Role of Genetic Redundancy in Polyhydroxyalkanoate (PHA) Polymerases in PHA Biosynthesis in *Rhodospirillum rubrum*

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This study investigated the apparent genetic redundancy in the biosynthesis of polyhydroxyalkanoates (PHAs) in the *Rhodospirillum rubrum* genome revealed by the occurrence of three homologous PHA polymerase genes (*phaC1, phaC2*, and *phaC3*). In *vitro* biochemical assays established that each gene product encodes PHA polymerase. A series of single, double, and triple *phaC* deletion mutants were characterized with respect to PHA production and growth capabilities on acetate or hexanoate as the sole carbon source. These analyses establish that *phaC2* contributes the major capacity to produce PHA, even though the PhaC2 protein is not the most efficient PHA polymerase biocatalyst. In contrast, *phaC3* is an insignificant contributor to PHA productivity, and *phaC1*, the PHA polymerase situated in the PHA biosynthetic operon, plays a minor role in this capability, even though both of these genes encode PHA polymerases that are more efficient enzymes. These observations are consistent with the finding that PhaC1 and PhaC3 occur at undetectable levels, at least 10-fold lower than that of PhaC2. The monomers in the PHA polymer produced by these strains establish that PhaC2 is responsible for the incorporation of the C\(_5\) and C\(_6\) monomers. The *in vitro* characterizations indicate that heteromeric PHA polymerases composed of mixtures of different PhaC paralogs are more efficient catalysts, suggesting that these proteins form complexes. Finally, the physiological role of PHA accumulation in enhancing the fitness of *R. rubrum* was indicated by the relationship between PHA content and growth capabilities of the genetically manipulated strains that express different levels of the PHA polymer.

*Polyhydroxyalkanoates (PHAs)* are polyester polymers that many microbes use as a means of storing carbon and energy. These molecules are deposited within cells mainly in the form of protein- and lipid-bound granular inclusions (15, 22, 36, 38). PHA biosynthesis and accumulation is enhanced when microbes are under conditions of excess carbon but are limited for other nutrient(s), such as phosphorus, nitrogen, or sulfur (15, 22, 36, 38). Additional functionalities that have been ascribed to PHAs include the transport of calcium phosphate and DNA across membranes. This is thought to be achieved by the formation of calcium polyphosphate membrane-spanning channels that can enhance transformation efficiency and regulate intracellular calcium concentrations. Such changes in intracellular calcium concentrations also influence calcium signaling (29–31) and resistance to stresses (e.g., radiation, desiccation, and osmotic pressure) (40).

PHAs have technological applications as biodegradable plastics (46). Because these applications are dependent on the chemical and physical properties of the PHA polymer, which are themselves dependent on the monomeric constituents and their relative order in the polymer, intensive studies have been conducted to understand the mechanisms of PHA biosynthesis (15, 22, 38). These studies have established that the type of polymer that is produced depends on the diversity of substrates that are available to the PHA polymerase (product of the *phaC* gene) that assembles the final polymer (2, 23). The substrate for all known PhaC enzymes is (R)-configured 3-hydroxyacyl-coenzyme A (CoA) of different acyl chain lengths (from 3 to 14 carbons) (26).

Most bacteria produce PHAs that are composed of monomers that are either of short chain length (C\(_3\) to C\(_5\)) or medium chain length (C\(_6\) to C\(_8\)) (1, 16, 37, 38). A few organisms produce PHAs with a broader range of monomer chain lengths (C\(_4\) to C\(_{12}\)) (4, 10, 21, 23). The purple and phototrophic bacterium *Rhodospirillum rubrum* is known to produce PHA composed of both short- and medium-chain-length monomers, and it can produce up to 50% (dry weight) PHA (4, 19, 45). This metabolically versatile bacterium can grow under aerobic or anaerobic conditions or in the presence or absence of light, and in the latter condition it can use a variety of different carbon substrates (39, 43). Previous studies and genome sequencing have revealed that *R. rubrum* can express three PHA polymerases (5, 12). Because of its metabolic flexibility, *R. rubrum* offers the potential for converting many different carbon sources to PHA, and therefore there are considerable industrial interests in exploring this flexibility (6, 35). In this study, we characterized the significance and roles of each *phaC* paralog in PHA production and growth of *R. rubrum*.

**MATERIALS AND METHODS**

*Chemicals and enzymes.* All chemicals were obtained from Sigma-Aldrich Corporation (St. Louis, MO) and Fisher Scientific Inc. (Pittsburgh, PA). All DNA-manipulating enzymes were obtained from Invitrogen Corporation (Carlsbad, CA).

*Bacterial strains and plasmids.* *Rhodospirillum rubrum* ATCC 11170 and *Escherichia coli* DH5\(\alpha\) were used in this study. The primers used in this study are listed in Table S1 in the supplemental material.

*Bacterial growth conditions.* *E. coli* was grown at 37°C in LB medium. *R. rubrum* was grown in supplemented malate-ammonium medium (SMN medium) (13). When needed, gentamicin (25 \(\mu\)g/ml) or kanamycin (25 \(\mu\)g/ml) was added to medium to maintain selection for plasmids. Isopropyl-\(\beta\)-D-thiogalactopyranoside (IPTG) and 5-bromo-4-chloro-3-
indolyl-β-n-galactopyranoside (X-Gal) were used at concentrations of 20 and 40 mg/ml, respectively.

PHA production was assessed in *R. rubrum* cultured in RRNCO medium (but omitting ammonium chloride, hydrogen sulfide, carbon monoxide, and carbon dioxide) (14). In these experiments, a 0.2-ml aliquot of a normalized SMN culture (5 optical density [OD] units) was collected by centrifugation at 13,000 × g for 2 min; the cells were washed once with RRNCO medium and resuspended in 20 ml RRNCO medium containing either 10 mM acetate or 5 mM hexanoate as the carbon source. Cultures were shaken at 150 rpm at 25°C under 5,000-lx light intensity. For anaerobic growth, *R. rubrum* cultures were grown with an argon headspace in 18- by 150-ml anaerobic tubes (Bellco Biotechnology, Vineland, NJ).

For biochemical analysis, aliquots of 4 ml were withdrawn from the cultures at 0, 72, 96, 120, and 192 h postinoculation. Cell density was determined by monitoring the absorbance using a Spectronic 20+ spectrophotometer (Thermo Fisher Scientific Inc., Waltham, MA). The doubling time (*T*) was determined from the initial four time points of cultures.

**GC-MS analysis of PHA.** Cells were collected from liquid cultures by centrifugation at 6,000 × g for 10 min, and following washing with 10 mM Tris-HCl buffer (pH 7.5), the cell pellets were lyophilized and stored at −70°C until analysis. PHA content and composition were determined using 4- to 20-μg dry cell samples as described by Brandl et al. (3). Adipic acid was added as an internal standard. Derivatized samples (methylated esters) were concentrated under a stream of nitrogen gas when necessary. A 1-μl portion of the methyl esters was assayed by splitless injection into a gas chromatograph-mass spectrometer (GC-MS; Agilent 6890 GC) interfaced to an electron impact ionization mass spectrometer detector (Model 5973; Agilent Technologies, Santa Clara, CA). The GC was equipped with a DB-WAX column (30 m by 0.25 mm inner diameter, 0.5 μm), separation was with helium as the carrier gas (1.2 ml/min), and the temperature gradient was programmed from 80 to 200°C at 10°C/min. In the MS, the operating parameters were set to 70 electron voltage (eV) for the ionization voltage, and the interface temperature was 280°C. The temperatures of the injector and detector were 250 and 240°C, respectively.

The GC-MS data files were deconvoluted with the NIST AMDIS software (http://chemdata.nist.gov/mass-spc/amdis/). The retention times of the ionization voltage, and the interface temperature was 280°C. The tempera-

tures of the injector and detector were 250 and 240°C, respectively. The GC-MS data files were deconvoluted with the NIST AMDIS software (http://chemdata.nist.gov/mass-spc/amdis/). The retention times of the ionization voltage, and the interface temperature was 280°C. The temperatures of the injector and detector were 250 and 240°C, respectively. The GC-MS data files were deconvoluted with the NIST AMDIS software (http://chemdata.nist.gov/mass-spc/amdis/). The retention times of the ionization voltage, and the interface temperature was 280°C. The temperatures of the injector and detector were 250 and 240°C, respectively. The GC-MS data files were deconvoluted with the NIST AMDIS software (http://chemdata.nist.gov/mass-spc/amdis/). The retention times of the ionization voltage, and the interface temperature was 280°C. The temperatures of the injector and detector were 250 and 240°C, respectively. The GC-MS data files were deconvoluted with the NIST AMDIS software (http://chemdata.nist.gov/mass-spc/amdis/). The retention times of the ionization voltage, and the interface temperature was 280°C. The temperatures of the injector and detector were 250 and 240°C, respectively. The GC-MS data files were deconvoluted with the NIST AMDIS software (http://chemdata.nist.gov/mass-spc/amdis/). The retention times of the ionization voltage, and the interface temperature was 280°C. The temperatures of the injector and detector were 250 and 240°C, respectively.

**RESULTS AND DISCUSSION**

**Three phaC paralogs in the *R. rubrum* genome.** Previous studies identified and characterized two PHA polymerases (phaCs) (5, 12) in *R. rubrum*. Using these two sequences as the query, homology searches of the *R. rubrum* genome of strain ATCC 11170 revealed three genes encoding PHA polymerases, Rru_A0275, Rru_A0276, and Rru_A0277. One of them (phaC1) is located adjoining the phaA and phaB homologous genes in the PHA biosynthetic operon. The three phaC genes encode proteins of 414, 598, and 600 amino acids, respectively, and they all belong to the class I PHA polymerase family (26). Genes in *R. rubrum* are nearly distinct from *PhaC* and *PhaC* (sharing 14.3 and 18.4% sequence identity, respectively).

**Enzymological and protein methods.** Recombinant His-tagged fusion proteins were purified via nickel-affinity chromatography (BD Biosciences, San Jose, CA) from extracts of *E. coli* BL21-AI or Arctic Express strains harboring the appropriate pDEST17-derivatized expression vector. To generate antibodies, the purified recombinant proteins were immunized into mice maintained at the Iowa State University Hybridoma Facility (http://www.biotech.iastate.edu/facilities/hybridoma/). PHA biosynthetic proteins were immunologically detected after protein extracts were separated by SDS-PAGE, and immunoblot analysis were performed as described previously (17); there was no detectable cross-reactivity among the three PhaC antibodies that were prepared. The PHA polymerase activity was assayed spectrophotometrically at 37°C in a 500-μl reaction mixture that contained 50 mM Tris-HCl (pH 7.5), 0.5 mM hydroxybutyryl-CoA substrate, 50 μl of cell lysate containing 5 μg of recombinant PhaC, and 1 mM DTNB. Polymerase activity was monitored by the rate of increase in absorbance at 412 nm due to the reaction of the CoA thiol with DTNB (24). The concentration of CoA was determined using an extinction coefficient (412 nm) of 13,700 M−1 cm−1 (8, 32). One unit of polymerase activity is defined as 1 μmol CoA released/min/μg polymerase.

**Conjugation.** The three phaC gene deletion plasmids were mobilized from *E. coli* strain 17-1 into *R. rubrum* by conjugation (18). *R. rubrum* conjugants were selected on gentamicin-containing MN medium (MN medium is SMN medium in which yeast extract and casein enzyme hydrolysate are omitted). Single-colony *R. rubrum* conjugants were isolated by repeated transfer to gentamicin-containing MN medium.

**Construction of *R. rubrum* deletion mutant strains.** Seven deletion strains of *R. rubrum* were generated by homologous recombination via a two-step procedure using the suicide vector pJQ2005SK (25). These consist of the ΔphaC1, ΔphaC2, and ΔphaC3 single-site deletion mutant strains, the ΔphaC1ΔphaC2, ΔphaC1ΔphaC3, and ΔphaC2ΔphaC3 double mutant strains, and the ΔphaC1ΔphaC2ΔphaC3 triple mutant strain. The molecular confirmation of each deletion allele was achieved by PCR amplifying each allele with flanking primers (see Table S1 in the supplemental material) and determining the nucleotide sequence of each amplification product. Primers imC1f and imC1r were used to confirm the ΔphaC1 allele, primers imC2f and imC2r were used to confirm the ΔphaC2 allele, and primers imC3f and imC3r were used to confirm the ΔphaC3 allele.
onstrating that each phaC gene encodes a functional PHA polymerase. Of the three gene products, PhaC1 is the most efficient catalyst and PhaC2 has the lowest specific activity, about 10% of that of PhaC1.

The occurrence of multiple PHA polymerases in a single genome has been noted before in a number of Pseudomonas andRalstonia species (11, 23, 42). A BLASTP analysis (conducted in October 2011) of sequenced microbial genomes identified 315 strains that contain PhaC homologs, and 156 of these strains contain two or more paralogs. The distribution of these multiple strains that contain PhaC homologs, and 156 of these strains contain two or more paralogs. The distribution of these multiple PhaC loci within individual genomes has some commonalities. For example, as with R. rubrum, often one phaC locus is situated in a PHA biosynthetic operon that also contains the phaB and phaA genes. The additional PhaC loci are sometimes situated in operons that also contain PHA-regulatory functions, such asphaD andphaZ (23). However, there are also examples ofphaC loci that reside in independent, non-operon loci, as is the situation for thephaC2 andphaC3 loci of R. rubrum. For example, the Pseudogulbenkiania sp. NH8B genome contains threephaC genes, one (NH8B_1486) of which is situated in an operon that also contains thephaA gene (NH8B_1485), but the other two paralogs (NH8B_2463 and NH8B_1804) are at independent non-operon loci.

To investigate the metabolic functions of the three phaC genes in R. rubrum, single-locus deletion mutants (DeltaphaC1, DeltaphaC2, and DeltaphaC3 strains), double-locus phaC deletion mutants (DeltaphaC1 DeltaphaC2, DeltaphaC1 DeltaphaC3, and DeltaphaC2 DeltaphaC3 strains), and the triple phaC deletion mutant (DeltaphaC1 DeltaphaC2 DeltaphaC3 strain) were generated as described in Materials and Methods. These mutant strains were characterized in terms of growth, PHA yields, and monomer composition of the polymer. These characterizations were conducted by growing these strains with either acetate or hexanoate as the sole carbon source.

**Effect of single-locus phaC deletions on PHA production and growth.** With the exception of the DeltaphaC2 strain, all single mutant strains grew with kinetics similar to those of the wild-type (WT) strain with acetate (Fig. 2B) or hexanoate (Fig. 2D) as the carbon source; the triple DELTAphaC strain showed growth with kinetics similar to those of the wild-type (WT) strain with either acetate or hexanoate. The most significant difference among these strains in terms of PHA production was that with either carbon source the DeltaphaC1 strain accumulated PHA, to about 10% of WT levels with either carbon source (<3% CDW). In contrast, the DeltaphaC1 and DeltaphaC3 mutant strains showed a PHA accumulation pattern that was similar to that of the WT with either acetate (Fig. 2A) or hexanoate (Fig. 2C) as the carbon source, reaching values of about 25 and 15% of dry weight, respectively. An interesting observation was that with either carbon source the DeltaphaC1 strain accumulated larger amounts of PHA than the WT, an increase of between 10 and 30% depending on the carbon source and phase of growth. These in vivo data indicate that PhaC2 is the major polymerase enzyme contributing to the synthesis of PHA. Furthermore, PhaC1 appears to have an inhibitory effect on PHA biosynthesis.

**Effect of single-locus phaC deletions on PHA monomer composition.** In parallel to the studies described above, we determined the effect of the individual phaC knockouts on PHA monomer composition (Table 2). These analyses indicate that in any individual mutant strain, PHA monomer composition was unaffected by the phase of growth of the culture; the data presented in FIG 1 Amino acid sequence alignment of PhaC3, PhaC2, and PhaC1 from R. rubrum constructed with ClustalW (41). Black-shaded residues are identical, and gray-shaded residues are similar.

### TABLE 1 PHA polymerase activity in vitro

<table>
<thead>
<tr>
<th>Enzyme(s)</th>
<th>Expected activity</th>
<th>Activity (U)</th>
</tr>
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<tbody>
<tr>
<td>PhaC1</td>
<td>NA</td>
<td>17.9 ± 2.4</td>
</tr>
<tr>
<td>PhaC2</td>
<td>NA</td>
<td>2.1 ± 0.4</td>
</tr>
<tr>
<td>PhaC3</td>
<td>NA</td>
<td>6.6 ± 0.6</td>
</tr>
<tr>
<td>PhaC1 + PhaC2</td>
<td>10.0</td>
<td>48.6 ± 1.8*</td>
</tr>
<tr>
<td>PhaC1 + PhaC3</td>
<td>12.3</td>
<td>22.7 ± 1.8*</td>
</tr>
<tr>
<td>PhaC2 + PhaC3</td>
<td>4.4</td>
<td>3.0 ± 1.3</td>
</tr>
</tbody>
</table>

*NA, not applicable. An asterisk indicates that the activity of the heteromeric enzymes is significantly higher than the expected activity (P < 0.05). Data represent averages from triplicate determinations ± standard errors.
FIG 2 PHA content (A and C) and growth curves (B and D, with doubling times indicated) of *R. rubrum* strains (WT and ΔphaC1, ΔphaC2, and ΔphaC3 mutants) grown in acetate (A and B) or hexanoate (C and D) as the carbon source. Data represent averages from triplicate biological samples, and error bars indicate the standard errors.

TABLE 2 PHA composition of *R. rubrum* strains

<table>
<thead>
<tr>
<th>Strain and carbon source</th>
<th>PHA composition (mol %) of:</th>
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<tbody>
<tr>
<td></td>
<td>3HB (C₄)</td>
</tr>
<tr>
<td>Acetate</td>
<td></td>
</tr>
<tr>
<td>WT</td>
<td>99.6 ± 0.2</td>
</tr>
<tr>
<td>ΔphaC1</td>
<td>99.5 ± 0.2</td>
</tr>
<tr>
<td>ΔphaC2</td>
<td>100</td>
</tr>
<tr>
<td>ΔphaC3</td>
<td>99.4 ± 0.3</td>
</tr>
<tr>
<td>ΔphaC1 ΔphaC2</td>
<td>NA</td>
</tr>
<tr>
<td>ΔphaC1 ΔphaC3</td>
<td>99.3 ± 0.3</td>
</tr>
<tr>
<td>ΔphaC2 ΔphaC3</td>
<td>100</td>
</tr>
<tr>
<td>ΔphaC1 ΔphaC2 ΔphaC3</td>
<td>NA</td>
</tr>
</tbody>
</table>

| Hexanoate                |           |           |           |
| WT                       | 95.1 ± 0.4 | 0.8 ± 0.1 | 4.1 ± 0.3 |
| ΔphaC1                   | 93.3 ± 0.5 | 0.9 ± 0.1 | 5.8 ± 0.4 |
| ΔphaC2                   | 100        | <0.1*     | <0.1*     |
| ΔphaC3                   | 93.2 ± 0.4 | 1.0 ± 0.1 | 5.8 ± 0.3 |
| ΔphaC1 ΔphaC2            | NA        | NA        | NA        |
| ΔphaC1 ΔphaC3            | 93.6 ± 0.4 | 0.8 ± 0.1 | 5.6 ± 0.3 |
| ΔphaC2 ΔphaC3            | 100        | ND        | ND        |
| ΔphaC1 ΔphaC2 ΔphaC3     | NA        | NA        | NA        |

a 3HB, 3-hydroxybutyrate; 3HV, 3-hydroxyvalerate; 3HHx, 3-hydroxyhexanoate; NA, not applicable; ND, not detected; *, less than the detection limit (0.5 μg/ml for each monomer). Samples were collected at the 72-h time point. Data represent averages ± standard errors from triplicate biological samples.

2 where gathered from cells collected at the 72-h time point. In all strains grown on acetate, the vast majority (>99%) of the PHA was composed of the 3-hydroxybutyrate monomer (3HB), but in hexanoate-grown cells, about 6% of the monomer was either 3-hydroxyvalerate (3HV) or 3-hydroxyhexanoate (3HHx). The most significant monomer compositional change was observed with the strain lacking the PhaC2 polymerase (ΔphaC2), which resulted in the loss of the longer chain monomers (i.e., 3HV and 3HHx). This was particularly discernible when these strains were grown on hexanoate as the carbon source. A somewhat more subtle effect was the increased proportion of the longer chain monomers that occurred in the ΔphaC1 and ΔphaC3 strains; although relatively small amounts were incorporated in the polymer, statistical Student *t* tests indicate that the 3HV and 3HHx monomer contents in these two strains are significantly increased (*P* = 0.008). These data indicate that PhaC2 is the unique polymerase responsible for integrating 3HV and 3HHx monomers into the PHA polymer, and this is a property that is absent from PhaC1 and PhaC3 polymerases.

These compositional changes in the PHA polymer may be due to the substrate specificities of the three polymerases or to the availability of the appropriate monomer precursors. The 3HV and 3HHx monomers may be derived from intermediates of fatty acid β-oxidation or, in the case of 3HV, may be generated by the condensation of a propionate and acetate moiety (34). Regardless of these two mechanisms, our data indicate that PhaC2 has a unique capability to access these intermediates and incorporate them into the PHA polymer, whereas the other two polymerases exclude the incorporation of the larger precursors. An analogous situation has been analyzed in a *Pseudomonas* species, in which the *phaC* gene that resides in the operon that also contains *phaA* and *phaB* is responsible for poly(3-hydroxybutyrate) (PHB) biosynthesis (i.e., incorporating 3HB monomers). In this case, two independent *phaC* paralogs are responsible for incorporating the C₆ to C₁₂ monomers, and these two paralogs are class II polymerases (23).

Effect of double and triple *phaC* deletions on PHA production. To further dissect the significance of the gene redundancy in PHA polymerase genes, we generated all possible double mutant combinations and the triple mutant, which lacked all three *phaC* genes. As would be expected, the ΔphaC1 ΔphaC2 ΔphaC3 triple mutant strain failed to accumulate any PHA when either carbon source was tested. This finding indicates that these are the only polymerases that assemble the PHA polymer. The inability to accumulate PHA influences the initial rate of growth of the culture with either acetate or hexanoate as the carbon source (Fig. 3A and B), and this is particularly more evident with acetate as the carbon source.

The double mutant strains provide a means of assessing the ability of individual PHA polymerases to support PHA production. Thus, these experiments are a corollary to the single-gene
deletion mutants and provide an independent means of evaluating the function of each phaC paralog in PHA production. The data gathered from these double mutants grown on either acetate or hexanoate indicate that phaC2 is the most effective gene in determining PHA levels, followed by phaC1 and phaC3; indeed, the latter strain, expressing only phaC3, does not accumulate any detectable PHA (Fig. 4A and C). These conclusions are consistent with those reached from the characterization of the single-gene deletion mutants.

The effects of these genetic manipulations influence the growth of the cultures, in that strains that fail to accumulate significant levels of PHA (\(<1\%\) dry weight) grow slower than the wild type (Fig. 4B and D). In contrast, the double mutant strain that expresses only the PhaC2 polymerase and accumulates elevated levels of PHA grows at the same rate as the wild-type strain.

Of the double and triple mutants, only the \(\Delta\)phaC1 \(\Delta\)phaC3 double mutant accumulated sufficient PHA for a reliable determination of the monomer composition of the polymer. In this strain, which expressed only the PhaC2 polymerase, the proportion of the 3HV and 3HHx monomers increased compared to the WT (\(P = 0.01\)) when it was grown on hexanoate (Table 2), consistent with previous findings that phaC2 is primarily responsible for the incorporation of 3HV and 3HHx monomers into the polymer.

**Characterization of the expression of the PHA biosynthetic proteins.** To ensure that the genetic deletion of phaC genes does not elicit an alteration in other PHA biosynthetic genes, particularly the phaC homologs, which would confound the interpretation of these experiments, we compared the expression of the PHA biosynthetic genes between the WT and mutant strains. These analyses were conducted by using antibodies raised against each PHA biosynthetic protein, and we evaluated whether the accumulation of each protein was altered by the genetic manipulations. To ensure that the Western blot analysis quantitatively detected each PHA biosynthetic protein, each antibody was used to detect different amounts of the respective recombinant protein in the range of 1 to 100 ng of protein (Fig. 5A). These analyses indicate that in our immunological assays the detection limits for PhaC1, PhaC2, PhaC3, PhaA, and PhaB are approximately 10, 1, 1, 1, and 1 ng, respectively (Fig. 5A).

Using this immunological assay, we quantified the expression of each PHA biosynthetic protein in the WT strain. These analyses indicate that of the three PhaC paralogs, the most abundantly expressed is PhaC2 (2 to 4 ng/\(\mu\)g total protein), and the other two paralogs were below the detection limit of the assay (\(<0.2\) ng/\(\mu\)g of total protein). This biochemical finding that the PhaC2 paralog is the most highly expressed polymerase protein, being at least 10-fold more abundant than the other two paralogs, is consistent with the genetic-based conclusions that PhaC2 plays a major role in producing the PHA polymer and the other paralogs play minor roles. Of the three proteins that are encoded by the PHA operon, the most abundantly expressed is PhaA (5 to 10 ng/\(\mu\)g total protein).

**FIG 3** Growth curves (A and B, with doubling times indicated) of *R. rubrum* strains (WT and \(\Delta\)phaC1 \(\Delta\)phaC2 \(\Delta\)phaC3 mutant) grown in acetate (A) or hexanoate (B) as the carbon source. Data represent averages from triplicate biological samples, and error bars indicate the standard errors.

**FIG 4** PHA content (A and C) and growth curves (B and D, with doubling times indicated) of *R. rubrum* strains (WT and \(\Delta\)phaC1 \(\Delta\)phaC2, \(\Delta\)phaC1 \(\Delta\)phaC3, and \(\Delta\)phaC2 \(\Delta\)phaC3 mutants) grown in acetate (A and B) or hexanoate (C and D) as the carbon source. Data represent averages from triplicate biological samples, and error bars indicate the standard errors.
form complexes. Ingesting heteromeric polymerase mixtures are more active than the two paralogs when mixed (Table 1). These results indicate that PhaC1-containing enzymes are more active than the two paralogs when mixed, but no such enhancement was obtained when PhaC2 and PhaC3 enzymes were mixed when PhaC1 and PhaC3 enzymes were mixed, but less synergistic enhancement in PHA polymerase-specific activity was found when PhaC1 and PhaC3 enzymes were mixed.

The finding that interactions among PhaC proteins enhance PHA polymerase activity makes it difficult to directly extrapolate from the in vitro results to explain the in vivo genetic deletion results. Most of our studies have considered the effect of modifying phaC paralogs on PHA production, but PHA accumulation is a more integrated system. For example, previous studies have shown that PHA biosynthesis and degradation occurs simultaneously, and PHA granule-associated proteins need to coordinate for optimal PHA accumulation (7, 27, 28, 44). Thus, in our ΔphaC mutants we need to consider not only the effect on PHA polymerization but also that the global PHA biosynthetic, catabolism, and storage machinery may modulate in response to the genetic modifications to accommodate the discrete ΔphaC mutant changes. However, our studies provide insights into the fact that PHA polymerase enzyme activity can be modulated at the biochemical level by the ability to produce heteromeric polymerase enzymes, and this attribute may have biotechnological applications to enhance PHA production.

The interrelationships among PHA monomer composition, PHA levels, and growth. In the characterizations of the mutant R. rubrum strains that express qualitative and quantitative changes in PHA, we noted correlations between these traits and the growth characteristics of the strains. Specifically, we observed a correlation between the occurrence of 3HHx monomer-containing polymer and the maintenance of PHA content into the late stationary growth phase of the culture. For example, thephaC2-containing strains, which accumulate significant levels of 3HHx monomers in the PHA polymer, maintain higher levels of PHA in the stationary phase of culture growth when grown in hexanoate as a carbon source. However, this maintenance of PHA into stationary phase does not occur in acetate-grown cultures, and the PHA that is produced does not contain 3HHx monomer. The likely explanation for this phenomenon is that the PHA depolymerase (encoded by phaZ1[Rru_A1585]) of R. rubrum has poor activity against PHA polymers that contain medium-chain-length monomers and greater specificity for degrading polymers that are composed of short-chain-length monomers (3HB and 3HV) (9).

Another observation that may be indicative of the physiological-
PHAs serve as an electron and energy sink by acting as a store for carbon and therefore provides a mechanism for growth of R. rubrum strains. The OD of strains grown on acetate as the carbon source was obtained after 72 h of cultivation (Fig. 6A) and a higher growth rate (Fig. 6B). These correlations are also dependent on the carbon source, there being a stronger growth dependency on PHA content for the cultures grown on acetate than on hexanoate.

Although the underlying mechanisms for these correlations are unclear, previous studies have shown that accumulation of PHA has positive contributions to growth by enhancing resistance to stresses (e.g., radiation, desiccation, and osmotic pressure) (40). PHAs serves as an electron and energy sink by acting as a store of highly reduced carbon and therefore provides a mechanism for cellular homeostasis of energy through carbon storage. Our data indicate that the capacity of the culture to accumulate PHA is correlated with the growth capabilities of the culture. A future direction of this research could be to investigate the role of the major regulator (guanosine-tetraphosphate) in coordinating growth and PHA production, as occurs in Pseudomonas oleovorans (20).

ACKNOWLEDGMENTS

This work was supported by the Bioeconomy Institute of Iowa State University and the U.S. National Science Foundation through its Engineering Research Center Program (award no. EEC-0813570), which funds the Center for Biorenewable Chemicals (CBiRC), headquartered at Iowa State University and including Rice University, the University of California, Irvine, the University of New Mexico, the University of Virginia, and the University of Wisconsin–Madison.

We acknowledge the suggestions made by the ad hoc reviewers concerning the in vitro characterization of the PhaC proteins.

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Role of Genetic Redundancy in Polyhydroxyalkanoate (PHA) Polymerases in PHA Biosynthesis in *Rhodospirillum rubrum*

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Volume 194, no. 20, pages 5522–5529, 2012. Page 5528, column 1: Lines 18–22 should read as follows. "A future direction of this research could be to investigate the role of the major regulator (guanosine-tetraphosphate) in coordinating growth and PHA production, as occurs in *Pseudomonas oleovorans* (32a)."

Page 5528: Reference 20 should be deleted.

Page 5529: The following reference was inadvertently omitted.