a 2.7 kb DNA fragment containing the *A. vinelandii phbZ1* gene (*avin03910)* for its expression.	This study
pJG03910::Gm	pJG03910 with the *Sal*I DNA fragment containing the gentamicin cassette from pBSL98 replacing an internal 0.4 kb *Sal*I fragment of *phbZ1*.	This study
$pPHB_{Av}$	Plasmid pTrc99A expressing the *phbBAC* operon from *A. vinelandii*.	Centeno-Leija et al. 2014
pET24a+	Expression vector with the selectable marker kanamycinan, C-terminal His*Tag sequence	Novagen

using the High Pure PCR Product Purification Kit (Roche), digested with the appropriate restriction enzyme(s) and finally purified from agarose gels.

PHB quantification

The quantification of PHB was performed as previously reported (Law and Slepecky 1961). The samples containing PHB were centrifuged for 10 min at 13700*g* and the supernatant was decanted. One milliliter of sodium hypochlorite at 30% was added and incubated 1 h at 37 °C. The PHB was washed with 1 ml of water and centrifuged for 10 min at 13700*g*. This process was repeated with acetone and ethanol and the samples were air dried. Finally, 1 ml of concentrated sulfuric acid was added and the sample was heated for 10 min at 90 °C. The samples were diluted in sulfuric acid, according to the PHB content, and measured spectrophotometrically at 235 nm.

Expression of the *A. vinelandii* PhbZ1 PHB depolymerase in *E. coli*

To express the PhbZ1 PHB depolymerase in *E. coli* BL21DE3, a 2.7-kb fragment, corresponding to the *phbZ1* gene (*avin03910)*, lacking the first and last codons, was amplified by PCR using primers Fw 5′-TAGGTACATATGAG CCAGACTTTTTTTG-3′ and Rv 5′-CTGTCAAAGCTTAG TATTGTCGCTAGGCTGAG-3′ with Phusion high-fidelity Taq polymerase (Thermo Fisher Scientific). The resulting PCR product was digested with *Nde*I and *Hind*III and cloned into plasmid pET24a (invitrogen). The resulting plasmid was named pJG03910. The DNA fragment was sequenced in order to confirm the presence of an intact *phbZ1* gene and the plasmid was transformed into *E. coli* BL21DE3. The expression of the protein was induced by the addition of IPTG (0.5 mM). After the 8-h induction at 27 °C, the cells were centrifuged, resuspended in buffer at 4 °C, and lysed by sonication at 8 kHz at 4 °C in a Vibra Cell (Sonics) in the presence of a protease

inhibitor (complete tablets; Roche Diagnostics). The protein extract was concentrated using Microcon YM-50 centrifugal filters (Amicon) and stored at 4 °C. Protein concentrations were determined by the method of Lowry et al. (1951) using BSA as a standard. SDS-PAGE of the purified protein revealed the expression of a protein.

SDS-PAGE and Western blot assays

Whole-cell extracts were prepared from *E. coli* BL21DE3 cells grown on LB and expressing PhbZ1 from plasmid pJG03910. Five micrograms of protein of each extract was subjected to electrophoresis on SDS-12% polyacrylamide gels and then transferred using a semidry transfer system (Thermo Fisher Scientific) to a nitrocellulose membrane (General Electric). The membranes containing the transferred proteins were blocked in 1% albumin and 0.1% of tween 20 overnight. Immunoblots were performed with anti-His monoclonal antibody (Qiagen) at 1:10,000 dilution. Horseradish peroxidase-conjugated anti-rabbit antibody (Pierce) was used as the secondary antibody at 1:10,000 dilution. Bands on the blotted membranes were developed by incubation with the Western Lightning Chemiluminescence Reagent Plus (Perkin-Elmer).

Construction of *A. vinelandii phbZ1*::Gm^r mutant strains

In order to generate a *phbZ1* mutant derivative of *A. vinelandii* UW136, plasmid pJG03910 was digested with *Sal*I (ThermoScientific) in order to delete a 0.4-kb fragment of the gene. This fragment was replaced by a *Sal*I DNA fragment containing the gentamicin cassette excised from plasmid pBSL98 (Alexeyev et al. 1995), resulting plasmid pJG03910::Gm that is unable to replicate in *A. vinelandii*. Competent cells of *A. vinelandii* UW136 were transformed with this plasmid and a gentamicin-resistant transformant was isolated and confirmed to carry the *phbZ1*::Gm mutation by PCR analysis using primers Fw 5′-AACA GCGAGGAGATCGAGAC-3′ and Rv 5′-CTCC TCGAACGCCTGTACGCCG-3 (data not shown). This strain was named PhbZ1. To construct a *phbZ1* mutant derivative of strain OP, competent cells of this strain were transformed with chromosomal DNA from mutant PhbZ1 and a gentamicin-resistant transformant was isolated and confirmed to carry the mutation by PCR analysis using the same primers (data not shown). This strain was named PhbZ1-OP.

To construct a *phbZ1-phbC* double-mutant derivative of UW136, competent cells of strain PhbZ1 were transformed with plasmid pCS18, which contains a spectinomycin-resistance cassette inserted into the *Kpn*I site of *phbC* (Segura et al. 2003). A spectinomycin-resistant transformant was confirmed to carry the *phbC*::Sp mutation by PCR analysis.

Complementation of mutant PhbZ1

For the complementation of the PhbZ1 mutant, this strain was transformed with plasmid pJG03910 for its co-integration into the chromosome, producing strain PhbZ1-C. The co-integration of the plasmid by a single recombination was verified by PCR analysis using primers Fw 5′- TCGTGTAC CCTCCGCTATAA-3′ and Rv 5′-CGTACAGGCGTTCG AGGAG-3′.

Isolation of native PHB granules

Native PHB (nPHB) granules were purified as previously described (Gebauer and Jendrossek 2006; Handrick et al. 2000). Briefly, *A. vinelandii* cells were grown for 48 h on PYS medium and were collected by centrifugation at 1448 g at 4 °C for 20 min. The cells were resuspended in buffer Tris-HCl 50 mM, pH 7.5, containing 1 mM DTT and were disrupted using a French press. The nPHB granules were separated using two glycerol gradients (first gradient, 87 and 50%, second gradient 87, 80, 60, and 40%) in buffer Tris-HCl 50 mM, pH 7.5. The purified granules were dialyzed with the corresponding reaction buffer. The nPHB granules from the *E. coli* strain MG1655 expressing the *A. vinelandii* PHB biosynthetic operon *phbBAC* from plasmid pPHB$_{Av}$ (Centeno-Leija et al. 2014) were purified in the same way, except that the cells were grown for 24 h in M9 medium.

Production of artificial PHB granules

Commercial PHB (Sigma Aldrich) was used for the generation of artificial PHB (aPHB) granules. One hundred and fifty milligrams per milliliter of PHB was dissolved in heated chloroform at 65 °C for 10 min and 20 volumes of CTAB 50 mM in water were added as surfactant agent. The mix was sonicated three times for 15 s in a Virsonic 60 sonicator and heated at 75 °C to evaporate the chloroform (Horowitz and Sanders 1994). These aPHB granules, nPHB granules produced in *E. coli* expressing plasmid pPHB$_{Av}$, and crystalline PHB (cPHB) granules were used as substrates for the PHB hydrolytic activity determinations with cell extracts of *E. coli* cells expressing PhbZ1 protein from plasmid pJG03910. The activity determinations were done in buffer Tris-HCl 50 mM pH 7.5.

Identification of proteins associated to the granules

For the proteomic analysis of the proteins present in the *A. vinelandii* nPHB granules, cells were grown for 48 h in PYS medium. The granules were isolated as described above. The granule suspension was centrifuged and resuspended in the loading buffer and was subjected to PAGE in 12% SDS. The protein spots were excised and analyzed at the

Laboratorio Universitario de Proteómica of the Instituto de Biotecnología for identification. Briefly, gel plugs were destained, reduced with dithiothreitol, and alkylated with iodoacetamide. The samples were subjected to overnight proteolytic digestion with 10 ng μl^{-1} trypsin at 37 °C (Trypsin Gold, Mass Spectrometry Grade, Promega) and the peptides generated were extracted and desalted with Zip Tip C18 (Millipore).

The tryptic peptide mixture was analyzed by online nanoflow liquid chromatography tandem mass spectrometry (LC-MS/MS) on Accela system (1/20 split-flow) connected to the LTQ Orbitrap Velos instrument (Thermo Fisher Scientific, Bremen, Germany) through a nano-electrospray ionization detector (ESI). The equipment was calibrated with a mixture of ten calibration molecules (Calmix) to allow determination of mass with accuracy within 5 ppm. The peptide mixture was sampled directly onto a capillary column in-house packed with 3-μm C18 beads with a flow of 300 nl min^{-1}. The peptides were eluted with a 120-min gradient from 5 to 70% acetonitrile in 0.1% formic acid. The effluent from the capillary column was directly electrosprayed into the mass spectrometer. For peptide fragmentation, collision-induced dissociation (CID) and high-energy collision dissociation (HCD) were used. Only 2+- and 3+-charged ions were selected for fragmentation events, while singly charged ions, 4+- or higher-charged ions, and ions for which no charge state could be determined were excluded from selection. All mass spectra were acquired in the positive-ion mode. MS data were acquired using a data-dependent method dynamically choosing the most abundant precursor ions from the survey scan for fragmentation. Mass spectra were collected using normalized collision energy of 35%, isolation width of 3.0 (m/z), activation Q of 0.25, activation time of 10 ms, and maximum ion injection time of 10 ms per micro-scan. Dynamic exclusion was used to reduce the redundancy of the data. Dynamic exclusion settings were (i) exclusion list size of 400, (ii) pre-exclusion duration of 30 s, and (iii) exclusion duration of 300 s. The MS data set produced by the amino acid sequence of the corresponding peptides was searched against the National Center for Biotechnology Information (NCBI) non-redundant database using Mascot software (Matrix Science, Boston, MA).

Determination of endogenous PHB hydrolytic activity of nPHB granules

In order to measure the contribution of the depolymerase PhbZ1 to the endogenous PHB hydrolytic activity of the granules, 5 mg of nPHB granules was used per reaction (1 ml) in 50 mM Tris-HCl buffer, pH 7.5. The reaction was incubated at 37 °C for 72 h, with constant agitation at 600 rpm and a sample of 100 μl was collected for the quantification of the remaining amount of PHB by spectrophotometric methods (Law and Slepecky 1961).

Determination of the PHB thiolytic activity

The thiolytic activity of the PhbZ1 protein was measured using nPHB granules, as described previously (Uchino et al. 2007), using 1 mg of granules. The reaction buffer was 50 mM potassium phosphate (pH 7) containing 50 mM DTT. CoA was added to complete 1 mM in a total volume of 1 ml. Of the samples, 100 μl was taken every 30 min. The samples were centrifuged for 3 min at 13700*g* before analysis by high-performance liquid chromatography (HPLC). The HPLC determination of 3-hydroxybutyryl-CoA and CoA was performed essentially as described (Uchino et al. 2007). Samples of 20 μl of the products of the enzymatic reaction were injected onto a Waters Xterra MS C18 column and were eluted with a gradient (0–40% A:B) of water acidified with 1% trichloroacetic acid (solution A) and pure methanol (solution B), at a flow rate of 0.8 ml/min, 1200 psi, at 30 °C with a detector of UV/vis at $\lambda = 260$ nm.

Determination of the PHB synthase activity

For the quantification of PHB synthase activity in crude extracts of *E. coli*-pPHBAv, E coli-JG03910 and *E. coliPET24a*+, the cultures were grown to an OD of 0.4–0.6 and were induced with 0.5 mM IPTG. After 3 h, the cells were washed twice with phosphate buffer (25 mM), pH7, and were disrupted by ultrasonic treatment at 4 °C and centrifuged at 9660 g for 15 min at 4 °C. For the determination of this activity in the *A. vinelandii* strains UW136, PhbZ1, PhbZ1-C, and the *phbC*$^-$ mutants CS18 and PhbZ1-phbC, the cells were grown on PYS medium for 48 h, washed twice with phosphate buffer (25 mM), pH 7, and disrupted by ultrasonic treatment at 4 °C and centrifuged at 9660 g for 15 min at 4 °C. The protein content of all cell extracts was quantified by the method of Lowry et al. (1951). The PHB synthase activity was assayed in all cases spectrophotometrically by quantifying at 412 nm the CoA released in a final volume of 1 ml of 25 mM phosphate buffer at pH 7, with 0.1 mM DTNB, 55 μM B-hydroxybutyryl-CoA, and 1 mg of total protein extract, as described by Millán et al. (2016).

Culture conditions in bioreactor under limited oxygen conditions

Batch cultures of *A. vinelandii* OP and its mutant derivative PhbZ1-OP$^-$ were conducted in a 3-l stirred tank glass bioreactor (Applikon Biotechnology, Netherlands), equipped with two Rushton turbines of 4.5 cm of diameter and operated at 500 rpm, as previously described (Millán et al. 2016). The bioreactor was initially loaded with 1.8 l

of PYS medium. The inoculum was previously grown in an Erlenmeyer flask with 100 ml of medium and incubated on rotatory shaker (New Brunswick Scientific Co., model G25) during 20–22 h at 29 °C and 200 rpm. In all the experiments, the temperature was controlled at 29 ± 0.5 °C and the pH of the medium was adjusted to 7.2 ± 0.09 by the automatic addition of a 2 N NaOH or HCl solutions using a peristaltic pump. The dissolved oxygen tension (DOT) was measured with an Ingold polarographic probe (AppliSens, Applikon, The Netherlands) that was controlled automatically by gas blending, varying the proportions of oxygen and nitrogen in the inflowing gas through two mass flow controllers (Brook Instruments B. V. model 5850F, The Netherlands). Both strains were grown under oxygen limited conditions at DOT = 1.0 ± 0.2% of saturation (Millán et al. 2016) and the results showed are the average of three independent runs.

Analysis of the molecular weight of the PHB produced

For the analysis of PHB molecular weight, the polymer was recovered by using organic solvents as previously described (Millán et al. 2016). The recovered PHB samples were dissolved in chloroform at a concentration of 2–3 mg ml^{-1} and were filtered through a 0.45-μm GHP membrane (PALL Acrodisc, cat. number 21854606). Then, the samples were injected into the HPLC. The HPLC system (Waters Alliance 2695, USA) used for the PHB molecular weight analysis consisted of two columns for gel permeation chromatography (GPC) (Styragel HR5E and HR6, Waters) connected in series and coupled with a refractive index detector (Waters 2414, USA). The mobile phase was chloroform at 30 °C at a flow rate of 0.7 ml min^{-1}. A calibration curve was constructed with polystyrene standards in the range of 2.94×10^3 to 5.97×10^6 g g_{mol}^{-1} and the data was processed with the Empower software.

Results

Identification of PhbZ1 as a PHB granule-associated protein

Using the sequences from several well-characterized PHB depolymerases in a *blast* search in the *A. vinelandii* genome, we found seven genes whose deduced proteins showed significant similarity with these enzymes. Because PHB depolymerases are usually granule-associated proteins (Abe et al. 2005; Kobayashi et al. 2003; Saegusa et al. 2001; Sznajder and Jendrossek 2014; Sznajder et al. 2015; Uchino et al. 2007), in order to further support the participation in PHB metabolism of the putative enzymes encoded in the genome, we isolated nPHB granules from *A. vinelandii* UW136 grown in Erlenmeyer flasks containing PYS medium, separated the associated proteins in SDS-PAGE and identified the main bands by mass spectrometry (Table 2). As expected, we found proteins involved in PHB biosynthesis, such as the acetoacetyl-CoA reductase (WP_012700951; Avin_23650), PHB synthase (WP_012700949; Avin_23630), and the phasin protein PhbP (WP_012700953; Avin_23670). Several other proteins (mainly membrane proteins), probably artifacts from the separation procedure, were also found attached to the purified granules. Some of these proteins have also been found on the granules of other bacteria, like porins and other membrane or hydrophobic proteins, and have been proposed to be there as product of contamination during nPHB granule isolation (Jendrossek and Pfeiffer 2014; Tirapelle et al. 2013). Interestingly, one of the putative PHB depolymerases identified in the genome was found associated to the granule, migrating in the gel with an apparent molecular mass of 100 kDa (Fig. 1). According to the DNA sequence of its gene, this protein (WP_012699077; Avin_03910), now named PhaZ1, has 914 amino acids, a molecular mass of 101,057.8 Da, and a theoretical pI of 5.52. When compared to the proteins encoded in the genome of *R. eutropha* H16, the model organism for PHB metabolism (Reinecke and Steinbüchel 2009), it shows an overall identity of 48% with an uncharacterized protein annotated as PHA synthase (H16_A0671) and 44% identity with the uncharacterized putative PHA depolymerase H16_B1632; however, the protein PhaZ1 from *A. vinelandii* UW136 shows a low similarity with the main PHB depolymerase from *R. eutropha* H16, PhaZa1 (H16_A1150) (Handrick et al. 2000; Saegusa et al. 2001; Uchino et al. 2008). The PhaZ1 protein shows a high identity with uncharacterized proteins present in several Proteobacteria, which are its probable orthologs that are annotated as PHB synthases or PHB depolymerases. It is interesting to note that it also shows similarity in its amino terminal half with the PhaC subunit of the heterodimeric form of polyhydroxyalkanoic acid (PHA) synthases class III. Interestingly, the *phbZ1* gene is located adjacent to *phbP2*, which encodes a protein (WP_012699079; Avin_03930) paralog of the main phasin PhbP. Due to these characteristics, this protein was chosen for further analysis to investigate its role in PHB metabolism.

The PhbZ1 protein expressed in *E. coli* has hydrolytic activity on amorphous PHB

In order to test the PHB hydrolysis activity of PhbZ1, its gene was cloned and expressed in pET24a + (plasmid

Table 2 Granule-associated proteins identified by ESI LC-MS

No.	Protein ID	Locus tag	Protein name	Short name	Theoretical MW (kDa)	Fragments	Coverage (%)
1	WP_012700116	Avin_14810	Ribonuclease E/G	RneE	128.96	18	17
2	WP_012699077.1	Avin_03910	PHB depolymerase	PhaZ1	101.06	16	20
3	CAA30987.1	Avin_44910	Dihydrolipoyltransacetylase component of the pyruvate dehydrogenase	Ipd	65.03	4	6
4	WP_012700949.1	Avin_23630	PHB synthase	PhbC	64.40	8	14
5	WP_012701529.1	Avin_29760	Dihydrolipoamide succinyltransferase component of the α-ketoglutarate dehydrogenase	SucB	41.98	10	35
6	WP_012700919.1	Avin_23330	Outer membrane porin	OprF	37.61	17	71
7	WP_012700953.1	Avin_23670	Phasin	PhbP	20.27	12	78
11	WP_012700951.1	Avin_23650	Acetoacetyl-CoA reductase	PhbB	26.71	3	20
12	WP_012700616.1	Avin_20000	Cytochrome C oxidase	CcoO/FixO	22.62	1	9
14	WP_012701814.1	Avin_32730	VacJ-like lipoprotein	–	25.97	7	57
15	WP_012702001.1	Avin_34720	Hypothetical protein	–	21.78	1	6
16	WP_012700896.1	Avin_23100	Outer membrane lipoprotein	OprI	8.73	2	24

pJG03910). Most of the protein remained insoluble, but by modification of the protocol, we were able to obtain an amount in the soluble fraction that was clearly detected by Western blot (data not shown). Several attempts to purify the initially soluble protein were unsuccessful, because most of it precipitated during purification; thus, the PHB hydrolytic activity was measured using the soluble *E. coli* cell extracts containing the protein. The enzymatic assay was done using three different substrates: nPHB granules purified from *E. coli* expressing the PHB biosynthetic operon of *A. vinelandii*, aPHB granules, and cPHB granules. The enzyme present had activity on nPHB (93.0 ± 1.4 μg of PHB h^{-1} μg of protein^{-1}) and aPHB (66.1 ± 3.6 μg of PHB h^{-1} μg of protein^{-1}), but no hydrolytic activity was detected on cPHB. No activity was detected in the corresponding controls with the empty plasmid.

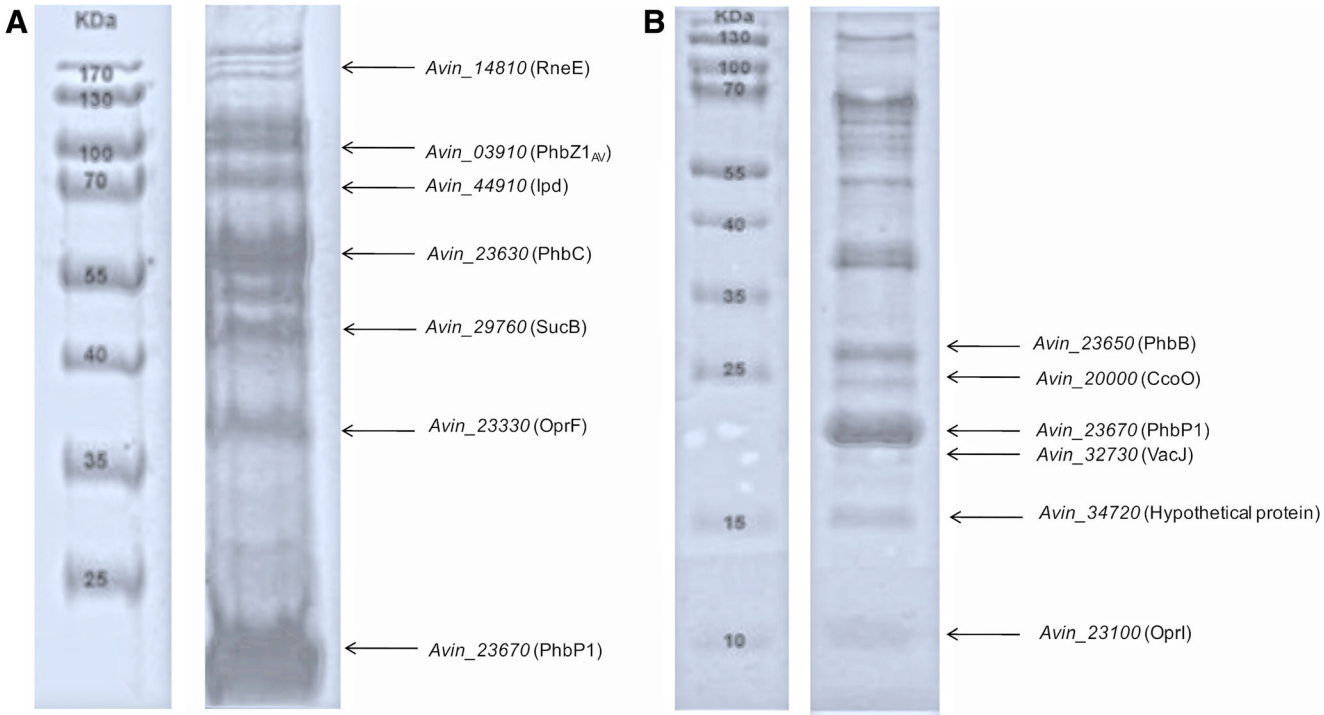

Fig. 1 Analysis of the granule-associated proteins of *A. vinelandii* UW136 (WT) separated by SDS-PAGE. The cells were grown in Erlenmeyer flasks with PYS medium and were collected at 48 h

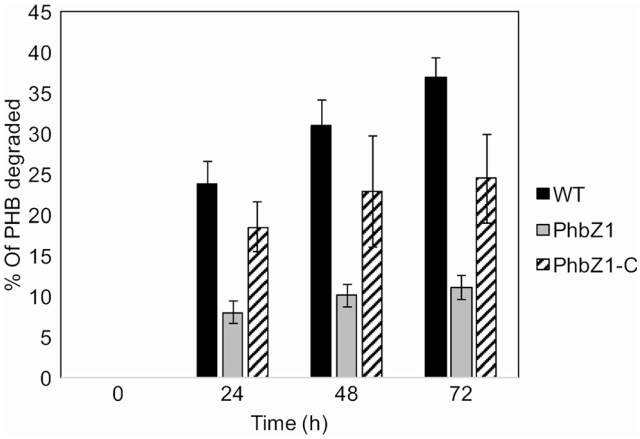

Fig. 2 Endogenous PHB hydrolytic activity of nPHB granules isolated from *A. vinelandii* UW136, its mutant derivative PhbZ1, and the complemented strain PhbZ1-C. The granules (5 mg of nPHB per 1 ml of reaction) were incubated in buffer Tris-HCl 50 mM at pH 7.5 and 37 °C. The PHB content of the granules was quantified at different times and the values are the mean of triplicates and the standard deviation is shown

Inactivation of the *phbZ1* gene negatively affects the PHB hydrolytic activity present in the nPHB granules of *A. vinelandii*

To further investigate the role of the granule-associated enzyme PhbZ1 in the metabolism of PHB, we inactivated the *phbZ1* gene. We purified nPHB granules from the wild-type strain and the PhbZ1 mutant after 48 h of growth on PYS medium and compared their PHB mobilization capacity in vitro. As shown in Fig. 2, the PHB granules from the wild type degraded 37.0 ± 2.5% of the polymer after 72 h of incubation, whereas the granules of the mutant degraded only 11.2 ± 1.5% of the initial content of PHB. The PhbZ1 mutant was complemented with the *phbZ1* wild-type allele by co-integration of the plasmid pJG03910 in the chromosome. The complemented strain partially recovered the PHB mobilization activity, degrading 24.5 ± 5.5% of the PHB present in the granules after 72 h (Fig. 2).

PhbZ1 participates in the thiolysis of nPHB in the presence of free CoA

In *R. eutropha*, the model organism for PHB metabolism, one PHB depolymerase (PhbZa1) has been shown to be able to perform a thiolytic reaction on PHB, in addition to the hydrolytic reaction. This thiolysis occurs in the presence of free CoA, producing hydroxybutyryl-CoA (Eggers and Steinbüchel 2013; Uchino et al. 2007). Because the PhbZ1 enzyme of *A. vinelandii* shows some similarity with PhbZa1, mainly with respect to the presence of the amino acid residues forming the putative catalytic triad in that protein (Kobayashi and Saito 2003), the thiolysis activity of PhbZ1 was assayed. This experiment was conducted using purified nPHB granules form the wild-type strain UW136, the PhbZ1 mutant and the complemented strain PhbZ1-C (Table 3). The granules of the wild-type strain were able to carry out the thiolytic reaction in the presence of CoA, forming hydroxybutyryl-CoA. In contrast, the nPHB granules from the mutant showed a 90% reduced activity, whereas the complemented strain PhbZ1-C restored most of the thiolytic activity of the wild-type strain (Table 3 and Fig. 3).

The PhbZ1 protein has no significant PHB synthase activity

Due to the similarity of the amino terminal half of PhaZ1 with the PhaC subunit of PHA synthases class III, and because the reverse reaction (thiolysis) has also been reported to occur with the PHA synthase PhbC from *R. eutropha* (Uchino and Saito 2006), we tested the PHA polymerization activity toward β-hydroxybutyryl-CoA of PhbZ1 expressed in *E. coli* from plasmid pJG03910 (Table 4). As controls, we used cell extracts of *E. coli* expressing the PHB synthase PhbC of *A. vinelandii* (WP_012700949) from plasmid $pPHB_{Av}$ and cell extracts of *E. coli* with the empty vector pET24a+. The presence of soluble PhbZ1 in the cell extract from *E. coli* containing plasmid pJG03910 was confirmed by Western blot (Fig. S1). The PHB polymerization activity (Table 4), measured as the release of free CoA, was clearly detected in the *E. coli* cells expressing the *A. vinelandii* PHB synthase PhbC, whereas a very low value was quantified in the cells expressing PhbZ1. This value was more than 20 times lower than the positive control and very similar to the negative control.

Although we did not detect significant PHB synthase activity for the PhbZ1 protein in this experiment, this could be due to the requirement of some additional factor not present in *E. coli*. To analyze this possibility, we tested the effect of the inactivation of *phbZ* on the PHB synthase activity of *A. vinelandii*. The activities of the wild type, the PhbZ1 and the complemented strains were quantified and compared to the activities of a PHB synthase mutant *phbC*⁻ (strain CS18, Segura et al. 2003) and a double-mutant *phbC-phbZ1*. As shown in Table 5, the PHB synthase activities of the wild-type strain, the PhbZ1 mutant, and the complemented strain were similar, whereas the *phbC-phbZ1* and the *phbC* mutants showed a considerably diminished activity and were similar.

Table 3 Thiolytic activity of native PHB granules purified from different *A. vinelandii* strains in the presence of coenzyme A

Strain	Specific activity (3HB-CoA μmol/h/mg of protein)
WT	20.0 ± 1.0
PhbZ1	1.8 ± 0.5
PhbZ1-C	18.6 ± 0.5

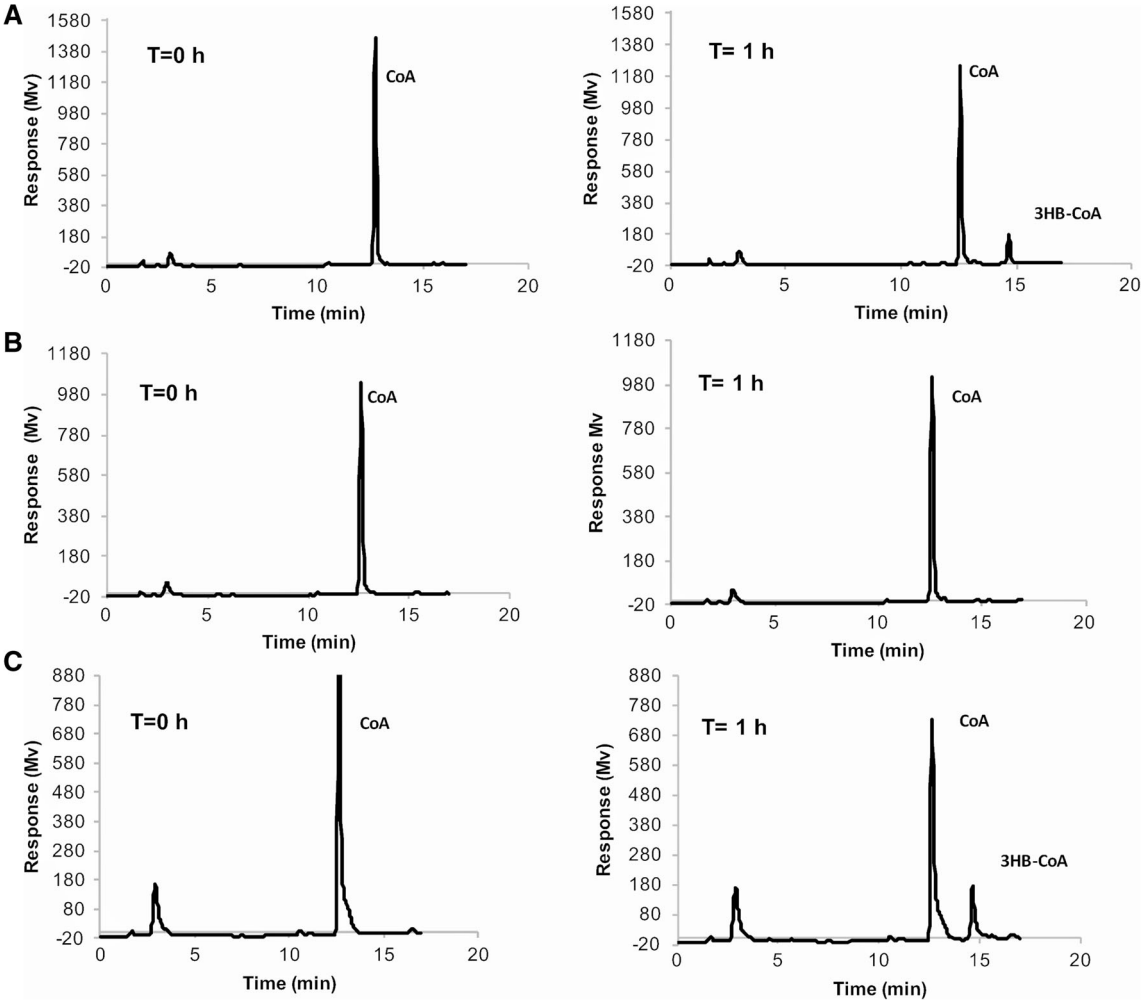

Fig. 3 HPLC analysis of the 3-hydroxybutyryl-CoA formed during the thiolysis of nPHB granules from **a** *A. vinelandii* UW136 (WT), **b** its PhbZ1 mutant, and **c** the complemented strain PhbZ1-C, in the presence of free CoA. A sample of 20 µl of the supernatant obtained after centrifugation was analyzed by HPLC at the indicated times (0 and 1 h)

The mutant PhbZ1 has no phenotype in flask cultures

To determine the effect of the inactivation of *phbZ1* on the metabolism of PHB, the accumulation of PHB of the mutant PhbZ1 was compared to that of the wild-type strain on PYS medium. The growth kinetics of both strains, measured as cellular protein, showed no difference (Fig. 4a). The maximum PHB accumulation, reached at the stationary phase (48 h; Fig. 4b) was very similar for both strains. The wild-type strain UW136 produced 1860 ± 49 µg ml^{-1}, whereas the PhbZ1 mutant accumulated 2029 ± 41 µg ml^{-1} of PHB. No significant difference in the maximum content of polymer was observed. To find out if the expression pattern of the *phbZ1* gene could explain the lack of phenotype, a transcriptional fusion was constructed with the reporter gene *gusA* and the ß-glucuronidase activity was measured at different times (Fig. 4c). The phbZ1 gene was found to have a higher expression during the stationary phase, when the PhbZ1 protein was found attached to the PHB granules (Fig. 1).

In order to compare the PHB mobilization phenotype, we analyzed the changes in the content of the polymer in both strains after the maximal PHB accumulation was reached and under carbon limitation. Cultures of the wild-type strain and the mutant, grown for 48 h to late stationary phase, were incubated for additional 48 h and the polymer content was measured every 8 h. No differences in PHB mobilization were observed between the two strains (not shown). We tried by transferring the cells after PHB accumulation, to different media and conditions, in order to determine if the mutant could show a phenotype. A defined medium with no carbon source and with different concentrations of sucrose, phosphate, ammonium, and different aeration conditions were tested. The sucrose concentration on PY medium was also modified. The mutant and the wild-type strains showed similar amount of PHB under all the conditions and times tested in Erlenmeyer flasks and no phenotype was observed (data not shown).

Table 4 PHB synthase activity of the PhbZ1 protein expressed in *E. coli*

Strain and plasmid	Specific activity (nmol of β-hydroxybutyryl-CoA monomer/minute/mg of protein)
E. coli—pET24a	93.1 ± 8.0
E. coli—pJG03910	80.0 ± 11.0
E. coli—pPHB$_{Av}$	1848.0 ± 97.0

Effects of *phbZ1* inactivation on the amount of PHB produced in bioreactors and its molecular weight

It was previously reported that in *A. vinelandii* OP cultures grown in a 3-l bioreactor (Millán et al. 2016), the mean molecular weight of the PHB produced during the exponential growth phase reached 4800–5500 kDa, but decreased to 3600 kDa after 20 h of culture, at the stationary growth phase. This decrease correlated with the appearance of a fraction of polymers of low molecular weight (lower than 1000 KDa) and also with an increase in the PHB depolymerase activity during the stationary phase of the culture. In order to determine if the PhbZ1 enzyme could be involved in the putative degradation of the PHB in the bioreactor, a PhbZ1-OP mutant derivative of *A. vinelandii* OP was constructed and studied in the bioreactor.

Both strains showed a similar growth in the bioreactor. The specific growth rate (μ) in the case of strain OP was 0.08 ± 0.01 h^{-1}, whereas for the PhbZ1 mutant, it was (0.09 ± 0.01 h^{-1}). When the growth was measured as protein (Fig. 5a), both strains also showed similar values, so the *phbZ1* gene inactivation had no significant effect on the growth capacity of the strain. However, when growth was measured as cell dry weight (CDW, Fig. 5b), some differences were observed. In the case of strain OP, the CDW was 3.9 ± 0.35 g l^{-1} at the end of the culture, while for PhbZ1-OP, it was of 5.2 ± 0.23 g l^{-1}. The accumulation of PHB also showed some interesting differences (Fig. 5c). The cultures of the OP strain had a maximum PHB accumulation of 79.6 ± 6.1% at 42 h and this value decreased to 70.7 ± 5.9% at the end of the culture. For the PhbZ1-OP mutant, the accumulation of PHB reached 90.0 ± 1.0% in the stationary phase, at a similar cultivation time, and the polymer content was stable during the rest of the culture.

Table 5 Effect of *phbZ1* gene inactivation on PHB synthase activity

Strain	Specific Activity (μmol of the β-hydroxybutyryl-CoA monomer/minute/mg of protein
WT	6.98 ± 0.08
PhbZ1	6.71 ± 0.10
PhbZ1-C	6.90 ± 0.04
PhbZ1-PhbC	0.17 ± 0.03
CS18	0.12 ± 0.01

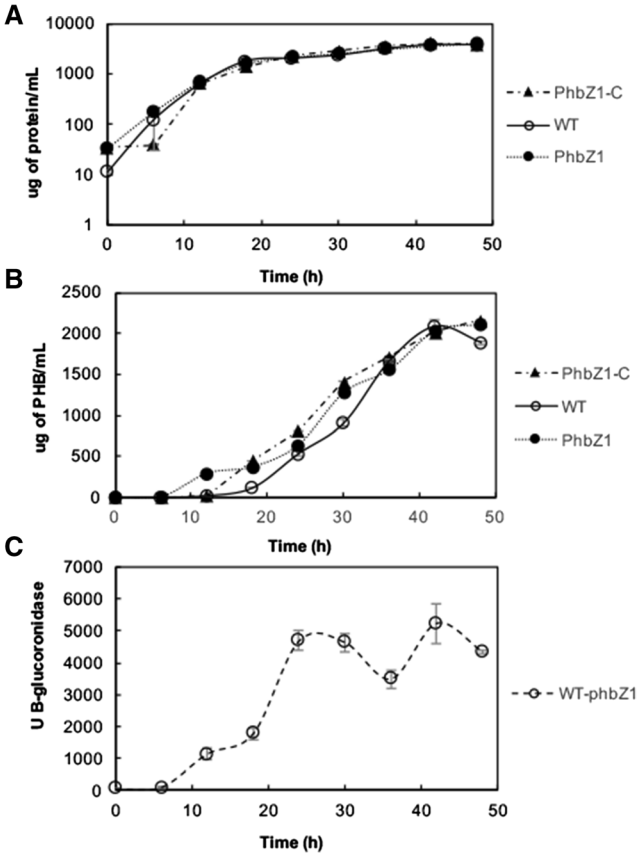

Fig. 4 Growth kinetics of strains WT, PhbZ1, and PhbZ1-C in Erlenmeyer flasks. **a** Growth quantified as protein. **b** Accumulation of PHB. **c** Transcriptional expression of the *phbZ1* gene using the reporter ß-glucuronidase gene *gusA*

The analysis of the PHB produced at different culture times (Fig. 6) showed that the polymer accumulated in the PhbZ1-OP mutant strain had a higher weight average molecular weight (Mw) (6100 kDa), when compared with that of the polymer produced by strain OP (5200 kDa). Furthermore, the Mw of the PHB produced by strain OP decreased during the exponential growth phase (from 5200 to 3600 kDa), remaining in values of 3500 kDa during the stationary phase. In the case of mutant PhbZ1-OP, the Mw of the polymer remained practically constant in the range of 5500 to 6200 kDa during the 48 h of cultivation. The molecular weight distribution (MWD) of the polymer accumulated by both strains was also different (Fig. 7). In the case of the PHB produced by strain OP, the polymer was constituted by fractions with molecular weights from 10 to 25,000 kDa at different times of the culture (Fig. 7a). Also, the fraction of PHB molecules with low molecular weights, in the range of 10 to 350 kDa, increased with respect to the cultivation time, reaching about 28% of the whole sample at the end of the culture (48 h) (Fig. 7a). In contrast, for the polymer produced

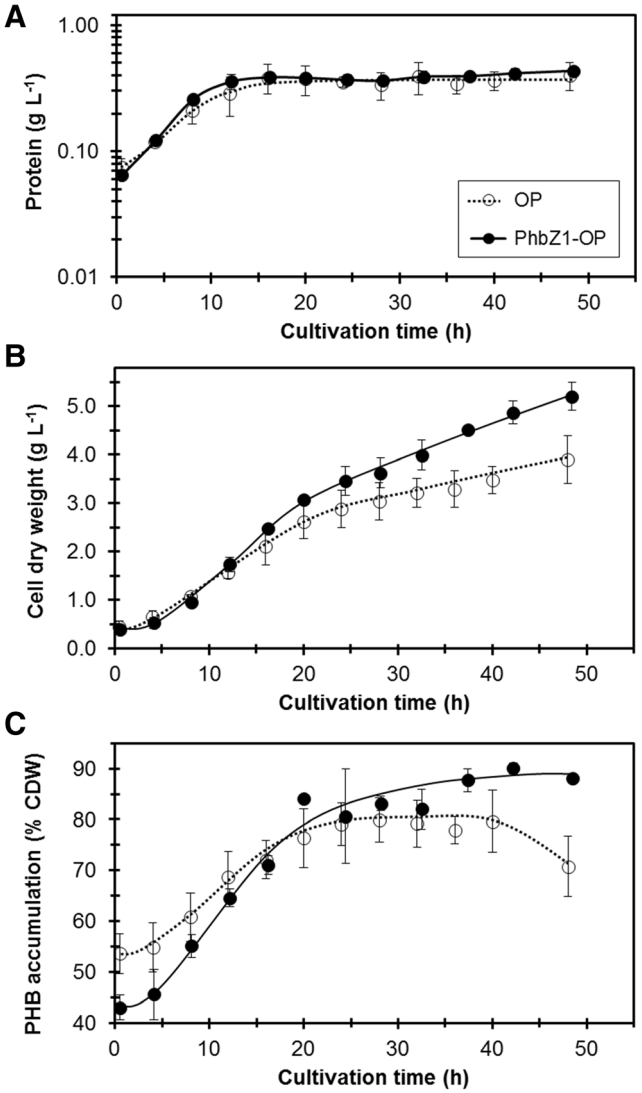

Fig. 5 Growth kinetics, measured as protein (**a**) and cell dry weight (**b**), and PHB accumulation (**c**) of *A. vinelandii* OP (white circles) and its mutant derivative *phbZ1* (black squares). Cultures were grown in a stirred tank bioreactor containing 2 l of PYS medium, as indicated in the "Materials and methods" section. Quantifications are the average of triplicates and the standard deviation for each point is shown

by the PhbZ1-*OP* mutant, the MWD showed molecular weights from 350 to 25,000 kDa and this distribution was similar for all the culture times analyzed (Fig. 7b). The fraction of 10–350 kDa was not observed in the samples obtained from strain PhbZ1-OP (Fig. 7c).

Discussion

The PHB biosynthetic process and the genetic elements involved are well known in *A. vinelandii* (Castañeda et al. 2000; Hernández-Eligio et al. 2011, 2012; Muriel-Millán et al. 2014; Noguez et al. 2008; Peralta-Gil et al. 2002); however, little is known about the mobilization of PHB. The localization in the

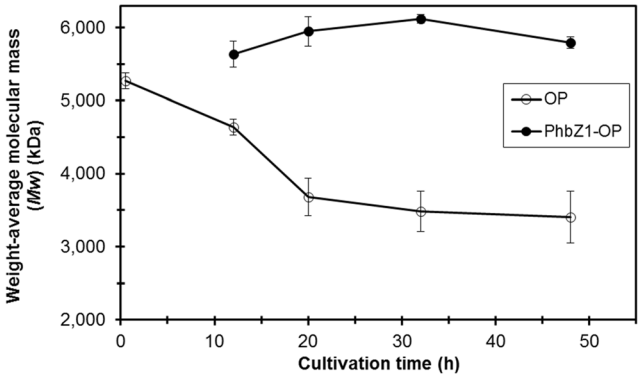

Fig. 6 Weight average molecular weight (Mw) of the PHB produced by *A. vinelandii* OP (white circles) and its mutant derivative PhbZ1-OP (black squares) at different culture times in a stirred tank bioreactor containing 2 l of PYS medium

nPHB granules of one of the putative PHB depolymerases identified in the genome sequence (Fig. 1), together with other proteins involved in PHB metabolism (Table 2), suggested that this protein could be a participant in the mobilization of the polymer. In accordance with this hypothesis, when expressed in *E. coli* and although most of the protein was insoluble, the soluble enzyme showed in vitro hydrolytic activity on nPHB and aPHB, but not of cPHB, as it is characteristic of intracellular PHB depolymerases that are active on

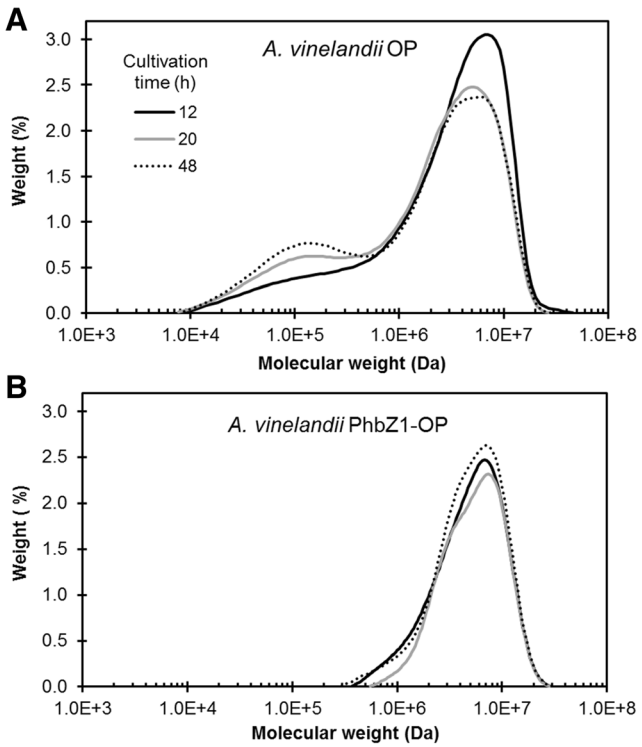

Fig. 7 Molecular weight distributions of PHB isolated from *A. vinelandii* OP (**a**), and its PhbZ1-OP mutant derivative (**b**) grown in a stirred tank bioreactor containing 2 l of PYS medium, as indicated in the "Materials and methods" section. The molecular mass was determined by gel permeation chromatography (GPC)

amorphous PHB (Saegusa et al. 2001; Sznajder and Jendrossek 2014).

Although the *phbZ1* gene was expressed in the wild-type strain in cultures grown in shake flasks (Figs. 1 and 4c), the *phbZ1* knockout mutant did not show a clear phenotype on PHB accumulation under this condition; However, the nPHB granules purified from mutant PhbZ1 had a clearly diminished in vitro auto-hydrolytic activity, when compared to those of the wild type (Fig. 2). This negative effect of the lack of PhbZ1 on the mobilization of PHB occurring in the isolated granules shows the participation of this enzyme in the degradation of the polymer. This result is similar to that reported for the mutant of PhaZa1, the main PHB depolymerase of *R. eutropha* (Handrick et al. 2000; Uchino et al. 2008). Both the lack of phenotype of the PhbZ1 mutant in shake flasks and the degradation still present in its granules (Fig. 2) suggest the presence of additional depolymerizing enzymes under this condition. We did not identify other PHB depolymerases in the mass spectrometry analysis because we only analyzed the main protein bands obtained from the granules. In the model organism *R. eutropha*, the existence of multiple PHA depolymerases is well documented, and at least seven PHB depolymerases and two oligomer hydrolases have been identified (Abe et al. 2005; Handrick et al. 2000; Kobayashi et al. 2003, 2005; Saegusa et al. 2001, 2002; Sznajder and Jendrossek 2014; Uchino et al. 2008; York et al. 2003). It is interesting to note that although the PHB depolymerizing activity in vitro has been reported for these enzymes of *R. eutropha*, the inactivation of their genes had no effect on PHB degradation, except for PhaZa1 that showed a partial phenotype on PHB mobilization (Handrick et al. 2000; Saegusa et al. 2001; Uchino et al. 2008; York et al. 2003). In the case of the *A. vinelandii* mutant PhbZ1, both its lack of a phenotype in flasks (although the PhbZ1 protein was present in the wild type; Fig. 1) and the residual auto-hydrolysis activity detected in its granules (Fig. 2) suggest a complexity of the mobilization system similar to that of *R. eutropha*.

The granules purified from *A. vinelandii* UW136 showed thiolytic activity in the presence of free CoA, forming hydroxybutyryl-CoA. The very low level of this activity in the granules of the PhaZ1 mutant and its reestablishment by complementation with *phbZ1* (Table 3 and Fig. 3) show that this activity is due to PhbZ1. This capacity has only been reported for the intracellular PHA depolymerase PhaZa1 from *R. eutropha* H16 (Eggers and Steinbüchel 2013; Uchino et al. 2007), an enzyme that lacks the lipase box sequence (Gly-X_1-Ser-X_2-Gly) present in many PHB depolymerases, having a cysteine instead of serine. This cysteine forms part of the catalytic triad (cysteine, aspartate, and histidine) (Kobayashi and Saito 2003), which is conserved and positioned similarly to the catalytic amino acids present in PHA synthases (Jia et al. 2000), which catalyze the reverse reaction. It is interesting to note that the *A. vinelandii* enzyme also lacks the lipase box and shows similarity in its amino terminal half with the PhaC subunit of the heterodimeric PHA synthases class III. From an alignment of PhbZ1 with PhaC from *Thiocystis violascens* and *Allochromatium vinosum*, it is interesting to note that although the overall identity with these proteins is low (21%), the amino acids of the catalytic triad (cysteine, aspartate, and histidine) seem to be conserved in PhbZ1 (positions 216, 371, and 410, respectively), as in PhaZa1. It would be interesting to know if this is a characteristic of other PHA depolymerases able to perform thiolytic mobilization.

Due to the similarity of the PhbZ1 protein with class III PHB synthases, the possible role of this protein as a PHB polymerizing enzyme was raised. The comparison of the PHB synthase activity of this protein with that of the PhbC enzyme of *A. vinelandii*, both expressed in *E. coli*, suggests that PhbZ1 is not a PHB polymerizing enzyme, because we did not detect any significant activity for PhbZ1 (Table 4). The small activity (free CoA released) obtained in the extracts containing PhbZ1 and in the negative control (empty plasmid) was very similar and could be due to other activities, like 3HB thiokinase or CoA transferase, that might be present in the *E. coli* extracts (Uchino and Saito 2006). Nevertheless, the lack of PHB synthase activity of the PhbZ1 protein in this experiment could also be due to the requirement of some additional factor not present in *E. coli*; however, when the PHB synthase activities of the *A. vinelandii* wild type, the PhbZ1 mutant and the complemented strain PhbZ-C were compared (Table 5), and no effect of the inactivation of *phbZ1* gene was observed. We also compared the PHA synthase activities of a PHB synthase (*phbC*) knockout mutant and a *phbC/phbZ1* double mutant of *A. vinelandii*, and in both strains, the activities were ten times lower, but similar, showing that this phenotype is due to the lack of PhbC and confirming that although the PhbZ1 enzyme has similarity with class III PHB synthases, it has no significant PHB polymerizing activity. Class III and IV PHA synthases require an additional polypeptide (PhaE and PhaR, respectively) that noncovalently associates with the catalytic subunit to have full functional activity (Rehm 2003). Homologs to such polypeptides were not found in the *A. vinelandii* genome.

In several bacteria, including *A. vinelandii* (Doi et al. 1990; Ren et al. 2009; Yan et al. 2000), PHB synthesis and mobilization occur simultaneously; therefore, the inactivation of the mobilization system could be expected to improve the accumulation of PHB. The presence of both the PHB synthase PhbC and PhbZ1 in the purified granules of *A. vinelandii* (Fig. 1) is in accordance with the concomitant synthesis and degradation of PHB. The inactivation of *phbZ1* did not affect cell growth (measured as protein, Fig. 5a) but, as expected, had a positive effect on the PHB content (Fig. 5c); the *phbZ1* mutant reached a 13% higher content of PHB than the wild-

type strain. The higher cell dry weight obtained for the *phbZ1* mutant (Fig. 5b), having a similar growth estimated as protein, is in accordance with this higher accumulation of PHB. Previous reports have shown increased production in mutants where *phaZ* genes have been knocked out, like *Pseudomonas putida* strain U (Arias et al. 2013) and *P. putida* KT2442 (Cai et al. 2009), although in other cases, no effect has been observed, like in *P. resinovorans* (Solaiman et al. 2003).

Because the mechanical strength of PHB has been positively correlated with its molecular size, a polymer of high molecular weight is preferred for those applications where a higher resistance is required (Tsuge 2016). *A. vinelandii* is able to produce a PHB of high molecular weight (Chen and Page 1994; Myshkina et al. 2008; Peña et al. 2014). However, a decrease in its mean molecular weight occurs during the stationary growth phase of cultures grown in bioreactors and this is concomitant with the appearance of a fraction of polymers of low molecular weight. This behavior correlated with an increase in the PHB depolymerizing activity (Millán et al. 2016). The present study confirmed that this decrease in the molecular weight (Fig. 6) and the corresponding appearance of PHB molecules of lower size (Fig. 7) in *A. vinelandii* strain OP are due to the degradation of the polymer by the activity of the PhbZ1 PHB depolymerase. In the case of the mutant strain, the change in the molecular weight distribution during stationary phase did not occur and the molecular size remained high and constant. A recent work where the genes of several PHA depolymerases of *R. eutropha* were deleted showed a similar result allowing the production of high-molecular-weight PHA (Arikawa et al. 2016).

In summary, the results presented show that PhbZ1 participates in the mobilization of PHB in *A. vinelandii* and is able to do it producing hydroxybutyryl-CoA. This enzyme is the main PHB depolymerase active during the stationary phase in bioreactors and its inactivation allows the production of a PHB of uniform high molecular weight.

Acknowledgements The authors are grateful to Soledad Moreno and Ramón de Anda for the technical assistance and Déborah Yanajara-Parra for her assistance in the purification of the PHB granules. L. Adaya is a doctoral student from Programa de Doctorado en Ciencias Biomédicas, Universidad Nacional Autónoma de México, and was supported by a scholarship from Consejo Nacional de Ciencia y Tecnología (México). This work was supported by grants 255158 and 238535 from Consejo Nacional de Ciencia y Tecnología and IT200415 form Programa de Apoyo a Proyectos de Investigación e Innovación Tecnológica (PAPIIT) DGAPA-UNAM, as well as by a grant of the Deutsche Forschungsgemeinschaft to D. J.

Compliance with ethical standards

Conflict of interest The authors declare that they have no conflict of interest.

Ethical statement This article does not contain any studies with human participants or animals performed by any of the authors.

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