Class I Polyhydroxyalkanoate Synthase from the Purple Photosynthetic Bacterium Rhodovulum sulfidophilum Predominantly Exists as a Functional Dimer in the Absence of a Substrate

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ABSTRACT: Polyhydroxyalkanoates (PHAs) are a family of biopolymers that accumulate as carbon and energy storage compounds in a variety of micro-organisms. The marine purple photosynthetic bacterium Rhodovulum sulfidophilum is capable of synthesizing PHA. In this study, we cloned a gene encoding a class I PHA synthase from R. sulfidophilum (phaC_Rs) and synthesized PhaC_Rs using a cell-free protein expression system. The specific activity of PhaC_Rs increased linearly as the (R)-3-hydroxybutyryl-coenzyme A (3HB-CoA) concentration increased and never reached a plateau, even at 3.75 mM 3HB-CoA, suggesting that PhaC_Rs was not saturated because of low substrate affinity. Size exclusion chromatography and native polyacrylamide gel electrophoresis analyses revealed that PhaC_Rs exists predominantly as an active dimer even in the absence of 3HB-CoA, unlike previously characterized PhaCs. The linear relationship between the PhaCRs activity and 3HB-CoA concentrations could result from a low substrate affinity as well as the absence of a rate-limiting step during PHA polymerization because of the existence of predominantly active dimers.

INTRODUCTION

Polyhydroxyalkanoates (PHAs) are biopolymers that accumulate in many organisms in the presence of excess carbon and limited nutrient sources. PHAs have attracted attention as an alternative source to petroleum-derived plastics, and their properties, such as biodegradability and biocompatibility, have been extensively studied. PHAs require a cost-consuming carbon source, such as sugars or plant oils. Therefore, many studies have focused on photosynthetic organisms as hosts for PHA production because carbon dioxide can be directly used as a carbon source. Most studies regarding PHA production via photosynthesis have been carried out using oxygenic phototrophs, such as higher plants and cyanobacteria. However, high levels of photosynthesis-mediated PHA production have not yet been achieved. Although anoxygenic photosynthetic bacteria produce higher amounts of PHA compared with plants and cyanobacteria, these bacteria, particularly marine photosynthetic bacteria, have not been extensively studied.

PHA synthase (PhaC) is a key enzyme involved in PHA biosynthesis, and it catalyzes the polymerization of (R)-3-hydroxyacyl-coenzyme A (CoA) to PHA. PhaC has been categorized into four classes based on its subunit composition, sequence similarity, and substrate specificity. Class I and II synthases consist of a single subunit, and class II PhaCs are composed of two different phaC genes (phaC1 and phaC2) separated by phaZ, which encodes PHA depolymerase. Class III synthases are composed of two subunits, PhaC and PhaE, whereas class IV synthases are composed of the subunits PhaC and PhaR. Classes I, III, and IV preferentially use short-chain-length (three to five carbon atoms) CoA thioesters, whereas class II synthases catalyze the polymerization of medium-chain-length (more than six carbon atoms) CoA thioesters. Ralstonia eutropha (R. eutropha) H16 is one of the best-known PHA-producing bacteria and has a class I PhaC. PhaC from the purple sulfur photosynthetic bacterium Allochromatium vinosum (A. vinosum) is categorized as a class III synthase whose biochemical properties have been extensively studied. In our previous studies, we reported the in vitro specific activity of class I PhaC from Aeromonas caviae (A. caviae) and class III PhaC and PhaE from Synechocystis sp. PCC6803 (Synechocystis) using a cell-free protein expression system. These studies demonstrated that the dimeric form of PhaC containing (R)-3-hydroxybutyryl (3HB)-CoA is an active form that catalyzes the polymerization. Recently, crystal structures of the C-terminal domain (residues 201–589) of PhaC from R. eutropha at 1.8 Å
resolution were reported.\textsuperscript{15,16} These analyses also revealed that the PhaC dimer is active during PHA polymerization.

Our previous study demonstrated that some species of marine purple photosynthetic bacteria could accumulate PHA.\textsuperscript{8} All purple nonsulfur bacteria accumulated PHA in the absence of nutrient deficiency, and some of them produced high-molecular-weight PHA, unlike the well-known PHA-producing bacteria. Although these PHA properties might be derived from the enzymatic characteristics of PhaC, those from marine purple nonsulfur bacteria have not yet been studied. \textit{Rhodovulum sulfidophilum}, a marine purple nonsulfur photosynthetic bacterium, was characterized in our study. \textit{R. sulfidophilum} can synthesize PHA,\textsuperscript{17} and its genome has been completely sequenced.\textsuperscript{18} In this work, we discovered a \textit{phaC} gene in the \textit{R. sulfidophilum} genome, and it was categorized as a class I synthase, unlike those of \textit{A. vinosum} and \textit{Synechocystis}. We previously reported that PHA production conditions and PHA compositions of purple nonsulfur bacteria differed from those of purple sulfur bacteria and cyanobacteria.\textsuperscript{8} Here, we characterized PhaC from \textit{R. sulfidophilum} (PhaCRs) in vitro using a cell-free protein expression system and demonstrated

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**Figure 1. PhaC from \textit{R. sulfidophilum}.** (A) Genetic organization of PHA biosynthesis genes in the \textit{R. sulfidophilum} genome. (B) Phylogenetic tree of PhaC from purple nonsulfur bacteria. The accession numbers are as follows: WP\_043812714 (\textit{Rubrivivax gelatinosus}), WP\_009856865 (\textit{Rubrivivax benzoaliticas}), WP\_01165085 (\textit{Ralstonia eutropha}), WP\_013502713 (\textit{Rhodopseudomonas palustris}), KAI9643 (\textit{Rhodocarcinum udaipurense}), ADP70651 (\textit{Rhodocarcinum vannielii}), EPY03196 (\textit{Phaeospirillum fulvum}), CCG41701 (\textit{Phaeospirillum molischianum}), WP\_02728919 (\textit{Rhodovibrio salinarum}), and CAB65395 (\textit{R. rubrum}). \textit{Synechocystis sp. PCC6803} was used as an outgroup. (C) Amino acid sequence alignment of PhaC from \textit{R. sulfidophilum}, \textit{R. capsulatus}, \textit{R. sphaeroides}, and \textit{R. eutropha}. The amino acid sequences were aligned using the MUSCLE algorithm. The amino acids of the catalytic triad are shown in red boxes. The conserved Cys residues are shown in blue boxes.

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that PhaC<sub>Rs</sub> exists preferentially in a constitutively active dimeric form even in the absence of a substrate, unlike the well-known PHA-producing bacteria. Elucidating these intriguing properties of PhaC from <i>R. sulfidophilum</i> will contribute to industrial PHA production and to a better understanding of the PHA polymerization mechanism.

## RESULTS

**PhaC from <i>R. sulfidophilum</i> and Cell-Free Synthesis.**

We found two homologous genes encoding PhaC proteins (WP_042463846 and WP_042457276), which are composed of 599 and 585 amino acids, respectively, in the <i>R. sulfidophilum</i> genome. The amino acid residues of the two proteins share 35 and 33% identity to that of PhaC from <i>R. eutropha</i>. PHA biosynthesis-related genes are clustered in bacterial genomes. In the <i>R. sulfidophilum</i> genome, the phaP gene encoding Phasin and the phaR gene encoding the PHA repressor protein are located downstream of the <i>pha</i>C gene encoding WP_042463846 (Figure 1A). The phaZ gene encoding the PHA depolymerase is located next to the <i>pha</i>C gene, and these genes are transcribed in opposite directions. The other <i>pha</i>C gene encoding WP_042457276 did not colocalize with any PHA biosynthesis-related genes. We analyzed the promoter regions using BPROM, a bacterial promoter prediction program. Recognition sites were found in the upstream region of the <i>pha</i>C gene encoding WP_042463846 but were not found in the gene encoding WP_042457276. Quantitative reverse transcription-polymerase chain reaction (RT-PCR) analysis was carried out to determine the expression levels of two <i>pha</i>C genes (WP_042463846 and WP_042457276). The expression level of the <i>pha</i>C gene encoding WP_042463846 was 1844-fold higher than that of the other <i>pha</i>C gene encoding WP_042457276. This observation strongly supports that the <i>pha</i>C gene encoding WP_042463846 is an actively expressed gene. Therefore, the <i>pha</i>C gene encoding WP_042463846 was used for further analysis and is hereafter referred to as PhaC<sub>Rs</sub>.

The phylogenetic tree was generated using PhaC amino acid sequences from 14 purple nonsulfur photosynthetic bacteria as well as from <i>A. caviae</i>, <i>R. eutropha</i>, and <i>Synechocystis</i> (Figure 1B). As with <i>R. sulfidophilum</i>, almost all purple nonsulfur bacteria have multiple homologous <i>pha</i>C genes in their genome. PhaCs that exhibit a higher amino acid sequence identity compared with that of <i>R. eutropha</i> were selected for alignment analysis. PhaC<sub>Rs</sub> is close to those of <i>Rhodobacter sphaeroides</i> (<i>R. sphaeroides</i>) and <i>Rhodobacter capsulatus</i> (<i>R. capsulatus</i>). The amino acid alignment of PhaC from <i>R. sulfidophilum</i>, <i>R. sphaeroides</i>, <i>R. capsulatus</i>, and <i>R. eutropha</i> is shown in Figure 1C. For <i>R. eutropha</i>, C319, D480, and H508 form a Cys-His-Asp catalytic dyad for the covalent catalysis. C319 is proposed to serve as a catalytic nucleophile, D480 acts as the general base catalyst, and H508 deprotonates C319 for nucleophilic attack from the HB-CoA thioester. C331, D486, and H515 in <i>R. sulfidophilum</i> were conserved in all PhaCs, suggesting that these three amino acids correspond to C319, D480, and H508 in <i>R. eutropha</i>; these amino acids are catalytic residues for PHA polymerization.

The genomic region of PhaC<sub>Rs</sub> was cloned from the genomic DNA extracted from <i>R. sulfidophilum</i> and was used for the cell-free synthesis. The PhaC<sub>Rs</sub> expression was evaluated by sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) (Figure S2), and the major band was approximately 66 kDa, which corresponds to PhaC<sub>Rs</sub>. To purify PhaC, an N11 tag with a tobacco etch virus (TEV) protease recognition sequence was inserted. After TEV treatment, PhaC<sub>Rs</sub> was purified with a nickel-nitrilotriacetic acid (Ni-NTA) column. A 36 mL reaction mixture yielded 2.8 mg of purified PhaC<sub>Rs</sub>.

**PhaC<sub>Rs</sub> Polymerization Activity.** The purified PhaC<sub>Rs</sub> was mixed with 100 mM sodium phosphate (pH 7.5) containing 0.12 mM Triton X-100 and characterized using a polymerization activity assay with 3HB-CoA. The polymerization activity of various concentrations of PhaC<sub>Rs</sub> (0.75, 7.5, 10, and 15 μM) was evaluated using 750 μM 3HB-CoA. The polymerization activity was increased at 0.75 μM PhaC<sub>Rs</sub> and showed similar levels at concentrations greater than 7.5 μM (Figure 2A). Consequently, the specific activity decreased as the PhaC<sub>Rs</sub> concentration increased (Figure 2B), unlike <i>Synechocystis</i> and <i>A. caviae</i>. In <i>Synechocystis</i> and <i>A. caviae</i>, the specific activity is saturated at approximately 1000 μM 3HB-CoA. The effects of various 3HB-CoA concentrations on the polymerase activity were evaluated using different concentrations of PhaC<sub>Rs</sub> (Figure 3). The specific activity increased sequentially as the 3HB-CoA concentration increased and was never saturated, even at 3750 μM 3HB-CoA. These results indicate that this substrate is not sufficient to fully activate PhaC<sub>Rs</sub>. PhaCs from <i>A. caviae</i> and <i>Rhodospirillum rubrum</i> have been shown to use the C6-monomer (3HHx-CoA) as a substrate; thus, 3HHx-CoA was used as a PhaC<sub>Rs</sub> substrate. PhaC<sub>Rs</sub> showed negligible activity toward 3HHx-CoA (Figure 4), indicating that 3HB-CoA is required for the polymerase reaction of PhaC<sub>Rs</sub>.

![Figure 2](image-url)
The dimeric formation of PhaC is important for its polymerization activity according to previous studies. The multimerization of PhaCRs was evaluated at various 3HB-CoA concentrations by size exclusion chromatography (Figure 5A). Four peaks were detected at 6.2, 6.7, 9.2, and 12.3 min. The peak at approximately 12.3 min corresponded to 3HB-CoA. According to the calibration curve generated from the elution times of the molecular standards, the molecular weights corresponding to peaks with elution times of 6.2, 6.7, and 9.2 min were estimated to be 185, 166, and 71 kDa, respectively. Interestingly, a peak shift was not observed when 3HB-CoA was added, although a slight change in the peak height was observed. Native PAGE analysis of each fraction resulting from size exclusion chromatography was performed to verify the multimer formation of PhaCRs (Figure 5B). Peak 1 (elution time of approximately 6 min) and peak 2 (elution time of approximately 9 min) fractions contained dimeric forms of PhaC based on their corresponding molecular weights. The peak 1 fraction also contained high-molecular-weight aggregates owing to multimeric PhaC. The molecular weight of peak 2 at 9.2 min was estimated to be 71 kDa, which likely corresponds to the PhaCRs monomer (67 kDa). Native PAGE analysis revealed that the peak 1 fraction contained the dimeric form of PhaCRs. This apparent difference may be explained by the compact structure of the PhaCRs dimer. As shown in Figure 5A, the elution times were almost identical in the presence and absence of 3HB-CoA. These results indicate that 3HB-CoA is not required for PhaCRs dimerization.

The cysteine (Cys) residue plays an important role in PHA polymerization. Two thiol groups from the Cys residues on each PhaC monomer are proposed to be required for the catalytic site formation. In addition to C331, PhaCRs contains four Cys residues (C452, C465, C479, and C483). Phylogenetic analysis revealed that PhaCRs was close to those of R. capsulatus and R. sphaeroides (Figure 1B). Amino acid sequences of R. sulfolobus, R. capsulatus, and R. sphaeroides were compared with that of R. eutropha (Figure 1C). C452, C479, and C483 were conserved in three purple nonsulfur bacteria, and these three Cys residues might affect PhaCRs dimerization. To verify this possibility, tris(2-carboxyethyl)-phosphine (TCEP) was used to reduce disulfide bonds, and the multimerization of PhaCRs was analyzed in the presence of 10 mM TCEP by size exclusion chromatography (Figure S3). The high-molecular-weight aggregation fraction (peak 1) was increased by TCEP treatment. However, no significant peak shift was observed by treatment with TCEP, suggesting that disulfide bonds are not required for dimerization because PhaCRs formed stable dimers even in the reduced state.

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Figure 3. Effect of 3HB-CoA concentrations on the specific activity of PhaCRs. The concentrations of PhaCRs ranged from 0.15 to 7.5 μM. The error bars represent the standard deviation of three independent experiments.

Figure 4. Time course of the release of CoA from 3HHx-CoA. The concentrations of 3HB-CoA and 3HHx-CoA were both 1.5 mM. The concentration of PhaCRs was 7.5 μM.

Figure 5. Analysis of PhaCRs, monomer-dimer formation. (A) Size exclusion chromatography profiles of PhaCRs. Concentrations of 3HB-CoA ranging from 0 to 750 μM were used for the analysis. The two peaks are labeled peak 1 and peak 2. The PhaCRs concentration was 7.5 μM. (B) Native PAGE analysis of samples collected from peaks 1 and 2. The PhaCRs and 3HB-CoA concentrations were 75 and 225 μM, respectively. Peak 1 and peak 2: dimeric PhaCRs; peak 3: 3HB-CoA.
DISCUSSION

In this study, we cloned a class I PhaC from R. sulfidophilum and synthesized PhaC<sub>Rs</sub> using a cell-free protein expression system. We demonstrated that the properties of PhaC<sub>Rs</sub> differ from those of other well-known PHA-producing bacteria. Monomeric and dimeric forms of PhaC exist as an equilibrium in vitro, and dimerization is induced in the presence of a substrate. Surprisingly, size exclusion chromatography and native PAGE analyses revealed that the dimeric form of PhaC<sub>Rs</sub> was dominant even in the absence of 3HB-CoA (Figure 5). Although a minor fraction of the PhaC dimer was previously observed in the absence of a substrate in other bacteria, to the best of our knowledge, the predominance of the PhaC dimer has not been described until now. We discovered four conserved Cys residues among three purple nonsulfur bacteria (C331, C452, C479, and C483) and demonstrated that PhaC<sub>Rs</sub> exists as a dimer in the reduced state (Figure S3). The lack of PhaC<sub>Rs</sub> monomers could be due to the fast association of the monomers with dimers and the stable formation of the dimer. Disulfide bonds are known to increase the conformational stability of many proteins. Conserved Cys residues in PhaC<sub>Rs</sub> might affect the stability of the dimer, which would lead to the predominance of the dimeric form. Cys residues also have important roles in sensing the changes in the cellular redox status. Many thioredoxin target proteins whose activities are controlled by the reduction of disulfide bonds have been identified in cyanobacteria and chloroplasts. In purple nonsulfur bacteria, the RegB/RegA two-component system, the AppA/PpsR transcription factor system, and the transcription factors CrtJ and Fnr are known to be associated with the redox-responding regulators via the thioldisulfide exchange reaction. The activity of PhaC<sub>Rs</sub> might be controlled by the redox state through the thioldisulfide exchange between conserved Cys residues. R. sulfidophilum has two homologousphaC genes in the genome, as previously described. Although the expression level of the secondphaC gene was quite low, it is still possible that PHA polymerization is regulated through the PhaC heterodimers.

PhaC<sub>Rs</sub> activity never reached a plateau, even at very high concentrations of 3HB-CoA, as shown in Figure 3, suggesting that this substrate was not sufficient to fully activate PhaC<sub>Rs</sub>. As a result, the specific activity decreased as the PhaC<sub>Rs</sub>-concentration increased (Figure 2B). The predominant dimeric form could affect these PhaC<sub>Rs</sub>-properties. The PHA polymerization process occurs in three steps: initiation, elongation, and termination. Regarding PhaC from R. eutropha, the chain initiation rate is slower than the chain elongation rate. The dimerization and binding of 3HB-CoA are assumed to be the rate-limiting steps in the initiation of PHA polymerization. Alternatively, PhaC<sub>Rs</sub> exists predominantly as a dimer; thus, dimerization does not appear to be the rate-limiting step. The specific activity against 3HB-CoA never reached a plateau, even at high concentrations of 3HB-CoA (Figure 3), suggesting that PhaC<sub>Rs</sub> has a low affinity for 3HB-CoA. The polymerization activity of PhaC<sub>Rs</sub> might follow a first-order reaction, which is proportional to the substrate concentration because of the predominance of its constitutively active dimeric form and its low substrate affinity.

PhaC<sub>Rs</sub> and PhaC from purple nonsulfur bacteria were classified as class I PhaCs (Figure 1). By contrast, PhaCs from A. vinosum and Synechocystis belong to class III. We verified that the whole genome sequences of PhaC from six purple sulfur bacteria strains (Thioflavicoccus mobilis, Thiocystis violascens, Marichromatium purpuratum, Thiorhodococcus drosesi, Thioacapsa marina, and Halorhodospira halophila) were available, and we determined that, similar to A. vinosum, five of the six strains have class III PhaCs, with H. halophila being the exception. In our previous study, certain differences in PHA production were found between purple nonsulfur bacteria and purple sulfur bacteria. Purple nonsulfur bacteria synthesized PHAs even when grown in the absence of nutrient deficiency, whereas purple sulfur bacteria synthesized PHAs only under nitrogen-limited conditions. We also demonstrated that purple nonsulfur bacteria synthesized 3HB and 3-hydroxyvalerate (3HV) copolymers, whereas purple sulfur bacteria produced 3HB homopolymers. Different PhaC properties between the purple nonsulfur bacteria and purple sulfur bacteria could result in differences in PHA production. Further analysis will be required to clarify these issues. The results of our study will contribute to a better understanding of PHA production.

MATERIALS AND METHODS

Cloning PhaC. R. sulfidophilum genomic DNA was isolated using a standard procedure. PhaC was cloned using a two-step PCR method according to previous reports. The forward (FW) and reverse (RV) primer sequences for the first PCR reaction are listed in Table S1 of the Supporting Information. The first PCR program was identical to that of our previous report, and the second PCR reaction was conducted according to previous methods established for the N11 tag. The N11 tag is a modified version of the natural polyhistidine tag. A TEV protease recognition sequence was inserted to cleave the target PhaC from its partner protein in N11-tagged constructs. Table S2 of the Supporting Information lists the tags used in this study. After the 10th PCR cycle, the annealing temperature was changed to 64 °C, and the duration of the extension was prolonged for 5 s per cycle. The last step was incubating the reaction mixture at 72 °C for 7 min. The resultant product was immediately cooled to 10 °C, and its concentration was determined using a PicoGreen dsDNA quantification kit (Invitrogen, Carlsbad, CA).

Cell-Free Synthesis of PhaC. The dialysis-mode cell-free protein synthesis method was used in this study, according to the methods presented in the literature. The internal solution (36 mL) was composed of the buffers, substrates, template DNA, and enzymes required for transcription and translation. The solution contained 55 mM HEPES-KOH buffer (pH 7.5) containing 1.7 mM dithiothreitol, 1.2 mM adenosine-5′-triphosphate (pH 7.0), 0.8 mM cytidine triphosphate (pH 7.0), 0.8 mM guanosine-5′-triphosphate (pH 7.0), 0.8 mM uridine-5′-triphosphate (pH 7.0), 80 mM creatine phosphate, 250 μg/mL creatine kinase, 4.0% polyethyleneglycol (average molecular weight 8000 g/mL), 0.64 mM 3′,5′-cyclic adenosine monophosphate, 68 μM 1-(-)-formyl-5,6,7,8-tetrahydrofolic acid, 0.05% sodium azide, 175 μg/mL Escherichia coli total transfer RNA (tRNA), 210 mM potassium glutamate, 27.5 mM ammonium acetate, 10.7 mM magnesium acetate, 1.0 mM each of the 20 amino acids, 6.7 μg/mL pK7-CAT plasmid, 93 μg/mL T7 RNA polymerase, and 9.0 μL of S30 extract. The S30 extract was prepared from the E. coli BL21 CodonPlus RIL strain (Stratagene, La Jolla, CA), as previously described. Cell-free giant-scale dialysis using a dialysis membrane with a molecular-weight cutoff (MWCO) of 15 kDa (Spectra/Por 7, Pierce, Rockford, IL) was performed using previously reported reaction conditions. The internal solution...
was dialyzed in a dialysis tube (Spectra/Por 7, MWCO of 15 kDa) against the external solution at 23 °C for 16 h while being shaken.38

**Purification of PhaC.** To purify PhaC, 36 mL of the internal solution containing PHA<sub>Rs</sub> with the N11 tag with a TEV protease recognition sequence was purified using an ÄKTA Xpress FPLC (GE Healthcare, Little Chalfont, U.K.), according to protocols presented in the literature.13 The buffers used for the purification were described in our previous study.13 Briefly, the protein solution was purified using a HisTrap (5 mL, Ni-NTA column, GE Healthcare). To remove the tag, TEV was added to the eluted protein fraction at a final concentration of 10 μg/mL.35 The protein in the flow-through fraction was loaded onto a HiLoad 16/60 Superdex 75 column (GE Healthcare) to concentrate the purified protein. The purified protein yield was determined using a Bio-Rad protein assay kit (Bio-Rad, Hercules, CA). Bovine serum albumin (BSA) was used as the protein standard. SDS-PAGE was performed using 15 to 20% precast Tris-HCl gels (DRC Co Ltd, Kyoto, Japan). The gels were stained with Coomassie brilliant blue.

**Specific Activity Assay for PhaC.** The specific activity of PhaC was determined by using the method of Gerngross et al.39 with modifications.13 Briefly, CoA released during the PhaC-catalyzed reactions can be measured using Ellman's reagent, S,S'-dithiobis(2-nitrobenzoic acid) (DTNB).40 The assay mixture (360 μL) contained 100 mM sodium phosphate (pH 7.5), 0.12 mM Triton X-100, an appropriate amount of 3HB-CoA (35, 70, 350, 700, 1000, 1400, or 2100 μM), and variable amounts of purified PhaC (0.75, 7.5, 10, or 15 μM). The reactions were initiated by the addition of PhaC. DTNB was added to the mixture, and then, the absorbance was measured at 405 nm. One unit was defined as the amount of enzyme required to catalyze the conversion of 1 μmol of substrate/min. The specific activity was determined from the CoA release ranging from 0 to 1 min of the reaction because the reaction obviously lacked a lag phase.

**Size Exclusion Chromatography and Native PAGE.** The reaction mixture consisting of PhaC, 3HB-CoA, and 100 mM sodium phosphate (pH 7.5) in a total volume of 10 μL was incubated for 15 min at 25 °C. For TCEP treatment, the reaction mixtures were incubated for 60 min at 37 °C in the presence of 10 mM TCEP. The reaction mixtures were then loaded onto a TSKgel G2000 SWXL column (TOSOH, Tokyo, Japan) equilibrated with 20 mM NaPi (pH 7.0) containing 200 mM Na<sub>2</sub>SO<sub>4</sub> at 25 °C. The molecular weight was determined from the calibration curve prepared using the following molecular weight standards: alcohol dehydrogenase (150 kDa, 7.7 min), BSA (66 kDa, 8.4 min), ovalbumin (44 kDa, 9.1 min), C2 (16 kDa, 10.9 min), and aprotinin (6.5 kDa, 11.8 min).

Native PAGE was carried out using precast gradient (4–12%) Bis-Tris gels (Invitrogen, Carlsbad, CA), according to standard protocols. The gels were visualized by silver staining using a SilverXpress Silver Staining Kit (Invitrogen, Carlsbad, CA).

**Phylogenetic Analysis.** The phylogenetic tree was prepared based on the PhaC amino acid sequences of the 13 selected purple photosynthetic bacteria and constructed using Phylogey [http://phylogey.lirmm.fr/phylo_cgi/index.cgi].<sup>31–45</sup> NJplot was used to display the phylogenetic tree.<sup>46</sup>

**Quantitative RT-PCR Analysis.** RNA of <i>R. sulfidophilum</i> was extracted under growth conditions. Total RNA was extracted from <i>R. sulfidophilum</i> cells using the RNAeasy Mini kit (QIAGEN, Tokyo, Japan). Using 1 μg RNA as the template, cDNA was synthesized by the QuantiTect Reverse Transcription Kit (QIAGEN, Tokyo, Japan) following the manufacturer's protocol. The PCR was performed using a SsoAdvanced Universal SYBR Green Supermix (Bio-Rad, Hercules, USA), and the product was analyzed by StepOne (Thermo Fisher Scientific, Yokohama, Japan), according to the protocol. To determine the expression levels of the two phac genes (WP_042463846 and WP_042457276), the following sets of primers were used: phaC (WP_042463846), phaC-F (5'-ATTGAGCCGCGTATCCTC-3') and phaC-R (5'-GCA GCC CCA TCA TTT CA-3'); phaC (WP_042457276), phaC2-F (5'-CGG CCT GGA TCA TGA AAC AC-3'), and phaC2-R (5'-ACA TGG CGA AGA CGG TAA AG-3'); rpoD, rpoD-F (5'-CTT GTC CTA GAT GAA ATC GC-3') and rpoD-R (5'-GTC CGC AAG GTG ATG AAG AT-3').

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