The role of genetic redundancy in Polyhydroxyalkanoate Polymerases in PHA biosynthesis in *Rhodospirillum rubrum*

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Running title: Genetic redundancy of *phaC* genes in *Rhodospirillum rubrum*

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This study investigated the apparent genetic redundancy in the biosynthesis of polyhydroxyalkanoates (PHAs) in *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 encode 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 PhaC2. The monomers in the PHA polymer produced by these strains establish that PhaC2 is responsible for the incorporation of the C₅ and C₆ 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 may form complexes. Finally, the physiological role of PHA accumulation in supporting growth 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 (17, 23, 38, 40). PHA biosynthesis and accumulation is enhanced when microbes are in conditions of excess-carbon, but limited for other nutrient(s), such as phosphorus, nitrogen, or sulfur (17, 23, 38, 40). 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 (30-32), and resistance to stresses (e.g., radiation, desiccation and osmotic pressure) (42).

PHAs have technological applications as biodegradable plastics (47). Because these applications are dependent on the chemo-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 (17, 23, 40). These studies have established that the type of polymer that is produced depends on the diversity of substrates that are availability to the PHA polymerase (product of the phaC gene) that assembles the final polymer (2, 24). The substrate for all known PhaC enzymes is (R)-configured 3-hydroxyacyl-CoA of different acyl-chain lengths (from 3 to 14 carbons) (27).

Most bacteria produce PHAs that are composed of monomers that are either of short chain length (C3-C5), or medium chain length (C6-C14) (1, 3, 39, 40). A few organisms that produce
PHAs with a broader range of monomer chain lengths (C_4-C_{12}) (5, 11, 22, 24). 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 of PHA (5, 12, 20). This metabolically versatile bacterium can grow under aerobic or anaerobic conditions, in the presence, or absence of light, and in the latter condition it can use a variety of different carbon substrates (41, 45). Previous studies and genome sequencing have revealed that *R. rubrum* can express three PHA polymerases (6, 14). Because of the metabolic flexibility of *R. rubrum*, it offers the potential for converting many different carbon-sources to PHA, and therefore there are considerable industrial interests in exploring this flexibility (7, 37). In this study we characterized the significance and roles of each *phaC* paralogs in PHA production and growth of *R. rubrum*. 
MATERIALS AND METHODS

Chemical 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, plasmids. The strains of *Rhodospillum rubrum* ATCC11170 and *Escherichia coli* DH5α were used in this study. The primers used in this study are listed in Table S1.

Bacteria growth conditions. *E. coli* was grown at 37 °C in LB medium. *R. rubrum* was grown in supplemented malate-ammonium medium (SMN medium) (15). When needed, gentamicin (25 µg/ml) or kanamycin (25 µg/ml) was added to media to maintain selection for plasmids. IPTG and 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) (16). In these experiments, a 0.2 ml aliquot of a normalized SMN culture (5 O.D.) 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 5000 Lux light intensity.

For anaerobic growth, *R. rubrum* cultures were grown with an argon head-space in 18×150 mm anaerobic tubes (Bellco Biotechnology, Vineland, NJ). For biochemical analysis, aliquots of 4-ml, were withdrawn from the cultures at 0, 72, 96, 120, 192-hours post-inoculation. Cell density was determined by monitoring A680 using a Spectronic 20D+ spectrophotometer (Thermo
Doubling time ($T_d$) was determined from the initial four time points of cultures.

**GC-MS analysis of PHA.** Cells were collected from liquid cultures by centrifugation at 6000×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 4-20 mg dry cell samples as described by Brandel et al. (4). 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 GC-MS; an Agilent 6890 GC, interfaced to an electron impact ionization Model 5973 mass spectrometer detector (Agilent Technologies, Santa Clara, CA). The GC was equipped with a DB-WAX column (30m×0.25 mm ID, 0.5 μm), separation was with helium as the carrier gas (1.2 ml/min), and the temperature gradient was programmed from 80 °C to 200 °C at 10 °C/min. In the MS, the operating parameters were set to 70 eV for the ionization voltage, and the interface temperature was at 280 °C. The temperatures of the injector and detector were 250 °C and 240 °C, respectively. The GC/MS data files were de-convoluted with the NIST AMDIS software (http://chemdata.nist.gov/mass-spc/amdis/). The retention times of the different, 3-hydroxy-carboxylic acid methyl esters (C₄, C₅ and C₆) were as follows: 6.75, 7.66 and 8.70 min, respectively. The PHA content was calculated as the percent of cell dry weight, using 3-hydroxybutyric acid (10 μg/ml-1000 μg/ml) to construct a standard curve, normalized for losses based on the recovery of the methyl ester of adipic acid.

**DNA isolation and manipulation.** Genomic DNA was isolated from *R. rubrum* as described
by Kerby et al. (15). Plasmids were isolated from *E. coli* cells grown in LB medium by using QIAprep Spin Miniprep Kit (Qiagen Inc., Valencia, CA). Agarose gel electrophoresis and transformation of *E. coli* were carried out as described by Sambrook and Russell (34). PCR products were cloned into pCR2.1-TOPO using a TOPO TA cloning kit (Invitrogen Corporation, Carlsbad, CA).

**Construction of gene-deletion-plasmids.** The 1018-bp DNA fragment upstream of the *phaC1* ORF was amplified by PCR with the primers mC1USf-*SacI* and mC1USr-*XbaI*, and this amplification product contained *SacI* and *XbaI* sites at the 5′ and 3′ ends. The PCR product was purified and ligated into the TA-cloning vector, pPCR2.1-TOPO. The PCR fragment was removed from the resulting vector by digestion with *SacI* and *XbaI*, gel-purified and cloned into pJQ200SK, at the *SacI* and *XbaI* sites, forming the vector pJQ200SKmC1U. The 1023-bp DNA fragment downstream of the *phaC1* ORF was cloned into the vector pJQ200SKmC1U via the same strategy, forming the ΔphaC1 gene-deletion-vector, pJQ200SKmC1UD. The same strategy was used to construct vectors pJQ200SKmC2UD and pJQ200SKmC3UD, which were used to create gene-deletion alleles for *phaC2* and *phaC3* genes, respectively. The primers used for amplifying *R. rubrum* genomic DNA fragments for these latter vectors are listed in Table S1.

**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 used to immunize mice maintained at the Iowa State University Hybridoma Facility.
PHB biosynthetic proteins were immunologically detected after protein extracts were separated by SDS-PAGE, and immunoblot analysis were performed as described previously (18); 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 5μg 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 (25). The concentration of CoA was determined using the extinction coefficient (412 nm) of 13,700 M⁻¹ cm⁻¹ (9, 33). 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 (19). 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 mutants strains. Seven deletion strains of R. rubrum were generated by homologous recombination via a two-step procedure using the suicide vector pJQ200SK (26). These consisted of three single site deletion mutant strains ΔphaC1, ΔphaC2, and ΔphaC3; three double mutant strains ΔphaC1ΔphaC2, ΔphaC1ΔphaC3, ΔphaC2ΔphaC3; and a triple mutant strain ΔphaC1ΔphaC2ΔphaC3. The molecular confirmation of each deletion allele was achieved by PCR amplifying each allele with flanking primers (Table S1), and
determining nucleotide sequence of each amplification product. Primers imC1f and imC1r were used to confirm the \( \Delta \text{phaC1} \) allele; primers imC2f and imC2r were used to confirm the \( \Delta \text{phaC2} \) allele; and primers imC3f and imC3r were used to confirm the \( \Delta \text{phaC3} \) allele.
RESULTS AND DISCUSSION

Three phaC paralogs in the R. rubrum genome. Previous studies identified and characterized two PHA polymerases (PhaCs) (6, 14) 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_A2413 and Rru_A1816, which we designated phaC1, phaC2, and phaC3, respectively. 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 (27, 35). Figure 1 shows the sequence comparison of the amino acids sequences of PhaC1, PhaC2 and PhaC3 proteins, and this analysis indicates that PhaC2 and PhaC3 share highest sequence conservation (50.2% identity), and PhaC1 is equally distinct from PhaC2 and PhaC3 (share 14.3% and 18.4% sequence identity, respectively).

Recombinantly expressing each phaC gene in E. coli and assaying the resulting cell lysate for PHA polymerase activity provided experimental evidence that these three homologs encode PHA polymerase enzymes (Table 1). All three individual proteins are capable of utilizing 3-hydroxybutyl-CoA to produce PHA, demonstrating that each phaC-gene encodes a functional PHA polymerase. But of the three gene products, PhaC1 is the most efficient catalyst, and PhaC2 has lowest specific activity, at about 10% of PhaC1.

The occurrence of multiple PHA polymerases in a single genome has been noted before in a number of Pseudomonas and Ralstonia species (13, 24, 44). 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 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 as phaD and phaZ (24). But there are also examples of phaC loci that reside in independent, non-operon loci, as is the situation for the phaC2 and phaC3 loci of R. rubrum. For example, the Pseudogulbenkiania sp. NH8B genome contains three phaC genes, one (NH8B_1486) of which is situated in an operon that also contains the phaA 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 phaCs in R. rubrum, single locus deletion mutants (ΔphaC1, ΔphaC2, ΔphaC3), double-loci phaC deletion mutants (ΔphaC1ΔphaC2, ΔphaC1ΔphaC3, and ΔphaC2ΔphaC3), and the triple phaC deletion mutant (ΔphaC1ΔphaC2ΔphaC3) were generated as described in the Materials and Methods. These mutant strains were characterized relative to 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 loci phaC deletions on PHA production and growth.** With the exception of the ΔphaC2 strain, all single mutant strains grew with similar kinetics to the wild-type strain with acetate (Fig. 2B) or hexanoate (Fig. 2D) as carbon sources; the ΔphaC2 strain grew significantly slower (doubling time is 14.2 h, as compared to 10.3 h for the WT strain) and to a lower cell
density when grown on acetate. The most significant difference among these strains in terms of PHA production was the observation that the \( \Delta \text{phaC2} \) strain lost ability to accumulate PHA, to about 10% of WT levels with either carbon sources (<3% cell dry weight (CDW)). In contrast, the \( \Delta \text{phaC1} \) and \( \Delta \text{phaC3} \) mutant strains showed a PHA accumulation pattern that was similar to the WT with both acetate (Fig. 2A) or hexanoate (Fig. 2C) as carbon sources, reaching values of about 25% and 15% of dry weight, respectively. An interesting observation was the fact that with either carbon-source the \( \Delta \text{phaC1} \) 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 \textit{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.

\textbf{Effect of single loci \textit{phaC} deletions on PHA monomer composition.} In parallel to the above study, we determined the effect of the individual \textit{phaC} knockouts on the 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 Table 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 hexanote 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 (\( \Delta \text{phaC2} \)), which resulted in the loss of the longer chain monomers (i.e., 3HV and 3HHx). This was particularly discernable 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 Δ
phaC3 strains; although relatively small amounts were incorporated in the polymer, statistical
Student t-tests indicate that the 3HV and 3HHx monomer content in these two strains are
significantly increased (p-value = 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 it may be generated by the condensation of a propionate and acetate moiety (36).
Regardless of these two mechanisms our data indicates 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* that resides in the operon that also
contains *phaA* and *phaB* is responsible for 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 (24).

**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

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expected, the triple mutant strain, ΔphaC1ΔphaC2ΔphaC3, 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 carbon source (Fig. 3A and 3B), 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 that express only phaC3 does not accumulate any detectable PHA (Fig. 4A and 4C). These conclusions are consistent with those reached from the characterization of the single-gene deletion mutants.

The effect 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 4D). 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 ΔphaC1ΔphaC3 double mutant accumulated sufficient PHA for a reliable determination of the monomer composition of the polymer. In this strain that expressed only the PhaC2 polymerase, the proportion of the 3HV and 3HHx monomer
increased as compared to the WT (p-value = 0.01), when it was grown on hexanoate (Table 2), consistent with previous finding 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 do 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 gene 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 1-100 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 10ng, 1ng, 10ng, 1ng, 1ng, respectively (Figure 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 (2-4 ng/μg total protein), and the other two paralogs were below the detection limit of the assay (<0.2 ng/μg 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 contributes the major capacity to produce 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 (5-10 ng/µg total protein), followed by PhaB (2-4 ng/µg total protein), and PhaC1 was below detection limit (<0.2 ng/µg total protein).

In response to the genetic manipulation of each *phaC* paralog, we analyze the expression level of those proteins that were detectable by this immunological assay (i.e., PhaA, PhaB and PhaC2) (Figure 5B). These analyses indicate that of the three proteins that were detectable, only the expression of PhaA and PhaC2 were altered in response to the genetic manipulations; PhaB accumulation was unaltered. PhaA levels increased in all three strains that carried a Δ*phaC2* deletion, but this occurred only in strains grown on hexanoate as a carbon source. This is consistent with the involvement of PhaC2 as the only polymerase that has the capacity to utilize 3HHx monomer generated from the β-oxidation of hexanoate; hence PhaA expression is enhanced in the Δ*phaC2* deletions to accommodate carbon flux through the PHA operon pathway (i.e., needing PhaA functionality).

**In vitro enzymological assay of PHA polymerase activities.** The observation that eliminating *phaC1* or *phaC1-phaC3* combination results in increased accumulation of PHA suggests that these two genes interact to affect PHA biosynthetic capability. Evidence for potential interactions between the PhaC-proteins was obtained by mixing *E. coli* cell lysates that contain each of the PhaC paralogs. For example, mixing lysates that contained 0.5 µg of PhaC1 and PhaC2, we expected a yield of about 10 units of PHA polymerase activity; however, we observed a specific activity of 49 units/µg (Table 1). This synergistically enhanced PHA polymerase activity is an indication that these two paralogs are interacting. This level of synergistic
activation of PHA polymerase activity was also observed when these two paralogs were mixed at a ratio of 1:10 (PhaC1:PhaC2) (data not shown), which is more reflective of the \textit{in vivo} accumulation of these two polymerases (see Figure 5). Similar, but less synergistic enhancement in PHA polymerase specific activity was found when PhaC1 and PhaC3 enzymes were mixed, but no such enhancement was obtained when PhaC2 and PhaC3 were mixed (Table 1). These results indicate that PhaC1-containing heteromeric polymerase mixtures are more active than the homomeric enzymes, indicating that these paralogs probably form complexes.

The finding that interactions among PhaC proteins enhance PHA polymerase activity makes it difficult to directly extrapolate from the \textit{in vitro} results to explain the \textit{in vivo} genetic deletion results. Most of our studies have considered the effect of modifying \textit{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 (8, 28, 29, 46). Thus, in our \textit{ΔphaC} mutants we not only need to consider the effect on PHA polymerization, but need to consider that the global PHA biosynthetic, catabolism and storage machinery may modulate in response to the genetic modifications to accommodate the discrete \textit{ΔphaC} 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, the *phaC2*-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) (10).

Another observation that may be indicative of the physiological function of PHA in the growth characteristic of *R. rubrum* is the correlation between PHA content and the initial growth rate and final density of the cultures. These correlations are illustrated in Fig. 6, which indicate positive correlations between PHA content and growth capabilities of the cultures, achieving higher cell density (Fig. 6A), and a faster 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 rather than on hexanoate.
Although the underlying mechanism 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) (42). PHA serves as an electron and energy sink by acting as a store of highly reduced carbon reservoir 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* (21).
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FIGURE LEGENDS

FIG. 1. Amino acid sequence alignment of PhaC3, PhaC2 and PhaC1 from *R. rubrum* constructed with ClustalW (43). Black shaded residues are identical, and gray shaded residues are similar.

FIG. 2. PHA content (A and C) and growth curves (B and D, with doubling time indicated) of *R. rubrum* strains (WT, ΔphaC1, ΔphaC2 and ΔphaC3) grown in acetate (A-B) and hexanoate (C-D) as a carbon source. Data represent average from triplicate biological samples, and error bars indicate the standard error.

FIG. 3. Growth curves (A and B, with doubling time indicated) of *R. rubrum* strains (WT and ΔphaC1ΔphaC2ΔphaC3) grown in acetate (A) and hexanoate (B) as a carbon source. Data represent average from triplicate biological samples, and error bars indicate the standard error.

FIG. 4. PHA content (A and C) and growth curves (B and D, with doubling time indicated) of *R. rubrum* strains (WT, ΔphaC1ΔphaC2, ΔphaC1ΔphaC3 and ΔphaC2ΔphaC3) grown in acetate (A-B) and hexanoate (C-D) as a carbon source. Data represent average from triplicate biological samples, and error bars indicate the standard error.
FIG. 5. Immunological characterization of PHA biosynthetic proteins. (A) Immunological
detection limit of each PHA biosynthetic protein. The indicated amounts of each purified
recombinant protein were subjected to SDS-PAGE and western blot analysis. (B) Western blot
analysis of PhaA, PhaB and PhaC2 proteins in extracts of *R. rubrum* strains, grown in acetate as
carbon source (after 72-hour cultivation), and hexanoate as carbon source (after 192-hour
cultivation). Not shown in B are immunoassays for PhaC1, PhaC3, which were undetectable and
below the detection limit of the assay. For PhaA, PhaB and PhaC2, each lane contained 5 μg of
total protein, and for PhaC1 and PhaC3, each lane contained 50 μg of total protein.

FIG. 6. Physiological consequence of genetic changes in PHA accumulation. (A) Correlation
between final culture cell density and PHA content of *R. rubrum* strains. The O.D. of strains
grown on acetate as carbon source was obtained after 72-hour cultivation (Δ), and the O.D.
of strains grown on hexanoate as carbon source was obtained after 240-hour cultivation (□).
(B) Correlation between culture doubling time and maximum PHA content of *R. rubrum* strains
when grown on acetate (Δ) or hexanoate (□) as carbon source. Data represent averages
from triplicate biological samples, and error bars indicate the standard error.
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<th>Enzyme mixture</th>
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<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, non applicable; *heteromeric enzymes exhibit significantly higher activity than the expected activity (p-value < 0.05).

Data represent average of triplicate determinations ± the standard error.
## TABLE 2. PHA composition of *R. rubrum* strains

<table>
<thead>
<tr>
<th>Carbon source</th>
<th>Strains</th>
<th>PHA composition (mol %)</th>
<th>3HB (C₄)</th>
<th>3HV (C₅)</th>
<th>3HHx (C₆)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Acetate</td>
<td>WT</td>
<td>99.6% ± 0.2%</td>
<td>0.4% ± 0.2%</td>
<td>&lt; 0.1%*</td>
<td></td>
</tr>
<tr>
<td></td>
<td>ΔphaC1</td>
<td>99.5% ± 0.2%</td>
<td>0.5% ± 0.2%</td>
<td>&lt; 0.1%*</td>
<td></td>
</tr>
<tr>
<td></td>
<td>ΔphaC2</td>
<td>100%</td>
<td>&lt; 0.1%*</td>
<td>&lt; 0.1%*</td>
<td></td>
</tr>
<tr>
<td></td>
<td>ΔphaC3</td>
<td>99.4% ± 0.3%</td>
<td>0.6% ± 0.3%</td>
<td>&lt; 0.1%*</td>
<td></td>
</tr>
<tr>
<td></td>
<td>ΔphaC1ΔphaC2</td>
<td>NA</td>
<td>NA</td>
<td>NA</td>
<td></td>
</tr>
<tr>
<td></td>
<td>ΔphaC1ΔphaC3</td>
<td>99.3% ± 0.3%</td>
<td>0.7% ± 0.3%</td>
<td>&lt; 0.1%*</td>
<td></td>
</tr>
<tr>
<td></td>
<td>ΔphaC2ΔphaC3</td>
<td>100%</td>
<td>ND</td>
<td>ND</td>
<td></td>
</tr>
<tr>
<td></td>
<td>ΔphaC1ΔphaC2ΔphaC3</td>
<td>NA</td>
<td>NA</td>
<td>NA</td>
<td></td>
</tr>
<tr>
<td>Hexanoate</td>
<td>WT</td>
<td>95.1% ± 0.4%</td>
<td>0.8% ± 0.1%</td>
<td>4.1% ± 0.3%</td>
<td></td>
</tr>
<tr>
<td></td>
<td>ΔphaC1</td>
<td>93.3% ± 0.5%</td>
<td>0.9% ± 0.1%</td>
<td>5.8% ± 0.4%</td>
<td></td>
</tr>
<tr>
<td></td>
<td>ΔphaC2</td>
<td>100%</td>
<td>&lt; 0.1%*</td>
<td>&lt; 0.1%*</td>
<td></td>
</tr>
<tr>
<td></td>
<td>ΔphaC3</td>
<td>93.2% ± 0.4%</td>
<td>1.0% ± 0.1%</td>
<td>5.8% ± 0.3%</td>
<td></td>
</tr>
<tr>
<td></td>
<td>ΔphaC1ΔphaC2</td>
<td>NA</td>
<td>NA</td>
<td>NA</td>
<td></td>
</tr>
<tr>
<td></td>
<td>ΔphaC1ΔphaC3</td>
<td>93.6% ± 0.4%</td>
<td>0.8% ± 0.1%</td>
<td>5.6% ± 0.3%</td>
<td></td>
</tr>
<tr>
<td></td>
<td>ΔphaC2ΔphaC3</td>
<td>100%</td>
<td>ND</td>
<td>ND</td>
<td></td>
</tr>
<tr>
<td></td>
<td>ΔphaC1ΔphaC2ΔphaC3</td>
<td>NA</td>
<td>NA</td>
<td>NA</td>
<td></td>
</tr>
</tbody>
</table>

* a 3HB, 3-hydroxybutyrate; 3HV, 3-hydroxyvalerate; 3HHx, 3-hydroxyhexanoate; NA, non
applicable; ND, non detected. * less than detection limit (0.5 μg/ml for each monomer).

b Samples were collected at 72-hour-time point. Data represent averages ± the standard error from triplicate biological samples.
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.