Three novel proteins co-localise with polyhydroxybutyrate (PHB) granules in *Rhodospirillum rubrum* S1

Tanja Narancic, Elisa Scollica, Gerard Cagney and Kevin E. O’Connor

**Abstract**

Polyhydroxybutyrate (PHB), a biodegradable polymer accumulated by bacteria, is deposited intracellularly in the form of inclusion bodies often called granules. The granules are supramolecular complexes harbouring a varied number of proteins on their surface, which have specific but incompletely characterised functions. By comparison with other organisms that produce biodegradable polymers, only two phasins have been described to date for *Rhodospirillum rubrum*, raising the possibility that more await discovery. Using a comparative proteomics strategy to compare the granules of wild-type *R. rubrum* with a PHB-negative mutant housing artificial PHB granules, we identified four potential PHB granules’ associated proteins. These were: Q2RSI4, an uncharacterised protein; Q2RWU9, annotated as an extracellular solute-binding protein; Q2RQL4, annotated as basic membrane lipoprotein; and Q2RQS1, annotated as glucose-6-phosphate isomerase. *In silico* analysis revealed that Q2RSI4 harbours a Phasin_2 family domain and shares low identity with a single-strand DNA-binding protein from *Sphaerochaeta coccoides*. Fluorescence microscopy found that three proteins Q2RSI4, Q2EWU9 and Q2RQL4 co-localised with PHB granules. This work adds three potential new granule associated proteins to the repertoire of factors involved in bacterial storage granule formation, and confirms that proteomics screens are an effective strategy for discovery of novel granule associated proteins.

**INTRODUCTION**

Polyhydroxylalkanoate (PHA) accumulating bacteria, including *Rhodospirillum rubrum*, store this polyester in the form of intracellular granules [1, 2]. These granules, also called carbonosomes, are covered by a surface layer of up to 4 nm thickness [3–5]. The surface of the granules contains proteins which can be structural or catalytic and involved in the metabolism of PHA [6]. Besides PHA synthetases and depolymerases, other proteins named phasins are shown to be present on the granules [3]. The localisation of these proteins on the surface of PHA granules was shown by fluorescence microscopy using fusions of green fluorescent protein variants and the target proteins or by immuno-gold-labelling and transmission electron microscopy [3]. Phasins and other PHA metabolism related proteins have been extensively studied to better understand the PHA metabolism in bacteria [6].

In *Cupriavidus necator* (previously *Ralstonia eutropha*), a model organism for short chain length PHA, polyhydroxybutyrate (PHB) accumulation, seven phasins have been identified [7–10]. Multiple roles have been assigned to phasins in different bacteria: promoting the synthesis of PHA, determining the number, size and formation of granules [11, 12], facilitating the PHA depolymerisation and controlling degradation by limiting the access of PHA depolymerases to the polymer [8, 13], intracellular localisation and distribution of PHA granules to daughter cells during cell division [14, 15].

Little is known about the proteins present on the surface of the PHB granules in *R. rubrum*. Two proteins have been annotated as phasins, *Rru_A3283* and *Rru_A2817*. The phasin encoded by the gene *Rru_A3283* has been implicated in the activation of PHB depolymerisation [16, 17]. This phasin has a high similarity to magnetosome-associated proteins expressed by magnetotactic bacteria and a low similarity to PHB-granule-bound proteins from other bacteria [17]. An additional role of increasing the number of PHB granules in the cell has also been assigned to this protein [17]. The function(s) of the second annotated phasin are not known. Since PHA accumulating organisms were found
to express significantly larger number of phasins [18] it is likely that the proteome of PHB granules in *R. rubrum* contains more than two phasins. In order to identify any such phasin proteins, PHB granules were isolated from *R. rubrum* and analysed by high resolution Orbitrap mass spectrometry. *In silico* analysis implicated several new proteins as potential granule associated proteins, and co-localisation with PHB granules was confirmed using fluorescent microscopy (Fig. 1).

**METHODS**

**Bacterial strains, plasmids and culture conditions**

*Rhodospirillum rubrum* S1 (ATCC 11170) wild-type strain, and the recombinant *R. rubrum* strains (Table 1) were maintained on tryptic soy (TS; Oxoid) agar plates (1.5% w/v) supplemented with kanamycin (25 mg l$^{-1}$) when required. *Escherichia coli* DH5$\alpha$ strain was used in cloning experiments and was routinely grown in lysogenic broth (LB, Sigma) medium, with the appropriate antibiotic when required. For long term storage, the strains were grown in TS (*R. rubrum* and *R. rubrum* recombinant strains) or LB (*E. coli* strain) medium with the appropriate antibiotic, then mixed with sterile glycerol to a final concentration of 15% glycerol (w/v), and stored at $-80^\circ$C.

**Preparation of artificial PHB granules**

Artificial PHB granules were prepared as described by Horrowitz and Sanders [19]. In brief, 0.5 g of pure PHB extracted from lyophilised cells of *R. rubrum* using Soxhlet extraction method [20] was dissolved in 10 ml chloroform and the solution was heated to 60°C. The solution was emulsified by

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**Fig. 1.** Discovery of novel phasins in *Rhodospirillum rubrum* S1. *R. rubrum* S1 and *R. rubrum ΔphaC1ΔphaC2* were grown under polyhydroxybutyrate (PHB) accumulating conditions. All proteins that associated with artificial PHB granules (aPHB), added to the PHB-mutant after cell lysis, were considered a background and used to identify the proteins exclusively present on native PHB granules.
the addition of 20 volumes 50 mM cetrimonium bromide (CTAB) and 3 min sonication on ice at 20 Hz using 65 % amplitude, 2 s on/2 s off (Sonic dismembrator, Thermo-Fisher Scientific). The sample was heated to 75 °C on a hot plate with stirring for 90 min to allow for the evaporation of the chloroform and subsequently concentrated by centrifugation (30 min, 8000 × g, room temperature; benchtop 5430R centri- fug; Eppendorf). After centrifugation, the supernatant was discarded and the artificial granules were resus- pended in 500 µl water to prevent crystallisation and stored at room temperature.

**Isolation of PHB granules**

For polyhydroxybutyrate (PHB) granules isolation *R. rubrum* was cultivated as described before [21]. In brief, to stimulate PHB accumulation, *R. rubrum* was cultivated in 400 ml SMN medium [22] with 0.31 g l⁻¹ disodium malate (4 g l⁻¹) and 3.7 g l⁻¹ as a nitrogen source (nitrogen limiting conditions), supplemented with disodium malate (4 g l⁻¹) and 3.7 g l⁻¹ sodium acetate added after 24 h of cultivation. *R. rubrum* cultures were grown for 72 h in 21 flasks wrapped in aluminium foil, at 30 °C and 200 r.p.m. in the dark. *R. rubrum* ΔphaC1ΔphaC2 used as a control for the identification of proteins associated with PHB granules was cultivated under the same conditions. PHB content was deter- mined as described previously [21].

The isolation of native PHB granules was carried out as described by Handrick and colleagues [23]. *R. rubrum* and *R. rubrum* ΔphaC1ΔphaC2 cells were harvested by centrifugation at 7000 g for 10 min at 4 °C (benchtop 5430R centri- fug; Eppendorf) and washed twice with 50 mM phosphate buffer (pH 7). The cells were lysed using BugBuster Master Mix (Merck Millipore). Using polyallomer ultracentrifuge tubes (Beckman Coulter), a glycerol gradient was prepared in 100 mM Trizma buffer (pH 8; Sigma) and adding 5 ml of 90 % glycerol (v/v), followed by 10 ml of 50 % glycerol (v/v). Artificial PHB granules corresponding to the PHB content of the *R. rubrum* grown under PHB accumulating conditions (0.24 g l⁻¹ PHB) were added to the cell lysate of *R. rubrum* ΔphaC1ΔphaC2 and incubated for 10 min at room temperature. The cell lysates were loaded on top of the glycerol gradient and centrifuged for 40 min at 50 000 × g, 4 °C (SW 32Ti swinging rotor and Optima Preparative Ultracentrifuge; Beckman Coulter). The layer between 90 and 50 % glycerol was collected and diluted in three volumes of 100 mM Trizma buffer (pH 8) to give a final volume of 10 ml. The second glycerol gradient was prepared adding 5 ml of 90 % glycerol (v/v), 5 ml of 80 % glycerol (v/v), 5 ml of 60 % glycerol (v/v) and 5 ml of 40 % glycerol (v/v) in a fresh polyallomer tube. The samples were loaded on top of the glycerol gradient and centrifuged for 40 min under the same conditions as the first centrifugation step. The layer between 80 and 60 % glycerol was collected, diluted in 10 volumes of 100 mM Trizma (pH 8) and centrifuged for 30 min at 50 000 × g, 4 °C. The supernatant was discarded and the pellet, containing the PHB granules, was resuspended in 500 µl of 100 mM Trizma (pH 8). The total protein concentra- tion was determined using the bicinchoninic acid kit (Sigma-Aldrich).

**Proteomics**

The glycerol fraction containing PHB granules was subse- quently prepared for proteomic analysis as previously described [21]. Trypsin digested samples were run on the Q-Exactive Hybrid Quadrupole Orbitrap Mass Spectrometer

Table 1. Bacterial strains and plasmids used in this study

<table>
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<th>Strain designation</th>
<th>Relevant characteristics</th>
<th>Source</th>
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<td><em>R. rubrum</em> S1 PHB negative mutant</td>
<td>This study</td>
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<td>This study</td>
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<td>This study</td>
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</table>

<sup>*Kan<sup>®</sup> – kanamycin-resistant.</sup>
(MS; Thermo Scientific) connected to a Dionex Ultimate 3000 (RSLCnano; Thermo Scientific) chromatography system (Buffer A: 97% water, 2.5% acetonitrile, 0.5% acetic acid; buffer B: 97% acetonitrile, 2.5% water, 0.5% acetic acid; all solvents were LC-MS grade). Spectra were identified using the Andromeda peptide identification algorithm [24, 25], searching against the UniProt database of *R. rubrum* S1 (www.uniprot.org, downloaded in October 2016).

**Construction of EYFP C-terminal fusions**

*R. rubrum* genomic DNA was extracted using GeneJet Genomic DNA Purification Kit (ThermoScientific) according to manufacturer’s instructions. The genes of interest were amplified by PCR and the products of appropriate size (366 bp for *Rru_A2817*; 491 bp for *Rru_A3283*; 933 bp for *Rru_A2111*; 1007 bp for *Rru_A2784*; 1675 bp for *Rru_A0592*; 1678 bp for *Rru_A2947*) were ligated to pBRR1MCS-2-*P*\_phac-eyfp-c1 [26] to form in frame fusion with the *eyfp* gene (Table 1). The constructs were verified by sequencing (GATC Biotech Hamburg, Germany) and used to transform *R. rubrum* by electroporation [27]. The expression of the proteins was confirmed by Western blot using an anti-GFP IgG antibody (Roche Diagnostics), and anti-mouse IgG conjugated horseradish peroxidase (HRP; Merk Millipore). The chemiluminescence signal was detected by the Pierce ECL Western blotting substrate kit (ThermoFisher Scientific) and Fusion FX imager (Vilber Lourmat) and Fusion FX Visio Capt molecular imaging software (Vilber Lourmat).

**Nile red staining of PHB granules**

*R. rubrum* S1 wild-type and recombinant *R. rubrum* strains containing pBRR1MCS-2-*P*\_phac-eyfp-c1 or this plasmid derived constructs (Table 1) were grown under PHB accumulating conditions in 3 ml SMN medium for 48 h. The cultures were stained by mixing the culture with 0.1 vol of a Nile red solution containing 0.1 µg ml\(^{-1}\) Nile red dissolved in acetone [28].

**Microscopy imaging**

The microscope slides were prepared by adding 10 µl of stained cell culture and mixing it with 10 µl of Mowiol mounting medium, adding a cover slide and leaving the slides to solidify overnight. The Mowiol mounting medium was prepared by dissolving 2.4 g Mowiol 4–88 (Millipore) into 6 g of glycerol and 6 ml of water, and incubated 4–5 h at room temperature. Then 12 ml of 0.2 M Tris-Cl (pH 8.5) was added to the solution, heated to 50 °C for 10 min with occasional mixing. The solution was clarified by centrifugation (5000 g for 15 min).

PHB granules and EYFP-fused proteins were visualised using a confocal laser scanning microscope Fluoview FV1000 (Olympus, Japan). Images were acquired with a 60×UPLSAPO 1.35NA (Olympus, Japan) oil immersion objective at a resolution of 1024×1024 pixels in sequential scanning model and with EGF-specific filter set at 488 nm, and Nile red filter (AlexaFluor 568 filter set at 559 nm wavelength).

**RESULTS**

**Isolation of PHB granules of *Rhodospirillum rubrum* S1 and proteomic analysis**

The native PHB granules with a preserved surface layer were isolated from lysed cells of *Rhodospirillum rubrum* S1 using a glycerol gradient method [23, 29]. In order to ensure specificity of the proteins identified by mass spectrometry [29, 30], artificial PHB granules were prepared and added to the cell lysate of a PHB-negative mutant *R. rubrum* ΔphaC1ΔphaC2 [27]. These cells were grown in the same conditions as the wild-type *R. rubrum*, and proteins identified in this preparation were deemed to be background and removed from the final dataset.

The glycerol fraction containing native or artificial PHB granules was subsequently washed and prepared for mass spectrometry analysis as previously described [21]. The proteomes of *R. rubrum* wild-type native PHB granules fraction and *R. rubrum* ΔphaC1ΔphaC2 artificial granules fraction were compared using quantitative proteomics [31]. Four biological replicates were analysed per sample and only the proteins identified in at least three replicates in a sample were further analysed. All proteins that associated with the artificial granules in the PHB-negative mutant (1497 identifications; Fig. 1) were considered to be background and used to identify the proteins exclusively present on native PHB granules. This led to the identification of seven proteins exclusively detected in the wild-type *R. rubrum* under PHB accumulating conditions (Table 2). This list confirmed the presence of a phasin previously annotated in *R. rubrum* genome, while highlighting four potential novel granule-associated factors.

The proteins Q2RSI4, uncharacterised protein; Q2RWU9, annotated as extracellular solute-binding protein; Q2RQL4, annotated as basic membrane lipoprotein; and Q2RQ51 annotated as glucose-6-phosphate isomerase were exclusively present in the PHB granules fraction obtained from the wild-type *R. rubrum* (Table 2). Along with these four proteins we identified three additional proteins associated with PHB metabolism exclusively detected in the wild-type. While PHB synthase (Q2RRN2), PHB depolymerase (Q2RZN5) and one of the annotated phasins (Q2RQ11) were exclusively detected in the wild-type, the phasin Q2RP67 was detected in the PHB-negative mutant as well, although with nearly 26-fold lower expression level (Table 2).

In addition to phasin Q2RP67, other PHB metabolism related proteins were detected in *R. rubrum* ΔphaC1ΔphaC2 as well (Table 2). Acetyl-CoA transferase (Q2RXX6) which catalyses the condensation of two acetyl-CoA molecules into acetoacetyl-CoA showed 41-fold higher expression in the wild-type compared to the PHB-negative mutant. The acetoacetyl-CoA reductase (Q2RXX7) involved in the reduction of acetoacetyl-CoA to (R)-3-hydroxybutyryl-CoA showed a two-fold higher level of expression in the PHB-negative mutant (Table 2). In summary, using a quantitative proteomics strategy incorporating artificial PHB granules and a
PHB-negative mutant as a stringency control, we confirmed the presence of known PHB granules associated proteins phasins of *R. rubrum*, while highlighting four potential new factors.

**In silico analysis of phasins and PHB-granule associated proteins**

We next asked if the new PHB granule-associated factors we identified showed protein sequence features typical of phasin molecules. Phasins share weakly homologous hydrophobic domains that may interact with the polymer molecules [15]. The presence of conserved domains in proteins exclusively expressed in wild-type *R. rubrum* was analysed using the Sequence Similarity DataBase SSDB [32], while the predicted topology of the proteins based on amino acid sequence was analysed using the Phobius web server (http://phobius.sbc.su.se/ [33]).

Consistent with a protein that may be located in a polymer-containing granule, the previously uncharacterised protein Q2RSI4 is predicted to be non-cytoplasmic using Phobius analysis (Fig. 2a). Furthermore, it contains a conserved Phasin_2 motif in a stretch of 87 amino acids (position 174 to 261) (Fig. 3). This same motif is present in annotated phasins Q2RQ1 and Q2RP67, although closer to the N-terminus (Fig. 3). Interestingly however, Q2RSI4 does not show similarity with any of the known phasins and shares low amino acid sequence identity with DNA-binding proteins (Table S1, available in the online version of this article). Using Jpred4 [34] for the prediction of the secondary structure and HeliQuest [35] to calculate the helix properties it was shown that Q2RSI4 contains amphipathic α-helix structures (Fig. S1). It is believed that the hydrophobic side of the amphipathic α-helix faces the polymer, while the hydrophilic side faces the cytosol [18].

In contrast, no phasin-related motifs were found in Q2RWU9 (Fig. 3). Q2RWU9 is annotated as a membrane associated transporter potentially involved in antibiotic resistance and quorum sensing in *R. rubrum* S1 (www.kegg.jp), and shows below 65 % identity with other membrane associated transporters (Table S2). Analysis using Phobius suggests that the first 50 amino acids of Q2RWU9 most likely belong to a signal peptide, however there is a lower probability that the same region contains a transmembrane helix domain (Fig. 2b).

Q2RQL4, also predicted to be non-cytoplasmic and containing no predicted phasin domain is annotated as a basic membrane lipoprotein in *R. rubrum* S1 (www.kegg.jp). Similarly to Q2RWU9, the first 25 amino acids form a signal peptide, while the rest of the protein is predicted to be non-cytoplasmic (Fig. 2c). A Bmp (basic membrane lipoprotein) family domain (43–267 aa) (http://pfam.xfam.org), which is classified as an ABC transporter substrate-binding protein PnrA-like domain was identified (Fig. 3).

A protein blast analysis (http://www.genome.jp/tools/blast/) showed similarity of the protein with basic membrane proteins and ABC transporter substrate-binding proteins from other Gram-negative bacterial species and in particular a purine-binding protein precursor (Table S3).

The protein Q2RQ51 is annotated as the glucose-6-phosphate isomerase of *R. rubrum* S1. This protein has two hydrophobic regions, the N terminal (1–270 aa) and the C terminal region (330–547 aa; Fig. 2c). It shows the presence of a PGI family domain typical of glucose-6-phosphate isomerase enzymes (49–538 aa; Fig. 3). Q2RQ51 shares high similarity >70 % with glucose-6-phosphate isomerase enzymes from other Gram-negative bacterial species (Table S4).

### Table 2. The expression of proteins detected in polyhydroxybutyrate (PHB) granules fraction obtained by glycerol gradient centrifugation

Proteins exclusively present in the wild-type *R. rubrum* (WT) under PHB accumulating conditions were identified by comparing the proteomes of *R. rubrum* wild-type and PHB-negative mutant *R. rubrum ΔphaC1ΔphaC2* (KO). Values are presented as the average protein expression levels (LFQ: Label-free quantification (units of 10^6)), the sum of the ion signal recorded in the mass spectrometer for all peptides derived from each protein [31]) of four biological replicates and two technical replicates.

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<thead>
<tr>
<th>UNIPROT Gene</th>
<th>Annotation</th>
<th>KO LFQ value</th>
<th>WT LFQ value</th>
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<td>Phasin</td>
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</tr>
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<td><em>Rru_A2111</em></td>
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<td>ND</td>
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<td><em>Rru_A2947</em></td>
<td>Glucose-6-phosphate isomerase</td>
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<td>Q2RWU9</td>
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<td>Basic membrane lipoprotein</td>
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*indicates proteins expressed with significant (ANOVA Multi-sample test; P≤0.05) difference under tested conditions (fold change≥2). ND. Not detected.
The protein Q2RP67 was previously characterised as an activator of polymer mobilisation [17], and its co-localisation with PHB granules in *R. rubrum* was demonstrated [28]. The amino acid sequence of Q2RP67 has a topology prediction similar to the uncharacterised protein Q2RSI4 and the *Phasin_2* family domain (Fig. 3). Q2RP67 shows [85].

**Fig. 2.** Topology prediction for proteins detected as exclusively expressed under polyhydroxybutyrate (PHB) accumulating conditions in *Rhodospirillum rubrum* S1. (a) Q2RSI4, (b) Q2RWU9, (c) Q2RQL4 and (d) Q2RQ51 topology was predicted using the Phobius analysis tool (http://phobius.sbc.su.se/).

**Fig. 3.** Protein domains of the putative PHB granules associated proteins of *R. rubrum* S1. The presence of conserved domains was analysed by the SSEARCH program integrated in KEGG Sequence Similarity DataBase (SSDB; http://www.kegg.jp/kegg/ssdb/). Bmp – basic membrane lipoprotein domain, PnrA like family; SBP – extracellular solute binding proteins family; Gp41 – retroviral envelope protein domain; PGI – phosphoglucose isomerase.
the highest similarity with phasins from *Magnetospirillum* sp. ME-1 and *P. photometricum* and proteins annotated as a magnetic particle membrane specific GTPase from *M. gryphiswaldense* and *M. magnetotacticum*, but both containing a *Phasin_2* family domain (Table S5).

The protein Q2RQ1 is the second of the two annotated phasins in *R. rubrum*. Similarly to Q2RP67 and Q2RSI4, Q2RQ1 has the *Phasin_2* family domain (Fig. 3) and the same topology as these two proteins.

In conclusion, *in silico* analysis based on sequence homology implicated that three out of four proteins identified as exclusively present in the proteome of PHB granules, highlighted properties consistent with granule function for three proteins. We decided to investigate these proteins further using localisation studies.

**In vivo localisation of proteins putatively associated with granules**

To investigate the *in vivo* localisation of Q2RSI4, Q2RQ51, Q2RWU9, and Q2RQL4, C-terminal gene fusions with enhanced yellow fluorescent protein (EYFP) were constructed using plasmid pBRR1MCS-2_EYFP. Similar constructs were made for two annotated phasins as positive controls. Successful engineering and expression of the corresponding proteins were verified by sequencing and Western blot.

*R. rubrum* wild type and strains expressing the EYFP-tagged proteins were grown in SMN<sub>lim</sub> medium containing malate and acetate. These conditions stimulate PHB accumulation [21], hence granule production is expected to be high. The cells were harvested after 48 h of cultivation and stained with Nile red, a lipophilic dye that stains PHB granules [28].

Four to six granular structures were observed in all analysed strains (Fig. 4). The EYFP portion of the fused proteins was monitored using an EGFP-specific filter set at 488 nm. Discrete granular structures were observed in recombinant *R. rubrum* strains EYFP-Q2RQL4, EYFP-Q2RWU9, EYFP-Q2RP67, EYFP-Q2RQ11 and EYFP-Q2RSI4 (Fig. 4). Moreover, the co-localisation of the Nile red stained granules and EYFP signal is clear in all these samples, suggesting that EYFP-tagged proteins are indeed PHB-granules associated (Fig. 4).

When an empty vector pBRR1MCS-2-<sub>Pphas</sub>-*eyfp*-c1 is expressed in *R. rubrum* in PHB accumulating conditions, the EYFP protein is diffused throughout the cell, and no discreet structures could be observed (Fig. 4). However, the same pattern of PHB granules was observed in this strain (Fig. 4). The absence of EYFP signal is clear in wild type *R. rubrum* used as a negative control.

In the case of Q2RQ51 protein fusion with EYFP a diffused signal was recorded when EGFP filter was used, while granular structures were observed when Nile red filter was used. This indicates that Q2RQ51 is not a PHB granule associated protein (Fig. 4).

**DISCUSSION**

It is difficult to assess how many different proteins are present on the PHB granules’ surface *in vivo*, mainly because of a large number of proteins associating with granules during the process of isolation [2, 36]. To overcome this limitation we have synthesised artificial PHB granules which were added to the PHB-negative mutant of *Rhodospirillum rubrum* S1 [27] during the sample preparation for the proteomics. The artificial PHB granules are amorphous, and stable in suspension, and they are essentially indistinguishable from native PHB granules in morphology, size, density and mobility [21]. All proteins that associated with artificial PHB granules were considered a background and were used to filter out the proteins which were exclusively present on native PHB granules (Table 2). Predicted *R. rubrum* proteome contains 3830 proteins [37] and using a shotgun approach 39% of the predicted proteome was identified in *R. rubrum* wild-type and the PHB-negative mutant (SI dataset). Analysis of the PHB granules’ proteome of *Ralstonia eutropha* revealed more than 400 different proteins [2]. While 193 proteins identified in this study were exclusively detected in the PHB-negative mutant (SI dataset), this still represents a substantial portion of the *R. rubrum* proteome associated with PHB granules’ fraction.

Among the proteins exclusively detected in the wild-type, four were shown to be potentially associated with PHB granules in *R. rubrum* S1 for the first time: uncharacterised protein Q2RSI4, extracellular solute-binding protein Q2RWU9, basic membrane lipoprotein Q2RQL4 and glucose-6-phosphate isomerase Q2RQ51 (www.kegg.jp). Interestingly, while phasin Q2RQ11 was exclusively present in wild-type *R. rubrum*, phasin Q2RP67 was found to be expressed in the PHB-negative mutant as well, although at a 26-fold lower level (Table 2; S1 dataset). As previously stated, only the role of Q2RP67 in activation of PHB mobilisation in *R. rubrum* was demonstrated so far [17].

Acetyl-CoA acetyltransferase Q2XR6 and acetacetyl-CoA reductase Q2XR7 were also detected in both wild-type and the PHB-negative mutant (Table 2). However, since acetate was added to the medium to stimulate PHB accumulation, and these two proteins are shared between the PHB synthesis pathway and the ethylmalonyl-CoA pathway involved in acetate assimilation [21, 38], this is the likely reason for their expression. Only one of the three annotated depolymerases, Q2RNZ5 was detected at a low expression level in the wild-type, while the transcriptional regulator of PHB synthesis was absent from both samples (Table 2).

The proteins Q2RSI4, Q2RWU9, Q2RQL4 fused with the enhanced yellow fluorescent protein (EYFP) show clear co-localisation with Nile red stained PHB granules (Fig. 3). While glucose-6-phosphate isomerase Q2RQ51 appeared to be exclusively expressed in *R. rubrum* under PHB accumulating conditions, the signal recorded for the EYFP-fusion was diffused (Fig. 3, line 4B). Glucose-6-phosphate isomerase, or phosphoglucose isomerase is a ubiquitous glycolytic enzyme which catalyses the reversible isomerisation of glucose-6-phosphate to fructose-6-phosphate. In addition
to its role in central metabolism a mammalian glucose-6-phosphate isomerase was shown to function as neuroleukin, autocrine motility factor, maturation factor and as a serine protease inhibitor, making this enzyme a moonlighting protein, i.e. a protein which can perform multiple unrelated functions [39]. The expression of Q2RQ51 was detected in all four biological replicates of the wild-type \( R. \) rubrum sample, while no expression was detected in any of the PHB-negative mutant samples (SI dataset). The reason for the exclusive expression of Q2RQ51 under the PHB accumulating condition, when malate and acetate were used as the substrates for aerobic growth of \( R. \) rubrum remains unclear.

One of the novel granules associated proteins, Q2RSI4 contains the characteristic Phasin\(_2\) domain, also present in two previously annotated phasins Q2RP67 and Q2RQI1. Furthermore, Q2RSI4 shows the presence of amphipathic \( \alpha \)-helices, which are characteristic for phasins [18]. However, the relative abundance of this protein is significantly lower compared to the annotated phasins (Table 2). While this protein shows no similarity to other annotated phasins, it shows a low identity with a single-strand DNA-binding protein from \( S. \) coccoides (Table S1). In \( E. \) coli, a similar single-strand DNA binding protein binds tightly and cooperatively to single-stranded DNA and it is involved in replication, repair, and recombination of the DNA strands [40]. PhaM from \( R. \) eutropha has been shown to interact with PHA synthases, other phasins and genomic DNA, ensuring an equal distribution of PHB granules to daughter cells during cell division [2, 26]. Since Q2RSI4 shows similarity with a DNA binding protein, it is possible that this putative phasin has a role similar to PhaM. However, further characterisation is required to reveal the exact function of Q2RSI4.

Other PHB granules associated proteins exclusively expressed in the wild-type \( R. \) rubrum, Q2RWU9 and Q2RQL4 did not show the presence of the characteristic phasin domain (Fig. 2). This domain is detected in most phasin proteins, although it is not essential to characterise this family of proteins. For example, PhaM from \( R. \) eutropha, shown to be involved in granules segregation and activation of PHB synthase [41] also lacks known phasin motives. PhaM has two potential transmembrane domains in the N-terminus which could interact with hydrophobic moieties such as the cytoplasm membrane or the PHB granule surface [41]. However, both Q2RWU9 and Q2RQL4 contain a signal peptide sequence, which is not usually associated with phasins, and hydrophobic regions which could be involved in the protein–PHB interaction (Fig. 1b, c). It is possible that Q2RWU9 and
Q2RQL4 are somehow linked to the PHB accumulation, and while EYFP-tagged Q2RWU9 and Q2RQL4 co-localise with PHB granules they don’t have to be specifically involved in the PHB metabolism.

Unravelling novel PHB granules associated proteins and their functions will contribute to the understanding of the structure of PHB granules, or carbonosomes [8]. Furthermore, this knowledge will open up novel possibilities to use of PHB granules as nanobeads for high-affinity bioseparation, enzyme immobilisation, protein delivery, and diagnostics [42].

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Conflicts of interest
The authors declare that there are no conflicts of interest.

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