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Phasin Proteins Activate *Aeromonas caviae* Polyhydroxyalkanoate (PHA) Synthase but Not *Ralstonia eutropha* PHA Synthase

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In this study, we performed *in vitro* and *in vivo* activity assays of polyhydroxyalkanoate (PHA) synthases (PhaCs) in the presence of phasin proteins (PhaPs), which revealed that PhaPs are activators of PhaC derived from *Aeromonas caviae* ($PhaC_{Ac}$). In *in vitro* assays, among the three PhaCs tested, $PhaC_{Ac}$ was significantly activated when PhaPs were added at the beginning of polymerization (prepolymerization $PhaC_{Ac}$), whereas the prepolymerization $PhaC_{Re}$ (derived from *Ralstonia eutropha*) and $PhaC_{Da}$ (*Delftia acidovorans*) showed reduced activity with PhaPs. The PhaP-activated $PhaC_{Ac}$ showed a slight shift of substrate preference toward 3-hydroxyhexanoyl-CoA (C_6). $PhaP_{Ac}$ also activated $PhaC_{Ac}$ when it was added during polymerization (polymer-elongating $PhaC_{Ac}$), while this effect was not observed for $PhaC_{Re}$. In an *in vivo* assay using *Escherichia coli* TOP10 as the host strain, the effect of $PhaP_{Ac}$ expression on PHA synthesis by $PhaC_{Ac}$ or $PhaC_{Re}$ was examined. As $PhaP_{Ac}$ expression increased, PHA production was increased by up to 2.3-fold in the $PhaC_{Ac}$-expressing strain, whereas it was slightly increased in the $PhaC_{Re}$-expressing strain. Taken together, this study provides evidence that PhaPs function as activators for $PhaC_{Ac}$ both *in vitro* and *in vivo* but do not activate $PhaC_{Re}$. This activating effect may be attributed to the new role of PhaPs in the polymerization reaction by $PhaC_{Ac}$.

Polyhydroxyalkanoates (PHAs) are aliphatic polyesters that are synthesized and accumulated by a wide range of microorganisms as their carbon- and energy-storage materials (1, 2). PHAs have attracted attention as biodegradable thermoplastics for industrial applications. A key enzyme in PHA biosynthesis is PHA synthase (PhaC), which is classified into four groups (classes I to IV) based on substrate specificity and subunit composition (see the review by Rehm [1] for a detailed classification of PHA synthases).

Class I PhaCs are homomeric enzymes comprised of PhaC subunits with molecular mass of 60 to 70 kDa; these enzymes prefer to polymerize monomers with a short acyl chain length (C_3 to C_5). As a typical class I enzyme, *Ralstonia eutropha* PHA synthase ($PhaC_{Re}$) has been well studied and characterized. *Delftia acidovorans* synthase ($PhaC_{Da}$) is also a class I synthase but has a unique insertion sequence of 40 amino acids located at the C terminus of the active center cysteine at position 322 (3). PhaC derived from *Aeromonas caviae* ($PhaC_{Ac}$) is not a typical class I enzyme because it is able to polymerize not only the C_4 and C_5 substrates but also C_6 substrates. $PhaC_{Ac}$ shows a relatively low amino acid sequence identity to $PhaC_{Re}$ (37.4%) and $PhaC_{Da}$ (31.1%), whereas $PhaC_{Re}$ and $PhaC_{Da}$ are relatively similar (50.7%).

A number of enzymes and nonenzymatic proteins are involved in PHA biosynthesis. Phasin proteins (PhaPs), which are PHA granule-associated proteins localized on the surfaces of the PHA granules in bacterial cells (4, 5), are well known PHA synthesis-related proteins. The primary function of PhaPs is control of the surface properties of PHA granules. PhaPs bind strongly to the hydrophobic surfaces of growing PHA granules to block the binding of other proteins. Accordingly, PhaP expression levels in bacterial cells affect the hydrophobicity of the surface of PHA granules, thereby influencing granule development via hydrophobic aggregation of small granules (4, 6).

Most phasins, including *R. eutropha* phasin ($PhaP1_{Re}$), have a molecular mass of approximately 20 kDa (5). The *A. caviae* phasin ($PhaP_{Ac}$) is a small protein having a molecular mass of 13 kDa and low identity to $PhaP1_{Re}$ (13.1%) (7). Several interesting observations suggested a second function of $PhaP_{Ac}$. These observations include $PhaP_{Ac}$ expression leading to enhanced accumulation of PHA and/or altering the copolymer composition, likely by influencing the activity and substrate specificity of $PhaC_{Ac}$ (8, 9). The same phenomenon was observed in recombinant bacteria between the PhaC and PhaP derived from *Aeromonas hydrophila* (10). However, these *in vivo* studies have not focused on the role of PhaP at the molecular level.

The aim of our study was to investigate the role of PhaP in the modulation of PhaC activity. For this purpose, we used purified proteins to assess the *in vitro* activity of PhaCs derived from *A. caviae*, *R. eutropha*, and *D. acidovorans* in the presence of PhaPs derived from *A. caviae* and *R. eutropha*. *In vivo* PHA production was also conducted using PhaCs from *A. caviae* and *R. eutropha* with or without the expression of $PhaP_{Ac}$. Based on the *in vivo* and *in vitro* results, we propose a new physiological role of PhaPs in the polymerization reaction catalyzed by $PhaC_{Ac}$.

MATERIALS AND METHODS

Plasmid construction. Plasmids to express His-tagged $PhaC_{Ac}$, $PhaP_{Ac}$, and $PhaP1_{Re}$ were constructed based on the pColdI vector (TaKaRa Bio, Inc., Otsu, Japan) to obtain purified proteins. For construction of pColdI::

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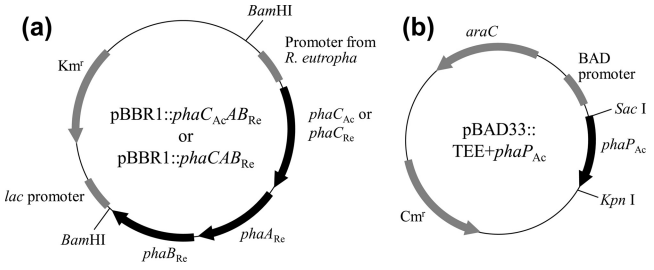

FIG 1 Map of plasmids used for *in vivo* experiments. (a) pBBR1-MCS2-derived plasmid for PHA biosynthesis. (b) pBAD33-derived plasmid for $PhaP_{Ac}$ expression.

$phaC_{Ac}$ and pColdI::$phaP_{Ac}$, $phaC_{Ac}$ and $phaP_{Ac}$ genes were obtained by PCR with pBBR1$phaPCJ_{Ac}AB_{Re}$ (11) as the template. The following PCR primers were used: forward for $phaC_{Ac}$, 5′-GGGTGAAGGAGAGCATATGAGCCAACCATCTTATGG-3′, reverse for $phaC_{Ac}$, 5′-GACCACGGATCCTGCGCTCA-3′, forward for $phaP_{Ac}$ 5′-TGGAGACCGCATATGAATATGGACGTGATC-3′, and reverse for $phaP_{Ac}$, 5′-CAGGGATCCTCAGGCCTTGCCCGTCGTTTT-3′ (underlined sequences indicate the NdeI and BamHI sites, respectively). These gene fragments were digested by NdeI and BamHI and were introduced into the same sites of the pColdI vector. For pColdI::$phaP1_{Re}$, genomic DNA of *R. eutropha* H16 was used as the template. The following PCR primers were used: forward, 5′-ACTGGAGACCACATATGATCCTCACCCCGG-3′, and reverse, 5′-GGATCCTCAGGCAGCCGTCGTCTTCTTTGC-3′ (underlined sequences indicate the NdeI and BamHI sites, respectively). The amplified DNA fragment carrying the $phaP1_{Re}$ gene was digested by NdeI and BamHI and was introduced into the same sites of the pColdI vector. Three plasmids, named pBAD::TEE+$phaP_{Ac}$, pBBR1::$phaC_{Ac}AB_{Re}$, and pBBR1::$phaCAB_{Re}$, were newly constructed for *in vivo* PHA production experiments. Maps of the plasmids used for *in vivo* experiments are shown in Fig. 1. In the construction of pBAD::TEE+$phaP_{Ac}$, the $phaP_{Ac}$ gene fragment, which includes the Shine-Dalgarno sequence, translation-enhancing element (TEE) sequence, and His tag sequence of pColdI::$phaP_{Ac}$, was amplified by PCR using pColdI::$phaP_{Ac}$ as the template. The following PCR primers were used: forward, 5′-TTAGAGCTCAAGAGGTAATACACCATGAAT-3′, and reverse, 5′-TTCGGTACCTCAGGCCTTGCCCGTGCTTTT-3′ (underlined sequences indicate the SacI and KpnI sites, respectively). For pBBR1::$phaC_{Ac}AB_{Re}$ and pBBR1::$phaCAB_{Re}$, the BamHI-digested fragments from pGEM′-$phbCAB_{Re}$ (12) and pGEM-$phaC_{Ac}AB$ (13), respectively, were ligated with BamHI-digested pBBR1-MCS2 (14). These PHA biosynthesis genes were located downstream from the promoter of the PHA biosynthesis operon ($phaCAB_{Re}$) from *R. eutropha*, and they were directionally opposite from the *lac* promoter present in pBBR1-MCS2.

Preparation of PhaCs and PhaPs. $PhaC_{Ac}$ with the His tag removed was prepared by using cell-free protein enzymes as reported previously (15). $PhaC_{Ac}$ was eluted with 20 mM Tris-HCl buffer (pH 7.5) containing 150 mM NaCl. His-tagged $PhaC_{Re}$ and $PhaC_{Da}$ were expressed in the recombinant *Escherichia coli* BL21(DE3) strain (Novagen, Madison, WI) with the pET-15b::$phaC_{Re}$ and pET-15b::$phaC_{Da}$ vectors, respectively (16, 17). $PhaC_{Re}$ and $PhaC_{Da}$ were purified from the cells using a HisTrap HP column (GE Healthcare), followed by desalting using a HiTrap desalting column with desalting buffer [20 mM Tris-HCl, 150 mM NaCl, 5% (vol) glycerol, and 0.05% (wt) 6-*O*-(*N*-heptylcarbamoyl)methyl α-D-glucopyranoside (Hecameg), pH 7.5]. All processes were conducted at 4°C. Aliquots of purified PhaCs were frozen in liquid nitrogen and stored at −80°C.

His-tagged $PhaC_{Ac}$, $PhaP_{Ac}$, and $PhaP1_{Re}$ were expressed in the recombinant *E. coli* BL21(DE3) strain with pColdI::$phaC_{Ac}$, pColdI::$phaP_{Ac}$, and pColdI::$phaP1_{Re}$ vectors, respectively. All strains were grown in Luria-Bertani (LB) broth (1% Bacto-tryptone, 0.5% yeast extract, and 1% NaCl, pH 7.0) with 100 μg/ml ampicillin. These expression strains were grown at 37°C to an optical density at 600 nm (OD_{600}) of ≈0.6 with shaking. The cells were incubated at 16°C for 30 min without shaking, and 0.1 mM isopropyl-β-D-thiogalactopyranoside (IPTG) was added. After the addition of IPTG, the cells were cultivated at 16°C for 24 h with shaking. Proteins were purified using a HisTrap HP column (GE Healthcare). Finally, purified $PhaC_{Ac}$ was desalted using desalting buffer, as described for $PhaC_{Re}$ and $PhaC_{Da}$; PhaPs were desalted using ultrapure water. These purified and desalted proteins were frozen in liquid nitrogen and stored at −80°C. All proteins were purified to near homogeneity (see Fig. S1 in the supplemental material). Protein concentrations were determined using a Quant-iT protein assay kit (Invitrogen, Carlsbad, CA).

Monomer preparation. To assay PhaC activity, (*R*)-3-hydroxybutyryl-coenzyme A (*R*-3HB-CoA) and (*R*)-3-hydroxyhexanoyl-CoA (*R*-3HHx-CoA) were prepared as substrates. *R*-3HB-CoA was chemically synthesized and purified as previously described (18). *R*-3HHx-CoA was synthesized from hexenoyl-CoA using *A. caviae* (*R*)-specific enoyl-CoA hydratase as previously reported (19) and purified using high-performance liquid chromatography (HPLC) in the same manner as for *R*-3HB-CoA. Substrate concentrations were quantified relative to an HPLC standard curve of racemic 3HB-CoA (Sigma; St. Louis, MO).

Activity assay of PhaCs in the presence of PhaPs. For the PhaC activity assay, a decrease in the absorbance at 236 nm, which occurs due to the cleavage of the thioester bond in *R*-3HB-CoA (ε_{236} = 4,500 [$M^{-1} \cdot cm^{-1}$]), was measured (20). The assay was carried out for the three PhaCs in the presence or absence of the PhaPs at 30°C in 50 mM sodium phosphate buffer (pH 7.0) with *R*-3HB-CoA or *R*-3HHx-CoA. Detailed assay conditions are described below. Specific activities were determined using the maximum velocities of each reaction.

PHA biosynthesis *in vivo*. For poly[(*R*)-3-hydroxybutylate] [P(3HB)] synthesis, *E. coli* TOP10 cells (Invitrogen, Carlsbad, CA) harboring a pBBR1-MCS2-derived plasmid (pBBR1::$phaC_{Ac}AB_{Re}$ or pBBR1::$phaCAB_{Re}$) and pBAD::TEE+$phaP_{Ac}$ (or the pBAD33 empty vector as a control) were cultivated as described below. These strains were inoculated into LB medium (containing 50 μg/ml kanamycin and 30 μg/ml chloramphenicol) in test tubes and cultured at 37°C for 15 h. These cultures (1 ml) were inoculated into 500-ml shake flasks containing 100 ml LB medium, 50 μg/ml kanamycin, 30 μg/ml chloramphenicol, 20 g/liter D-glucose, and 0 to 1% (wt/vol) L-arabinose. These flasks were cultivated at 37°C for 72 h using a reciprocal shaker (130 strokes/min). After cultivation, cells were centrifuged, washed with pure water, and lyophilized.

TABLE 1 PhaC activity assay toward *R*-3HB-CoA in the presence of PhaP[a]

Assay conditions	Sp act (mean U/mg PhaC ± SE) (fold activation by PhaP) of:			
	$PhaC_{Ac}$			
	Without His tag	With His tag	$PhaC_{Re}$	$PhaC_{Da}$
PhaP free	0.76 ± 0.08 (1.0)	0.72 ± 0.03 (1.0)	3.51 ± 0.21 (1.0)	1.40 ± 0.14 (1.0)
+$PhaP_{Ac}$	2.31 ± 0.07 (3.0)	2.24 ± 0.11 (3.1)	0.36 ± 0.07 (0.1)	0.15 ± 0.03 (0.1)
+$PhaP1_{Re}$	1.82 ± 0.10 (2.4)	1.76 ± 0.03 (2.4)	0.39 ± 0.02 (0.1)	0.64 ± 0.06 (0.5)

[a] Assay conditions: PhaCs, 350 nM ($PhaC_{Ac}$ with/without His tag) or 200 nM ($PhaC_{Re}$ and $PhaC_{Da}$ with a His tag); *R*-3HB-CoA, 100 μM; PhaP, 0 or 1 μM. n = 3 replicates.

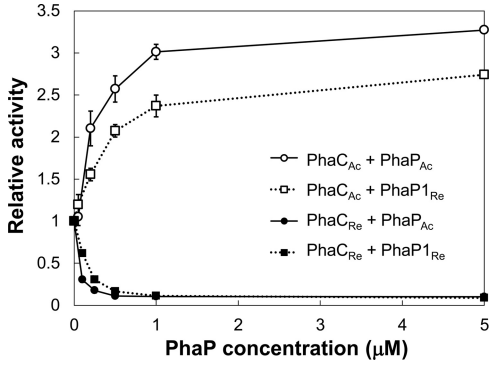

FIG 2 Relative activities of PhaC$_{Ac}$ and PhaC$_{Re}$ for the *R*-3HB-CoA substrate at different concentrations of PhaP$_{Ac}$ and PhaP1$_{Re}$. Relative activities were determined using the change in absorbance at 236 nm at maximum velocity. Reaction mixtures containing PhaC (350 nM PhaC$_{Ac}$ without a His tag or 200 nM PhaC$_{Re}$), 100 μM *R*-3HB-CoA, and 0 to 5 μM PhaP (PhaP$_{Ac}$ or PhaR1$_{Re}$) were used. Results are expressed as the means ± standard errors ($n = 3$), except for results with 5 μM PhaP ($n = 2$).

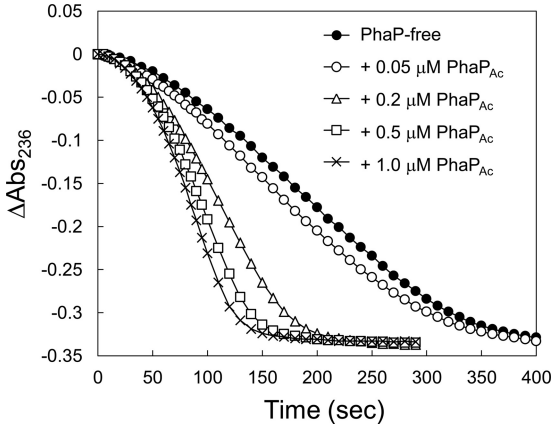

FIG 3 Effects of PhaP$_{Ac}$ on the PhaC$_{Ac}$ reaction rate for the *R*-3HB-CoA substrate. The assay was carried out by monitoring the changes in absorbance at 236 nm of reaction mixtures containing 100 μM *R*-3HB-CoA, 350 nM PhaC$_{Ac}$ without a His tag, and the indicated concentrations of PhaP$_{Ac}$.

Analyses of PHA produced *in vivo*. The PHA content in the cells was determined by gas chromatography (GC) after methanolysis in the presence of 15% (vol/vol) sulfuric acid, as described previously (21). Synthesized PHA was extracted from cells by stirring with chloroform for 72 h at room temperature, followed by filtration of cell debris. The extracted PHA solutions were precipitated into methanol.

The molecular weights and their distribution were determined by gel permeation chromatography (GPC) at 40°C using a Shimadzu 10A GPC system equipped with a 10A refractive index detector with two Shodex K-806 M joint columns. Chloroform was used as the eluent, with a flow rate of 0.8 ml/min; the sample concentrations were 1.0 mg/ml. Low polydispersity polystyrenes were used as the molecular-weight standard. All PHA samples for molecular-weight measurements were obtained from cultivations with 1.0% (wt/vol) L-arabinose.

Western blotting of PhaCs. The amounts of PhaC$_{Ac}$ and PhaC$_{Re}$ in cells were analyzed by Western blotting as described previously (17). Briefly, *E. coli* cells were cultivated in the LB-plus-glucose medium described above (containing 1.0% [wt/vol] L-arabinose), and the cells were harvested after 6 h of cultivation and disrupted by sonication. The disrupted cells were centrifuged at low speed (1,500 × *g* for 5 min at 4°C) to separate the soluble cell extract containing PHA granules and an insoluble precipitate. The protein concentrations in the soluble/insoluble fractions were determined using the Quant-iT protein assay kit (Invitrogen). Sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) was performed using 8 μg of protein in each soluble/insoluble sample. The detection of PhaC$_{Ac}$ and PhaC$_{Re}$ was carried out with specific rabbit antisera to PhaC$_{Ac}$ (22) and PhaC$_{Re}$ (23), respectively. Protein bands were visualized using goat anti-rabbit antibodies conjugated with horseradish peroxidase (HRP) (Santa Cruz Biotechnology, CA, USA) as a secondary antibody and an ECL (enhanced chemiluminescence) plus Western blotting detection reagent (GE Healthcare).

RESULTS

Activity assay of PhaCs in the presence of PhaPs *in vitro*. *In vitro* polymerization activity assays using purified PhaCs and PhaPs were conducted. The results of the activity assay are shown in Table 1. In the PhaP-free assay, PhaC$_{Ac}$ without a His tag showed the lowest activity (0.76 U/mg PhaC) toward *R*-3HB-CoA among the PhaCs examined, while PhaC$_{Re}$ showed the highest activity (3.51 U/mg PhaC). Interestingly, the addition of 1 μM PhaP$_{Ac}$ into the reaction mixture exhibited two different effects on PhaC activity; the activity of PhaC$_{Ac}$ increased to 2.31 U/mg PhaC in the presence of PhaP$_{Ac}$, which was 3.0-fold higher than that in the PhaP-free assay. In contrast, the addition of 1 μM PhaP$_{Ac}$ decreased the activities of PhaC$_{Re}$ and PhaC$_{Da}$ by approximately 10-fold; the calculated activities of PhaC$_{Re}$ and PhaC$_{Da}$ were 0.36 U/mg PhaC and 0.15 U/mg PhaC, respectively, in the presence of PhaP$_{Ac}$.

Furthermore, a similar effect was observed by adding another phasin, PhaP1$_{Re}$. In the presence of 1 μM PhaP1$_{Re}$, PhaC$_{Ac}$ activity increased to 1.82 U/mg PhaC, whereas PhaC$_{Re}$ and PhaC$_{Da}$ activities decreased to 0.39 U/mg PhaC and 0.64 U/mg PhaC, respectively. These results suggest that PhaPs function as modulators of PhaC activity but that the modulatory action (activating or inhibiting) is PhaC dependent. Namely, PhaPs enhance the activity of PhaC$_{Ac}$ but inhibit the activity of PhaC$_{Re}$ and PhaC$_{Da}$. This trend was also observed in the parallel assay using His-tagged PhaC$_{Ac}$ purified from *E. coli* BL21(DE3) cells harboring pColdI::*phaC*$_{Ac}$ (Table 1).

Effect of PhaP concentration on PhaC$_{Ac}$ activation *in vitro*. We assayed the activity of PhaC$_{Ac}$ relative to its activity under the PhaP-free condition by varying the concentrations of PhaPs added into the reaction mixture (Fig. 2; reaction progress curves are shown in Fig. 3). PhaC$_{Ac}$ activity increased 3.0-fold with increasing PhaP$_{Ac}$ concentration up to 1 μM; a further increase in the activity of PhaC$_{Ac}$ was not observed with the addition of 5 μM PhaP$_{Ac}$. These data suggest that the activating effect of PhaP$_{Ac}$ reached a plateau around a concentration of 1 μM, where the molar ratio of PhaP$_{Ac}$ to PhaC$_{Ac}$ was 2.9 (mol/mol).

The effect of another phasin, PhaP1$_{Re}$, on the activity of

TABLE 2 PhaC$_{Ac}$ activity toward C$_4$ and C$_6$ substrates *in vitro*[a]

Assay conditions	Sp act (mean U/mg PhaC$_{Ac}$ ± SE) (fold activation by PhaP) with:		C$_6$/C$_4$ activity ratio
	R-3HB-CoA (C$_4$)	*R*-3HHx-CoA (C$_6$)	
PhaC$_{Ac}$	1.27 ± 0.03 (1.0)	0.16 ± 0.01 (1.0)	0.13
PhaC$_{Ac}$ + PhaP$_{Ac}$	3.15 ± 0.13 (2.5)	0.69 ± 0.03 (4.3)	0.22

[a] Assay conditions: PhaC$_{Ac}$ without a His tag, 700 nM; substrate (*R*-3HB-CoA or *R*-3HHx-CoA), 150 μM; PhaP$_{Ac}$, 0 or 2.5 μM. $n = 3$ replicates.

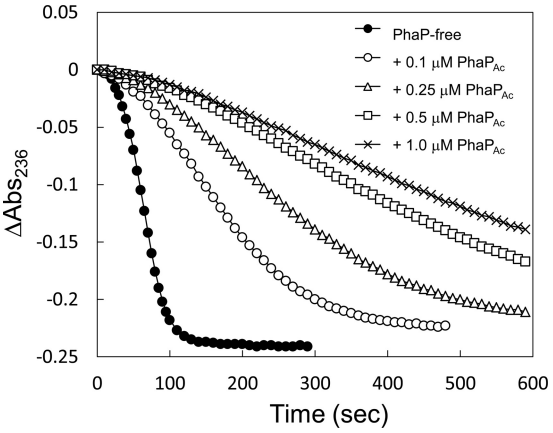

FIG 4 Effects of PhaP$_{Ac}$ on the PhaC$_{Re}$ reaction rate for the *R*-3HB-CoA substrate. The assay was carried out by monitoring the changes in absorbance at 236 nm of reaction mixtures containing 100 μM *R*-3HB-CoA, 200 nM PhaC$_{Re}$, and the indicated concentrations of PhaP$_{Ac}$.

PhaC$_{Ac}$ was also investigated (Fig. 2). PhaP1$_{Re}$ increased PhaC$_{Ac}$ activity up to 2.7-fold compared to its activity in the absence of PhaP; however, the activating effect was slightly lower than that of PhaP$_{Ac}$. The enhancing effect reached a plateau at around 1 μM PhaP1$_{Re}$ (the molar ratio of PhaP1$_{Re}$ to PhaC$_{Ac}$ was 2.9 [mol/mol]), which is identical to that of PhaP$_{Ac}$, regardless of the molecular mass of the PhaP. At the initial stage of polymerization, the maximum polymerization activity may be achieved at a PhaP/PhaC$_{Ac}$ molar ratio of 3:1.

Reactivity of activated PhaC$_{Ac}$ toward 3HHx *in vitro*. PhaC$_{Ac}$ that was activated by 2.5 μM PhaP$_{Ac}$ was spectrophotometrically assayed using *R*-3HHx-CoA (C_6 substrate) and *R*-3HB-CoA (C_4 substrate). Table 2 shows the specific activities of PhaC$_{Ac}$ with a high concentration (150 μM) of C_4 and C_6 substrates, which clearly show a weak activity toward the C_6 substrate. The activities for the C_4 and C_6 substrates were increased by the addition of 2.5 μM PhaP$_{Ac}$. The specific activities of the C_6 substrate in the presence and absence of PhaP$_{Ac}$ were 0.69 U/mg PhaC and 0.16 U/mg PhaC, respectively. This activation fold increase was calculated to be 4.3, which was higher than that of the C_4 substrate (2.5-fold), and it resulted in a higher C_6/C_4 activity ratio. These data indicate that PhaP$_{Ac}$ increased the specificity of PhaC$_{Ac}$ against the C_6 substrate compared to its activity against the C_4 substrate.

Effect of PhaP concentration on PhaC$_{Re}$ inhibition *in vitro*. The activities of PhaC$_{Re}$ and PhaC$_{Da}$ were inhibited by 1 μM PhaP (Table 1), which was in contrast to the activation observed for PhaC$_{Ac}$. To investigate the effect of PhaP concentration, activity assays of PhaC$_{Re}$ with various PhaP concentrations were conducted (Fig. 2; the reaction progress curves are shown in Fig. 4). The inhibitory effect on PhaC$_{Re}$ activity was dependent on the concentration of the PhaPs, and it reached a plateau at around 0.5 μM PhaP. PhaP$_{Ac}$ showed a slightly stronger inhibitory effect than PhaP1$_{Re}$ (Table 1 and Fig. 2).

Effect of PhaP on the activity of polymer-elongating PhaC *in vitro*. We conducted activity assays of polymer-elongating PhaCs in the presence or absence of PhaP$_{Ac}$. In this assay, the polymerization reaction by PhaC$_{Ac}$ (350 nM) or PhaC$_{Re}$ (200 nM) was started with 50 μM *R*-3HB-CoA. After 300 s of incubation with almost no change in absorbance at 236 nm, 1 μM PhaP$_{Ac}$ was added to the reaction mixture, followed by an additional 50 μM *R*-3HB-CoA. The progress curves of this assay using PhaC$_{Ac}$ and PhaC$_{Re}$ are shown in Fig. 5. An activation effect of PhaP$_{Ac}$ on the polymer-elongating PhaC$_{Ac}$ was observed, similar to the prepolymerization PhaC$_{Ac}$ (Table 1 and Fig. 2). On the other hand, polymer-elongating PhaC$_{Re}$ was not affected by the presence of PhaP$_{Ac}$ (Fig. 5b), unlike the prepolymerization PhaC$_{Re}$, which was inhibited by PhaP$_{Ac}$ (Fig. 2). The relative activities (U/mg PhaC) of polymer-elongating PhaCs were as follows: PhaC$_{Ac}$ alone, 0.66 ± 0.03 (mean ± standard deviation); PhaC$_{Ac}$ plus PhaP$_{Ac}$, 2.07 ± 0.11; PhaC$_{Re}$ alone, 1.74 ± 0.12; PhaC$_{Re}$ plus PhaP$_{Ac}$, 1.65 ± 0.12.

Effect of PhaP on PHA production *in vivo*. To evaluate the effect of PhaP on PHA production *in vivo*, *E. coli* TOP10 cells harboring P(3HB) biosynthesis genes (*phaC*$_{Ac}$/*phaC*$_{Re}$, *phaA*$_{Re}$, and *phaB*$_{Re}$) under *pha*$_{Re}$ promoter control and the *phaP*$_{Ac}$ gene under L-arabinose-inducible promoter control were cultured in LB medium containing 20 g/liter glucose (Fig. 6). The P(3HB) content was increased at high concentrations of L-arabinose (0.2% or 1.0% [wt/vol]) in both PhaC$_{Ac}$- and PhaC$_{Re}$-expressing strains. The maximum P(3HB) content was 2.3-fold (PhaC$_{Ac}$) or 1.2-fold (PhaC$_{Re}$) higher than the levels without PhaP$_{Ac}$ expression. The molecular weights of P(3HB) extracted from 1.0% L-arabinose culture are listed in Table 3. These molecular weights were re-

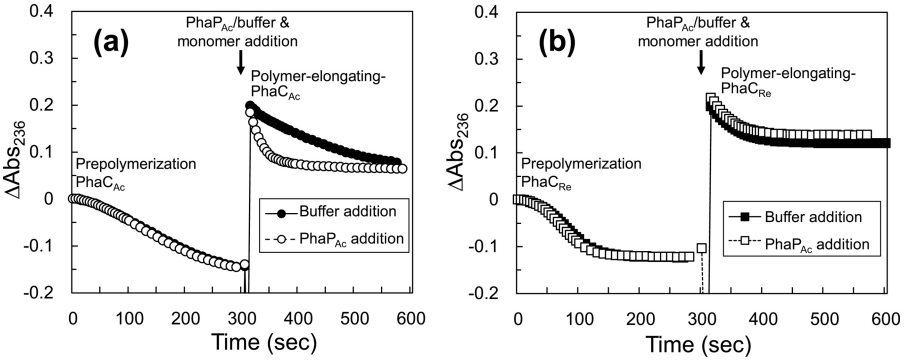

FIG 5 Reaction progress curves obtained with 350 nM PhaC$_{Ac}$ with a His tag (a) and 200 nM PhaC$_{Re}$ (b) during two-step addition of *R*-3HB-CoA. Reactions were initiated with 50 μM *R*-3HB-CoA, followed by the addition of PhaP$_{Ac}$ (1 μM in final concentration) or buffer (control) and 50 μM *R*-3HB-CoA with a 300-s incubation.

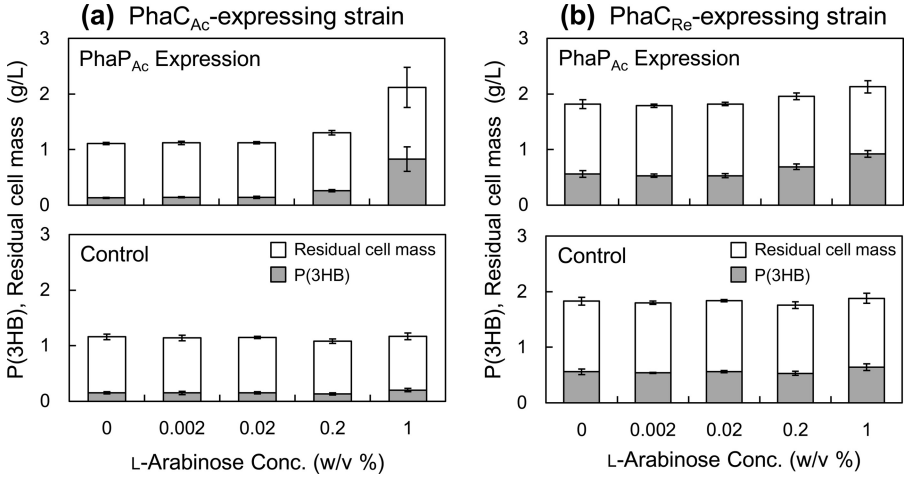

FIG 6 Effect of PhaP$_{Ac}$ expression on P(3HB) accumulation in the PhaC$_{Ac}$-expressing strain (pBBR1::*phaC$_{Ac}$AB$_{Re}$*) (a) and the PhaC$_{Re}$-expressing strain (pBBR1::*phaCAB$_{Re}$*) (b). A higher L-arabinose concentration induced a stronger expression of PhaP$_{Ac}$ in the *araBAD* system. Empty plasmid pBAD33 was retained in each control strain instead of using pBAD::TEE+*phaP$_{Ac}$*. Residual cell mass was calculated as the dry cell weight minus P(3HB). Error bars indicate the standard deviations ($n = 3$).

duced in both strains by the expression of PhaP$_{Ac}$. In particular, the PhaC$_{Re}$-expressing strain exhibited a remarkable reduction in molecular weight. Western blotting was performed to estimate the amounts of PhaCs in the soluble fractions obtained by low-speed centrifugation (Fig. 7). Based on the band intensity, the amount of soluble PhaC$_{Ac}$ was decreased to one-half by the expression of PhaP$_{Ac}$ (Fig. 7a), while the amount of PhaC$_{Re}$ in the soluble fraction was increased approximately 3-fold by PhaP$_{Ac}$ expression (Fig. 7b).

DISCUSSION

Previous studies have indicated that PhaPs have an enhancing effect on PHA biosynthesis *in vivo* (9, 10, 24, 25). To further investigate this effect, we performed *in vitro* polymerization activity assays in this study. Our results demonstrated that both PhaP$_{Ac}$ and PhaP1$_{Re}$ increased PhaC$_{Ac}$ activity, providing convincing evidence of PhaP-mediated PhaC$_{Ac}$ activation at the molecular level. In contrast, PhaC$_{Re}$ and PhaC$_{Da}$ showed reduced activities in the presence of PhaPs (Table 1). Some detergents, such as Triton X-100 and Hecameg, have been shown to activate PhaC$_{Re}$ and PhaC$_{Da}$ *in vitro* (17, 18). We tested the ability of these detergents to activate PhaC$_{Ac}$, but an activating effect was not observed (data not shown). Although these detergents and PhaPs are both amphiphilic molecules, the behavior of PhaCs was very different depending on the additive used. Therefore, PhaPs and the detergents appear to have different activation mechanisms for PhaCs. Most recently, PhaM, a DNA- and PHA-binding protein, has been shown to have an activating effect for PhaC$_{Re}$ (26). PhaPs and PhaM have the same ability to bind PHA granules; however, their effects on PhaC$_{Re}$ activity were opposite. The underlying mechanism for this difference between PhaPs and PhaM would be an interesting subject for study.

With respect to P(3HB-*co*-3HHx) production in the PhaC$_{Ac}$-expressing strain, several studies have reported that PhaP$_{Ac}$ affects not only PHA accumulation levels but also the 3HHx fraction in the polymer (8, 9). We hypothesized that the enhanced 3HHx fraction was also the result of the PhaP-activating effect of PhaC$_{Ac}$. To test this hypothesis, the effect of PhaP$_{Ac}$ on the substrate specificity of PhaC$_{Ac}$ was investigated using *R*-3HHx-CoA (C_6 substrate) and *R*-3HB-CoA (C_4 substrate). The PhaC$_{Ac}$ activity toward *R*-3HHx-CoA was increased 4.3-fold by the presence of PhaP$_{Ac}$, which was a significantly higher fold increase in activation than was observed for the C_4 substrate (2.5-fold), suggesting that the substrate preference of the activated PhaC$_{Ac}$ is slightly shifted toward the C_6 substrate. This shift may explain the increased 3HHx incorporation previously observed *in vivo* (8, 9).

Regarding the activity assays of PhaC$_{Re}$ in the presence of PhaPs, the *in vitro* results were not consistent with the *in vivo* result (Fig. 2 and 6b). In the presence of PhaPs, PhaC$_{Re}$ showed reduced activity *in vitro*, but the activity was not reduced *in vivo*. Cho and coworkers (27) reported a similar inhibitory effect of PhaP1$_{Re}$ on PhaC$_{Re}$ activity *in vitro*. According to their report (27), PhaP1$_{Re}$ reduced the *in vitro* activity of PhaC$_{Re}$ mainly by increasing the lag phase at the start of polymerization. However,

TABLE 3 P(3HB) synthesis in recombinant *E. coli* TOP10 cells cultured in the presence of L-arabinose[a]

Expressed protein(s)	Dry cell wt (g/liter)	P(3HB) content (wt%)	M_n ($\times 10^5$)	M_w ($\times 10^5$)	M_w/M_n
PhaC$_{Ac}$	1.2 ± 0.1	17 ± 2	3.5 ± 0.6	12.0 ± 0.9	3.4
PhaC$_{Ac}$ + PhaP$_{Ac}$	2.1 ± 0.4	39 ± 4	2.4 ± 0.6	7.4 ± 0.5	3.1
PhaC$_{Re}$	1.9 ± 0.1	35 ± 2	24.9 ± 3.5	39.9 ± 6.3	1.6
PhaC$_{Re}$ + PhaP$_{Ac}$	2.1 ± 0.1	43 ± 1	9.3 ± 1.1	24.4 ± 1.4	2.6

[a] Strains were the same as in the experiments whose results are shown in Fig. 6. The L-arabinose concentration was 1.0% (wt/vol). Results are expressed as the means ± standard errors ($n = 3$).

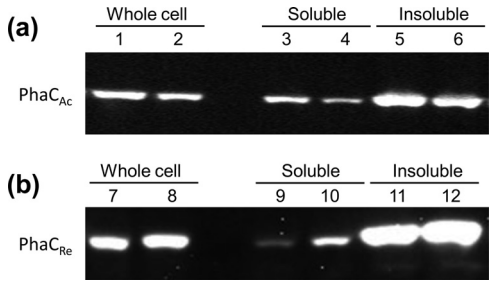

FIG 7 Western blots of PhaCs in 6-h-cultured PhaC$_{Ac}$-expressing strains (a) and PhaC$_{Re}$-expressing strains (b) with and without PhaP expression. The L-arabinose concentration was 1.0% (wt/vol). Lanes 1, 3, 5, 7, 9, and 11 are PhaP$_{Ac}$-free strains. Lanes 2, 4, 6, 8, 10, and 12 are PhaP$_{Ac}$-expressing strains. The soluble cell extract, containing PHA granules, and the insoluble precipitate were separated by low-speed centrifugation (1,500 × *g*, 5 min, 4°C). The amount of protein in each lane was 8 μg.

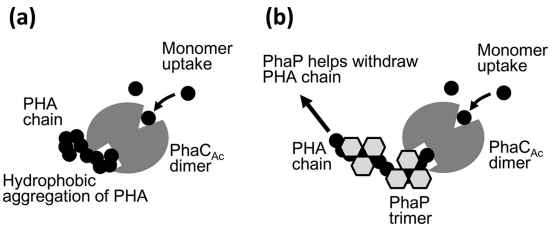

FIG 8 Proposed model of PhaP function for assisting in the initial stage of PHA polymerization by PhaC$_{Ac}$. PhaC$_{Ac}$ and PhaP are illustrated as dimer and trimer forms, respectively. (a) PhaC$_{Ac}$ alone. (b) PhaC$_{Ac}$ with PhaPs. See text for details.

our observations indicated that the maximum velocity in the activity assay was mainly repressed by the presence of PhaPs (Fig. 4). The reason for this difference might be attributed to the difference in PhaP1$_{Re}$ concentrations; namely, the PhaP concentration in our study (5 μM) was higher than the concentration used in their study (0.14 μM).

In addition, Cho and coworkers (27) reported that the PhaC$_{Re}$-PHA-PhaP1$_{Re}$ complex showed higher polymerization activity than the prepolymerization PhaC$_{Re}$ even though the PhaC$_{Re}$-PHA-PhaP1$_{Re}$ complex contained a high molar ratio of PhaP1$_{Re}$ (PhaP1$_{Re}$-PhaC$_{Re}$-PHA chain = 9:3:1). This observation has led to the hypothesis that the PHA chain prevents the inhibitory effect of PhaPs on PhaC$_{Re}$ polymerization activity. To test this hypothesis, we conducted an activity assay using polymer-elongating PhaCs. The polymer-elongating PhaC$_{Re}$ prepared by the two-step addition of substrate *in vitro* showed almost the same polymerization activity regardless of PhaP addition (Fig. 5b), suggesting that the PHA chain plays an important role in preventing the reduction of PhaC$_{Re}$ activity by PhaPs. The inhibitory effect by PhaP only occurred in the initial stage of the polymerization reaction; therefore, it is not likely to be observed in *in vivo* studies.

As shown in Fig. 6, the PhaP$_{Ac}$ expression level was controlled by the L-arabinose concentration. The P(3HB) content was increased in both PhaC$_{Ac}$- and PhaC$_{Re}$-expressing strains when the L-arabinose concentrations were over 0.2% (wt/vol). The highest P(3HB) accumulations were observed at 1.0% (wt/vol) L-arabinose, leading to 2.3- and 1.2-fold increases in the P(3HB) content for the PhaC$_{Ac}$- and PhaC$_{Re}$-expressing strains, respectively, compared with the expression levels in the corresponding PhaP$_{Ac}$-free strains. These data imply that a higher expression level of PhaP$_{Ac}$ results in a greater positive effect on PHA production *in vivo*. From the Western blot analysis of 6-h-cultured cells (Fig. 7), the amount of soluble PhaC$_{Ac}$ was slightly decreased by the expression of PhaP$_{Ac}$, while the amount of soluble PhaC$_{Re}$ was markedly increased. The difference in the levels of soluble PhaC$_{Ac}$ would result from differences in the cell growth phase, that is, the PhaP$_{Ac}$-free strain was still actively growing at 6 h of cultivation (polymer content, 9% [wt]), while the PhaP$_{Ac}$-expressing strain was already beginning to accumulate P(3HB) (22% [wt]) due to the activated PhaC$_{Ac}$. Thus, the protein expression pattern in these PhaC$_{Ac}$-expressing strains might be slightly different. On the other hand, the difference in soluble PhaC$_{Re}$ is attributed to the increased solubility of PhaC$_{Re}$ by the presence of PhaP$_{Ac}$. In general, as the amount of soluble PhaC$_{Re}$ increases in cells, the PHA chain number increases, whereas the molecular weight decreases (23). The P(3HB) molecular weight data (Table 3) are consistent with the fact that the amount of soluble PhaC$_{Re}$ increased. These observations may suggest that PhaP$_{Ac}$ plays a chaperone-like role in PhaC$_{Re}$ folding, which is a previously unrecognized function of PhaPs. Further studies are needed to confirm the role of PhaPs in protein folding.

PhaPs have been reported to form trimers or tetramers as shown by X-ray analysis (28, 29). On the other hand, PhaCs were observed in oligomeric form at the initial stage of PHA polymerization by atomic force microscope imaging (19, 30), but the PhaC dimer is thought to be a minimal functional unit (1, 15). Based on the observations in this study and previous works (19, 27–30), we propose a role for PhaPs in the initial stage of PHA polymerization by PhaC$_{Ac}$, as shown in Fig. 8. In this model, we assume that PHA synthase has a monomer uptake site and a polymer release site, which are distantly located. In the PhaP-free model, a synthesized PHA chain would generate hydrophobic aggregation near the polymer release site. Hydrophobic aggregation of the PHA chain might be stuck and block the release site, thereby preventing effective polymerization by PhaC$_{Ac}$ (Fig. 8a). In the presence of PhaP, the PhaP molecules adsorb to the PHA chain, which increases PHA's hydrophilicity, thus leading to prevention of PHA chain aggregation (Fig. 8b). In fact, the specific activity of the prepolymerization PhaC$_{Ac}$ was lower than that of the prepolymerization PhaC$_{Re}$ (Table 1), which was most likely due to inefficient polymer release of the prepolymerization PhaC$_{Ac}$. To further verify the validity of this model, detailed structural information of PhaCs and PhaPs is necessary, which is currently unresolved.

In conclusion, this study demonstrated that PhaPs could function as activators of PhaC$_{Ac}$, in addition to controlling the surface properties of PHA granules. PhaC$_{Ac}$ was activated *in vitro* by the presence of PhaPs both in the prepolymerization state and the polymer-elongating state. The PhaP$_{Ac}$-activated PhaC$_{Ac}$ exhibited a substrate preference that was slightly shifted toward the C$_6$ substrate. In contrast, the activities of prepolymerization PhaC$_{Re}$ and PhaC$_{Da}$ were decreased by PhaP$_{Ac}$, whereas the activity of polymer-elongating PhaC$_{Re}$ was not affected. PhaP$_{Ac}$ expression *in vivo* increased P(3HB) accumulation 2.3-fold and 1.2-fold in the PhaC$_{Ac}$ strain and PhaC$_{Re}$ strain, respectively, compared with its accumulation in the corresponding PhaP$_{Ac}$-free strains. The enhanced P(3HB) accumulation may be attributed to the role that PhaP$_{Ac}$ plays in assisting the initial stage of PHA polymerization by PhaC$_{Ac}$ and its chaperone-like role in PhaC$_{Re}$ folding. These

observations provide new insights into the functions of PhaPs and highlight the importance of PhaPs for effective PHA production.

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