Identification and characterization of PhbF: A DNA binding protein with regulatory role in the PHB metabolism of *Herbaspirillum seropedicae* SmR1

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**Abstract**

**Background:** *Herbaspirillum seropedicae* SmR1 is a nitrogen fixing endophyte associated with important agricultural crops. It produces polyhydroxybutyrate (PHB) which is stored intracellularly as granules. However, PHB metabolism and regulatory control is not yet well studied in this organism.

**Results:** In this work we describe the characterization of the PhbF protein from *H. seropedicae* SmR1 which was purified and characterized after expression in *E. coli*. The purified PhbF protein was able to bind to eleven putative promoters of genes involved in PHB metabolism in *H. seropedicae* SmR1. In silico analyses indicated a probable DNA-binding sequence which was shown to be protected in DNA footprinting assays using purified PhbF. Analyses using *lacZ* fusions showed that PhbF can act as a repressor protein controlling the expression of PHB metabolism-related genes.

**Conclusions:** Our results indicate that *H. seropedicae* SmR1 PhbF regulates expression of *phb*-related genes by acting as a transcriptional repressor. The knowledge of the PHB metabolism of this plant-associated bacterium may contribute to the understanding of the plant-colonizing process and the organism’s resistance and survival in planta.

**Background**

Polyhydroxyalkanoates (PHA) are aliphatic polyesters biosynthesized by several bacteria [1-4] as a means of carbon storage and a source of reducing equivalents when other nutrients are limiting. The most frequently PHA produced is poly(3-hydroxybutyrate) or PHB [2]. The ability to produce PHB has been correlated with improved survival under stress conditions or in competitive environments [5,6]. PHB is generally produced in conditions of carbon oversupply and low levels of other nutrients such as nitrogen, phosphate or oxygen [7]. The biosynthesis of PHB is dependent on the activity of the following enzymes: (i) a 3-ketothiolase which condenses two acetyl-CoA yielding acetoacetyl-CoA (encoded by *phbA*), (ii) a NADPH-dependent acetoacetyl-CoA reductase which reduces acetoacetyl-CoA to (R)-3-hydroxybutyryl-CoA (encoded by *phbB*) and (iii) the PHB synthase (encoded by *phbC*) that catalyses the polymerization of (R)-3-hydroxybutyryl-CoA to form the polymer [8,9]. This polymer is stored intracellularly as insoluble inclusion bodies called PHB granules [1] which also contain about 2% protein as well as phospholipids [10]. The main protein associated with the PHB granules is phasin (encoded by *phaP*) which prevents coalescence of PHB granules by coating the granule surfaces [11-14]. However, other proteins have also been found associated with the granules, including transcriptional regulators such as PhaF from *Pseudomonas oleovorans* GPo1, PhaR from *Paracoccus denitrificans*, and PhaR from *Ralstonia eutropha* H16 [15-17].

Expression of enzymes involved in PHA/PHB biosynthesis and the granule-associated phasin are reported to be...
regulated at the transcriptional level [15,16,18-26]. This regulation may include repressors as well as activators [21]. The proteins PhbR from Azotobacter vinelandii UW136 [22] and PhaD from Pseudomonas putida KT2442 [24] are transcription activators. In contrast, PhaR of P. denitrificans represses phaR expression by binding to a TGC rich region which over-laps the -35/10 promoter [16]. In R. eutropha H16 the PhaR protein binds to the -35/10 phaP promoter at two sites: the transcriptional start site and upstream from the -35 at the promoter region, thereby blocking RNA polymerase transcriptional start site and upstream from the -35/10 promoters [16]. In R. eutropha H16 the PhaR protein binds to the -35/10 phaP promoter at two sites: the transcriptional start site and upstream from the -35 at the promoter region, thereby blocking RNA polymerase transcriptional start site and upstream from the -35 at the promoter region, thereby blocking RNA polymerase

The β-Proteobacterium Herbaspirillum seropedicae SmR1 is a plant-endophytic diazotroph found in association with economically important graminaceous species such as sugar cane, sorghum, rice and maize [27]. 

H. seropedicae SmR1 has been already described as a PHB producer using glucose as carbon source [28], however the molecular aspects of its PHB metabolism have not been addressed. The H. seropedicae SmR1 genome sequence analysis indicated several genes likely to be involved in PHB metabolism, including phbA, phbB and phbC encoding for 3-ketothiolase, acetoacetyl-CoA reductase and poly(3-hydroxybutyrate) synthase. The phbF gene encoding a putative regulator was located downstream from phbCB [29]. In this work we characterized the transcriptional regulator PhbF of Herbaspirillum seropedicae SmR1.

**Methods**

**Strains and plasmids**

All bacterial strains and plasmids used in this work are listed in Table 1.

**Media and growth conditions**

Escherichia coli strains were grown in LB or M9 minimal media at 37°C [30]. The H. seropedicae SmR1 strain was grown at 30°C in NFBHPN-Malate medium supplemented with 20 mM NH₄Cl [31]. Antibiotics were added as follows: ampicillin 100 μg.mL⁻¹, tetracycline 10 μg.mL⁻¹, streptomycin 20 μg.mL⁻¹ (E. coli) or 80 μg.mL⁻¹ (H. seropedicae SmR1), kanamycin 50 μg.mL⁻¹ (E. coli) or 500 μg. mL⁻¹ (H. seropedicae SmR1), chloramphenicol 30 μg.mL⁻¹ (E. coli) or 150 μg.mL⁻¹ (H. seropedicae SmR1) and nalidixic acid 10 μg.mL⁻¹.

**Plasmid Construction**

The phbF gene was amplified from the H. seropedicae SmR1 genome using the primers 5’GACTGGAACCTCA-TATGACTACTGCG3’ and 5’CAACAGGATCCGGCAGAATGC3’ carrying NdeI or HindIII restriction sites (underlined). The amplified product was cloned into pET-28a to yield plasmid pKADO3, which over-expresses the PhbF protein fused to a N-terminal six-histidine tag (His-PhbF). To express PhbF from a tac promoter, phbF was obtained in an XbaI/HindIII fragment from pKADO3 and cloned into pDK6, yielding plasmid pMMS31.

**Construction of transcriptional fusions phbF::lacZ and phaP1::lacZ**

The promoter regions of phbF (containing 353 bp including 54 bp of the phbF coding sequence) and phaP1 (containing 381 bp including 28 bp of the phaP1 coding sequence) were amplified from the H. seropedicae SmR1 genome and cloned into pMP220 [32], upstream from the promoter-less lacZ gene to yield the respective plasmids pKADO5 and pMMS35.

**β-galactosidase activity assay**

β-galactosidase activity was determined in E. coli ET8000 carrying transcriptional fusion plasmids (pKADO5 or pMMS35), in the presence or absence of plasmid pMMS31 (expresses PhbF), grown in M9 minimal medium as described [33]. Protein concentration was determined using the Bradford method [34] with bovine serum albumin as standard.

**In silico identification of DNA motif**

The MEME program [35] was used to detect a common motif among promoter regions of genes related to PHB metabolism in the H. seropedicae SmR1 genome [29]. The MEME program was set to identify not more than one motif with 6 to 50 bp in length. The conserved motif was represented in the LOGO format.

**Purification of His-PhbF**

E. coli strain BL21 (DE3) carrying pKADO3 was grown in LB medium at 37°C to an OD₆₀₀ of 0.6-0.8. The culture was then induced with 0.5 mmol/L IPTG at 20°C for 15 hours. After harvesting, cells were lysed by sonication in buffer A (100 mmol/L NaCl, 50 mmol/L Tris-HCl pH 7.5, 10 mmol/L imidazole and 0.05% Triton X-100). After clarification by centrifugation at 14000 × g for 30 minutes at 4°C, the protein extract was loaded onto a Hi-Trap Chelating Ni²⁺ column (GE Healthcare). Protein elution was carried out using a linear imidazole gradient, and His-PhbF was eluted with 300 mmol/L imidazole in buffer A. Protein fractions were pooled and, after dialysis against buffer A with 50% glycerol, were stored in liquid N₂.

**Electrophoretic Mobility Shift Assay (EMSA)**

The promoter regions of genes related to PHB biosynthesis were amplified using fluorescent (VIC and FAM)
| Strains       | Relevant genotype                                                                 | Reference/source |
|--------------|-----------------------------------------------------------------------------------|------------------|
| **E. coli**  |                                                                                   |                  |
| BL21(DE3)    | hsdS gal (Act ts 857 ind 1 Sam7 nin5 lacUV5-T7 gene 1).                           | Invitrogen       |
| ET8000       | rbs lacZ:IS1 gyrA hutCk k (wild-type).                                            | [42]             |
| **H. seropedicae** |                                                                                   |                  |
| SmR1         | Wild-type, Nif⁺, Sm⁺                                                            | [43]             |
| **Plasmids** |                                                                                   |                  |
| pET-28a      | Expression vector, T7 promoter, Km⁹.                                              | Novagen          |
| pDK6         | Expression vector tac promoter lacq, Km⁹.                                         | [44]             |
| pKADO3       | *H. seropedicae* SmR1 phbF cloned into pET-28a; expresses the His-tag PhbF protein. | This work        |
| pKADO5       | 353 bp containing phbF promoter region cloned into pMP220 resulting in the phbF: lacZ transcriptional fusion. | This work        |
| pMMS31       | Derivative of pDK6 encoding PhbF from *H. seropedicae* SmR1.                     | This work        |
| pMMS35       | 381 bp containing phaP1 promoter region cloned into pMP220 resulting in the phbP1: lacZ transcriptional fusion. | This work        |
| pMP220       | Vector used to construct transcriptional lacZ fusions; Tc⁶.                       | [32]             |
end-labeled primers. Alternatively, phbF and phaP1 promoters were amplified and end-labeled using \( ^{32}P \)-\( \gamma \)-ATP and T4 polynucleotide kinase [30]. DNA-binding assays were performed in 10 \( \mu \)L containing 20 mmol/L of end-labeled DNA, 100 ng of calf thymus DNA, and increasing amounts of purified His-PhbF in binding buffer (10 mmol/L Tris-HCl pH 7.5, 80 mmol/L NaCl, 1 mmol/L EDTA, 10 mmol/L \( \beta \)-mercaptoethanol and 5% (m/v) glycerol) following incubation at 30°C for 5 minutes. The fluorescent DNA was observed after excitation with UV light (254 nm) and the \( ^{32}P \)-labeled DNA was detected using a PhosphorImager screen and a STORM scanner.

**DNasel footprinting assay**

A 325bp DNA fragment containing the phbF promoter region was amplified using \( ^{32}P \)-labeled primer and genomic DNA as template [30]. The fragment was purified using the Wizard kit (Promega) and then incubated with His-PhbF in 50 mmol/L Tris-acetate pH 8.0, 8 mmol/L magnesium acetate and 10 mmol/L KCl at 30°C for 5 minutes. For partial hydrolysis, 1 unit of DNasel (Invitrogen) was added and the reaction incubated at 30°C for 1 minute. The reaction was stopped by adding 0.2 volume of 0.5 mmol/L EDTA and heating at 80°C for 5 minutes. After ethanol precipitation of DNA fragments in the presence of yeast tRNA, samples were resuspended in 6 \( \mu \)L of loading buffer (47% formamide (v/v), 10 mmol/L EDTA, 0.05% bromophenol blue (m/v), 0.05% xylene xylanol (m/v)), denatured at 80°C for 5 minutes and loaded on a 6% (m/v) polyacrylamide denaturing DNA sequencing gel [30]. The phbF promoter region was sequenced using the T7 sequencing kit (GE Healthcare). Images of the autoradiograms were obtained using a PhosphorImager screen and a Storm scanner. Densitometric profiles were analyzed using the ImageQuant v.5.2 program (Molecular Dynamics).

**Extraction of PHB granules**

PHB granules were extracted from H. seropedicae SmR1 grown in NFHBP-malate medium containing 5 mM glutamate at 30°C until \( OD_{600} = 1.0 \), following a described procedure [36]. After extraction, granules were washed twice with water and then with acetone. Granules were dried under a nitrogen gas stream at room temperature and stored at -20°C.

**PHB granule-binding of the His-PhbF protein**

The PHB granule-binding reaction was performed as described [37] with modifications. His-PhbF (25 \( \mu \)g) was incubated with 1 mg of purified H. seropedicae SmR1 PHB granules in a final volume of 100 \( \mu \)L in 50 mmol/L Tris-HCl pH 7.5. Samples were incubated at 37°C for 10 minutes and then centrifuged at 10,000 \( \times \) g for 1 minute. The supernatant was collected and the granules were washed twice with 400 \( \mu \)L of 50 mM Tris-HCl pH 7.5 and the supernatant from each wash step was also collected separately. Protein bound to the granules was dissociated by incubation in 2% (m/v) SDS, 10% (m/v) glycerol and 5% (m/v) \( \beta \)-mercaptoethanol at 90°C for five minutes. Samples were analyzed by SDS-PAGE [38].

**Results and discussion**

The H. seropedicae SmR1 PhbF protein was first identified in the cellular proteome by [39] using late log phase culture grown under ammoniotrophic conditions. The phbF gene (H_sero2997) is located downstream from phbC and phbB (GenBank: CP002039) and encodes a 188 amino acids protein with high similarity to R. eutropha H16 PhaR (183 amino acids, 83% identity, 90% similarity) [17], and, to a lesser extent, to Rhodobacter sphaeroides F1 (41% identity and 59% similarity) and P. denitrificans PhaR (restricted to the N-terminus with 37% identity and 56% similarity to the first 120 amino acids). In silico analysis indicated a helix-turn-helix motif located at its N-terminal sequence suggesting that PhbF is capable of DNA-binding and may act as a regulator of PHB biosynthesis genes in H. seropedicae SmR1.

To characterize the H. seropedicae SmR1 PhbF protein, it was overexpressed and purified as a His-tag fusion form (His-PhbF) from E. coli BL21(DE3) harboring the plasmid pKADO3 (Table 1). Most of the expressed His-PhbF was found in the soluble protein fraction when cells were induced at low temperature (20°C) and lysed in buffer containing Triton X-100 0.05% (m/v). This detergent at low concentration yielded a homogenous His-PhbF protein solution of 98% purity by Ni\(^{2+}\)-affinity chromatography. Circular dichroism analysis indicated that purified His-PhbF is folded in the presence of the detergent (Additional file 1, Figure S1). Also, gel filtration chromatography indicated that H. seropedicae SmR1 PhbF is tetrameric in solution with an apparent molecular weight of 104.3 kDa (Additional file 1, Figure S2). The PhaR from P. denitrificans is also a tetrameric protein of approximately 95 kDa in solution [16].

Twelve putative promoter regions were identified by DNA sequence analyses of genes potentially involved in PHA metabolism in H. seropedicae SmR1 (GenBank: CP002039, [29]) as shown in Additional file 1, Figure S3. All of these putative promoter regions, with the exception of phaP2, were assayed for DNA binding by His-PhbF. DNA band-shift assays showed that purified His-PhbF was able to bind specifically to these eleven promoter regions (Figure 1 and results not shown) but not to the unrelated nifB promoter [40](Additional file 1, Figure S4) indicating that the protein is active. The apparent dissociation constants observed varied from 150 nM (phaP1) to 450 nM (phbF).
Figure 1 The DNA-binding assays of purified His-PhbF from *H. seropedicae* SmR1 to the promoter regions of *phaP1, phbF, dskAphbC, fadBphbA, phbCphbB* and *H_sero3316phaB* were performed as described in Material and Methods. DNA promoter regions used in the assays are indicated by vertical black arrow heads and numbers indicate base position related to the translation start of each gene. **Panel A:** DNA labeled with [³²P]. Lanes 1 to 5 indicate increasing amounts of purified His-PhbF (0, 280, 570, 860 or 1100 nM). **Panel B:** Fluorescent labeled DNA. Lanes 1 to 8 indicate increasing amounts of purified His-PhbF (0, 62, 125, 250, 500, 750, 1000 or 1250 nM). Protein concentrations were calculated assuming His-PhbF as a tetrameric protein.
These twelve promoter regions (including \textit{phaP2}, additional file 1, Figure S3) were also analyzed \textit{in silico} using the MEME program [35] which indicated the sequence TG[N]TGC[N]GCAA as a probable DNA-binding motif for PhbF (Figure 2A). A similar sequence (CTGC[N]3GCAG) was also described in \textit{R. sphaeroides} FJ1 as the DNA-binding site for the regulator PhaR [41]. Both sequences show two highly conserved triplets (TGC and GCA) which seem to be essential for DNA-binding of \textit{R. sphaeroides} PhaR [41].

To verify if the TG[N]TGC[N]GCAA sequence is important for DNA-binding of \textit{H. seropedicae} SmR1 PhbF, a DNaseI footprinting assay was performed using the \textit{phbF} promoter region and purified His-PhbF (Figure 2B). A clear DNaseI protection site was observed when His-PhbF was present in the assay. The protected site

![Diagram of promoter regions and DNA sequence](image)

\textbf{Figure 2 Panel A: Sequence logo representing the consensus sequence of \textit{pha} promoter regions identified by the program MEME motif discovery tool.} In the y axis the information is represented in bits indicating the nucleotide frequency in the sequence at that position. The putative consensus sequence probably recognized by PhbF is indicated. \textbf{Panel B:} DNase I-protection footprinting assay was carried out as described in Material and Methods. The non-coding strand of the \textit{phbF} promoter was used as a probe. The assays were in the absence (lane 1) or presence 155 (lane 2) or 312 nM (lane 3) of the purified His-PhbF tetramer. Lane P indicates the undigested promoter region. The DNA sequencing reaction is indicated in lanes A, C, G, and T. The region showing protection from DNaseI digestion is indicated by **. The probable \( \sigma^{52} \) promoter is indicated by *. Numbers indicate base position corresponding to the translation start codon.
covers positions 181 to 204 upstream from the translation start site indicating that His-PhbF binds to a 24 bp region of its own promoter which includes the conserved TG[N]TGC[N]3GCAA motif indicated by the MEME program, reinforcing the suggestion that it is the DNA site recognized by the H. seropedicae SmR1 PhbF. Furthermore, a putative sigma 70-dependent promoter was also identified upstream from the PhbF DNA-binding site (position 208 to 212 from the translation start site) (Figure 2C). The proximity of both sites also suggests that H. seropedicae SmR1 PhbF may repress its own expression.

We verified the potential repressor activity of PhbF in E. coli ET8000 by using a gene reporter expression assay with phaP1 and phbF promoters fused to the lacZ gene. These genes were chosen because they have the putative PhbF-binding sequence highly similar to the consensus sequence, and also because EMSA assay showed clear interaction with these promoters. The β-galactosidase activities indicated that both phaP1 and phbF promoters were functional in E. coli (Figure 3). However, a clear decrease in β-galactosidase activity is observed if H. seropedicae SmR1 PhbF is present (expressed upon plasmid pMMS31), indicating that PhbF represses the expression of the phasin gene (phaP1) and also of its own gene promoter (phbF). Expression of an unrelated protein (NifH) did not affect β-galactosidase activity of E. coli bearing the phbF::lacZ and phaP1::lacZ fusions (data not shown), reinforcing the repressor effect of PhbF.

Protein domain analysis indicated that PhbF contains a DNA-binding motif and a domain possibly involved in binding PHB. Therefore, we tested if H. seropedicae SmR1 PhbF was able to interact with PHB granules in vitro. The purified His-PhbF was incubated with PHB granules extracted from H. seropedicae SmR1 and the protein remaining in solution was visualized by SDS-PAGE (Figure 4). When His-PhbF was incubated with PHB granules most of the protein was extracted from solution (Figure 4, lane 2). The protein remained bound to the granule even after two washing steps (lanes 3 and 4), and was released only after heating in the presence of SDS, indicating a strong interaction between His-PhbF and PHB.

Our results indicate that H. seropedicae SmR1 PhbF is capable of DNA binding and also of associating with PHB granules. In addition, expression of PhbF from H. seropedicae SmR1 leads to 10 and 4-fold reduction (P < 0.05) in expression of phbF and phaP1 promoters, respectively. These results strongly suggest that H. seropedicae SmR1 PhbF protein is a repressor which controls expression of genes involved in PHB production as well its own expression. In both respects it shows similarity with the PhaR regulator from R. eutropha [17] and from P. denitrificans [16].

The expression of phbF gene in H. seropedicae SmR1 increases sharply in the log phase (not shown) and PHB starts to accumulate in the log phase reaching maximum as the culture entry in the stationary phase [28], suggesting that the repressor activity of PhbF may be relieved as PHB oligomers levels increase inside the cell, as suggested in R. eutropha and P. denitrificans [11,16,17]. The expression of phaP1 has a similar pattern. We hypothesize that when
PHB oligomers levels increase, the PhbF protein is sequestered, allowing transcriptional initiation. Whether PhbF can be released from DNA by binding to PHB, thus allowing expression of pha/phb genes once PHB synthesis is favored is not known.

The production of reserve material such as PHB has important metabolic features, since stress endurance and survival is improved when bacteria produce PHB, as observed for Azospirillum brasilense [5], and cells with high PHB content were able to increase the population 2-3 fold and survive for longer periods of starvation as seen in Sinorhizobium meliloti [6]. Therefore, knowledge of the PHB metabolism of plant-associated bacteria may contribute to the understanding of the colonization process and improvement of their resistance and survival under colonizing conditions.

Conclusions

Our results show that PhbF from H. seropedicae SmR1 binds to eleven promoter regions of genes related to PHB metabolism. A DNA-binding consensus sequence was determined and confirmed by DNase-I footprinting assay. Furthermore, expression of phbF::lacZ and phaP1::lacZ fusions indicated that PhbF may act as a transcriptional repressor of genes involved in PHB metabolism in H. seropedicae SmR1.

Additional material

Additional file 1: Figure S1: Circular dichroism spectrum of purified H. seropedicae His-PhbF. Figure S2: Gel filtration chromatography of purified H. seropedicae His-PhbF. Figure S3: Schematic organization of genes probably involved in polyhydroxyalkanoate (PHA) pathway and regulation in H. seropedicae. Figure S4: The DNA-binding assays of purified His-PhbF from H. seropedicae to the ntrB promoter region (negative control).

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Authors' contributions

MASK carried out cloning, expression, purification and EMSA of PhbF, participated in experimental design and drafted the manuscript. MWS carried out cloning, in vivo assays, participated in experimental design and drafted the manuscript. FGM carried out the DNase I-footprinting assay. RAM participated in DNA sequence analysis. EMS, FOP and LSC participated in experimental design, discussion and manuscript writing. MGY participated in manuscript drafting and correction. MBRS conceived of the study and participated in its design and coordination. All authors read and approved the final manuscript.

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