MICROBIAL CELL FACTORIES

CrossMark

Compositional regulation of poly(3-hydroxybutyrate-*co*-3-hydroxyhexanoate) by replacement of granule-associated protein in *Ralstonia eutropha*

Yui Kawashima, Izumi Orita, Satoshi Nakamura and Toshiaki Fukui[*]

Abstract

Background: Phasin (PhaP), a kind of polyhydroxyalkanoate (PHA) granule-associated proteins, has a role in controlling the properties of PHA granules surface, and is thought to have influence on PHA biosynthesis in PHA-producing bacteria. This study focused on the $phaP1_{Re}$ locus in *Ralstonia eutropha* as a site of chromosomal modification for production of flexible poly(3-hydroxybutyrate-*co*-3-hydroxyhexanoate) [P(3HB-*co*-3HHx)] from soybean oil.

Results: Considering the high expression level of $phaP1_{Re}$, $phaJ_{Ac}$ [encoding (*R*)-specific enoyl-CoA hydratase from *Aeromonas caviae*] was inserted into the downstream of $phaP1_{Re}$ on chromosome 1 of *R. eutropha* strain NSDG harboring $phaC_{NSDG}$ (encoding PHA synthase with broad substrate specificity). The constructed strain efficiently accumulated P(3HB-*co*-3HHx) on soybean oil with higher 3HHx composition when compared to the previous strain having $phaJ_{Ac}$ within *pha* operon. Insertion of the second $phaC_{NSDG}$ along with $phaJ_{Ac}$ at the $phaP1_{Re}$ locus led to incorporation of much larger 3HHx fraction into PHA chains, although the molecular weight was markedly reduced. The *R. eutropha* strains were further engineered by replacing $phaP1_{Re}$ with $phaP_{Ac}$ (encoding phasin from *A. caviae*) on the chromosome. Interestingly, the phasin replacement increased 3HHx composition in the soybean oil-based PHA with keeping high cellular contents, nevertheless no modification was conducted in the metabolic pathways. Kinetic and Western blot analyses of PHA synthase using cellular insoluble fractions strongly suggested that the phasin replacement not only enhanced activity of PHA synthase from *A. caviae* but also increased affinity especially to longer (*R*)-3HHx-CoA. It was supposed that the increased affinity of PHA synthase to (*R*)-3HHx-CoA was responsible for the higher 3HHx composition in the copolyester.

Conclusions: The downstream of $phaP1_{Re}$ was a useful site for integration of genes to be overexpressed during PHA accumulation in *R. eutropha*. The results also clarified that polymerization properties of PHA synthase was affected by the kind of phasin co-existed on the surface of PHA granules, leading to altered composition of the resulting P(3HB-*co*-3HHx). The phasin replacement is a novel engineering strategy for regulation of composition of PHA copolyesters.

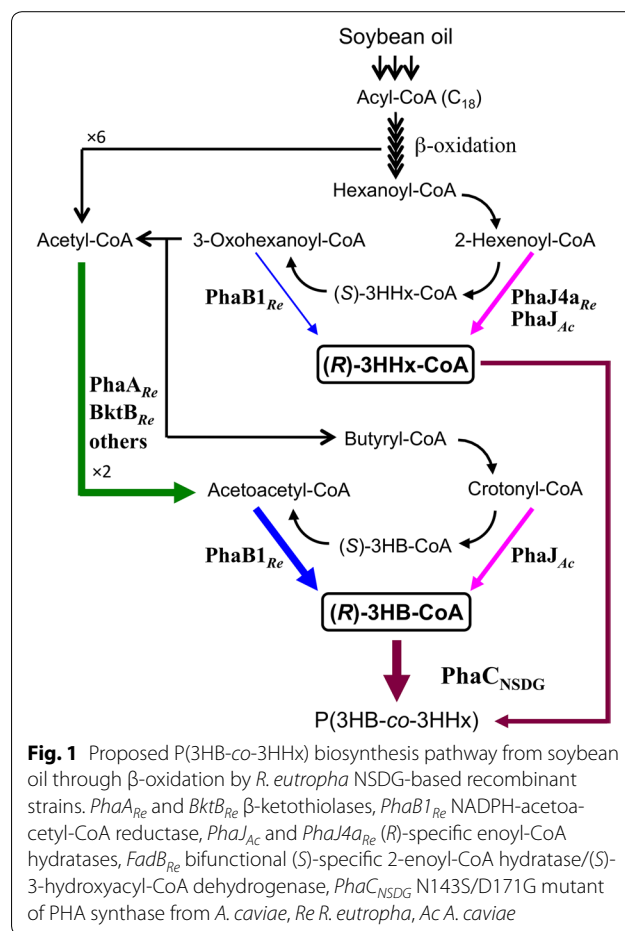**Keywords:** Polyhydroxyalkanoates, *Ralstonia eutropha*, PHA synthase, Phasin

*Correspondence: tfukui@bio.titech.ac.jp
Department of Bioengineering, Graduate School of Bioscience
and Biotechnology, Tokyo Institute of Technology, 4259 Nagatsuta,
Midori-ku, Yokohama 226-8501, Japan

BioMed Central

Kawashima *et al. Microb Cell Fact* (2015) 14:187

Page 2 of 12

Background

Polyhydroxyalkanoates (PHAs) are biopolyesters produced by a number of microorganisms as intracellular carbon and energy storage materials. PHAs have attracted industrial attentions as one of possible solutions for recent environmental problems caused by general petroleum-based plastics, because PHAs are eco-friendly polymeric materials that can be produced from renewable biomass resources, and show biodegradable and biocompatible properties [1, 2].

The most abundant PHA in nature is poly(3-hydroxybutyrate) [P(3HB)], which is generally synthesized from acetyl-CoA through three consecutive reactions by β-ketothiolase (PhaA), NADPH-dependent acetoacetyl-CoA reductase (PhaB) and PHA synthase (PhaC). *Ralstonia eutropha* (*Cupriavidus necator*) H16 is a well-studied P(3HB) producer [3, 4]. It has been known that the three genes for P(3HB) biosynthesis are clustered as $phaC$-A-$B1_{Re}$ operon, and $PhaC_{Re}$ shows polymerization activity toward short-chain-length (*R*)-3-hydroxyacyl (3HA)-CoAs of C_3–C_5. However, the application range of P(3HB) is very limited due to the brittle and struggle properties attributed to the high crystallinity and high melting temperature. Poly(3-hydroxybutyrate-*co*-3-hydroxyhexanoate) [P(3HB-*co*-3HHx)] is a PHA copolyester naturally produced by some bacteria such as *Aeromonas caviae* from vegetable oils and fatty acids [5]. This copolyester has been demonstrated to show more soft and flexible properties that are suitable for practical applications when compared to P(3HB) homopolymer [1, 5]. In *A. caviae*, (*R*)-3HA-CoA monomers of C_4 and C_6 are provided from 2-enoyl-CoA intermediates in β-oxidation by the function of (*R*)-specific enoyl-CoA hydratase ($PhaJ_{Ac}$), and successively polymerized by $PhaC_{Ac}$ having unique substrate specificity (C_4-C_7) [6, 7]. Based on these facts, efforts have been made to construct recombinant bacteria for efficient production of P(3HB-*co*-3HHx). Several previous studies engineered *R. eutropha* H16 by introduction of heterologous PHA synthase and (*R*)-specific enoyl-CoA hydratase, resulting in biosynthesis of P(3HB-*co*-3HHx) composed of adequately high 3HHx fractions from vegetable oils (Fig. 1) [8–12]. Tsuge et al. have reported that the Asn149Ser/Asp171Gly double mutant of *A. caviae* PHA synthase, named $PhaC_{NSDG}$, could accept more 3HHx unit than wild type $PhaC_{Ac}$ in vivo [8]. Insomphan et al. reported that deletion of one of *fadB* homologs in *R. eutropha* harboring $phaC_{NSDG}$ increased 3HHx fractions in the soybean oil-based P(3HB-*co*-3HHx) [13]. Recently, an artificial pathway for synthesis of this copolyester from structurally unrelated fructose was developed in *R. eutropha* [14].

Fig. 1 Proposed P(3HB-*co*-3HHx) biosynthesis pathway from soybean oil through β-oxidation by *R. eutropha* NSDG-based recombinant strains. $PhaA_{Re}$ and $BktB_{Re}$ β-ketothiolases, $PhaB1_{Re}$ NADPH-acetoacetyl-CoA reductase, $PhaJ_{Ac}$ and $PhaJ4a_{Re}$ (*R*)-specific enoyl-CoA hydratases, $FadB_{Re}$ bifunctional (*S*)-specific 2-enoyl-CoA hydratase/(*S*)-3-hydroxyacyl-CoA dehydrogenase, $PhaC_{NSDG}$ N143S/D171G mutant of PHA synthase from *A. caviae*, *Re R. eutropha*, *Ac A. caviae*

In PHA-accumulating bacterial cells, PHA chains are gathered and make granules in cytoplasm, and the PHA granules are covered with various proteins called PHA granule-associated proteins, PGAPs [15, 16]. Phasins (PhaPs) are amphiphilic small size proteins and known to be major PGAPs widely distributed in various PHA producers [17, 18]. The functions of PhaPs are thought to not only control the properties of PHA granules surface, but also have influence on PHA biosynthesis. $PhaP1_{Re}$ is the most abundant phasin in *R. eutropha* H16 [17]. The expression level is very high owing to the strong *phaP1* promoter [19, 20], by which transcription is regulated by PHA granule-binding transcriptional factor $PhaR_{Re}$ [15, 21]. $PhaP1_{Re}$ occupied approximately 5 % of the total proteins in the crude extract of the cells cultivated on fructose [17], and was estimated to cover 27–54 % of surface of the PHA granules [22]. It was demonstrated that $PhaP1_{Re}$ participated in control of the size and number of intracellular PHA granules and consequent amount of PHA, as the $phaP1_{Re}$-deficient strain of *R. eutropha* stored approximately half amount of P(3HB)

Kawashima *et al. Microb Cell Fact* (2015) 14:187

Page 3 of 12

as many smaller granules in comparison with the wild strain [17, 23]. Interestingly, PhaP1$_{Re}$ did not bind to PhaC1$_{Re}$ directly in two-hybrid assay [24], but formed a high-molecular weight complex along with PhaC1$_{Re}$ and soluble P(3HB) oligomer in *R. eutropha* [25]. The PhaC1$_{Re}$-PhaP1$_{Re}$-PHB complex showed no lag phase in PhaC activity assay, which suggested that PhaC$_{Re}$ had an active form in the complex.

Aeromonas caviae also has phasin (PhaP$_{Ac}$) of which gene is organized as *phaP-C-J$_{Ac}$* operon on the chromosome [6]. PhaP$_{Ac}$ belongs to a different class of phasin from PhaP1$_{Re}$, as they share no significant homology. When *phaPCJ$_{Ac}$* genes were highly expressed in *A. caviae*, a numerous number of small PHA granules were accumulated within the cells [26]. Moreover, a recombinant strain of *A. caviae* overexpressing *phaPC$_{Ac}$* synthesized P(3HB-*co*-3HHx) with much higher 3HHx composition (46–52 mol %) than the strain overexpressing *phaC$_{Ac}$* alone (16–21 mol %) [26]. Although the detail for this phenomenon has not been elucidated, the result suggested some effects of PhaP$_{Ac}$ on catalytic properties of PhaC$_{Ac}$. Recent in vitro analyses demonstrated that DNA/ PHA-binding protein PhaM$_{Re}$ activated polymerization activity of PhaC1$_{Re}$, and formed a high-molecular-weight complex with PhaC1$_{Re}$ [27]. Another enzyme assay indicated that PhaP1$_{Re}$ and PhaP$_{Ac}$ inhibited PhaC1$_{Re}$ activity, whereas they enhanced the activity of PhaC$_{Ac}$ up to 2.4 to 3-fold [28].

In this study, we focused on the *phaP1$_{Re}$* locus as a site for chromosomal modification in *R. eutropha* for production of P(3HB-*co*-3HHx). The effects of insertion of PHA biosynthesis genes at the downstream of *phaP1$_{Re}$*, as well as those of replacement of *phaP* on biosynthesis of PHA copolyester from soybean oil were investigated.

Results

Effects of insertion of *phaJ$_{Ac}$*/*phaC$_{NSDG}$* at downstream of *phaP1$_{Re}$* on PHA biosynthesis

Previous studies have demonstrated that introduction of PhaJ capable of accepting the C_6-substrate was effective to increase 3HHx composition of P(3HB-*co*-3HHx) produced by *R. eutropha* from vegetable oils [10–12]. While, the expression of *phaP1$_{Re}$*, regulated by PhaR$_{Re}$ [15, 21], is highly induced at the PHA accumulation phase and the induced transcription level is one of the highest in *R. eutropha* H16 [19, 20]. We here examined insertion of *phaJ$_{Ac}$* and/or the second copy of *phaC$_{NSDG}$* into the downstream of *phaP1$_{Re}$* on chromosome 1 of *R. eutropha* strain NSDG possessing *phaC$_{NSDG}$* instead of native *phaC$_{Ac}$* in *pha* operon. The gene organization in the constructed strains NSDG-P1$_{Re}$C, NSDG-P1$_{Re}$J, and NSDG-P1$_{Re}$CJ are illustrated in Fig. 2.

These strains were cultivated in a nitrogen-limited synthetic medium containing 1 % (v/v) soybean oil as a sole carbon source, and the results of PHA biosynthesis are shown in Table 1. The insertion of the additional copy of *phaC$_{NSDG}$* at the downstream of *phaP1$_{Re}$* gave almost no influence on production and composition of PHA. NSDG-P1$_{Re}$J, constructed by insertion of *phaJ$_{Ac}$*, produced similar amount of P(3HB-*co*-3HHx) with four-fold higher 3HHx composition compared to the parent strain NSDG. This increase of 3HHx fraction was slightly larger than that observed by the strain MF02 harboring *phaJ$_{Ac}$* within *pha* operon. Enzyme assay indicated that enoyl-CoA hydratase activities toward crotonyl-CoA (C_4) and 2-hexenoyl-CoA (C_6) in NSDG-P1$_{Re}$J (C_4: 27.2 U/ mg, C_6: 12.0 U/mg) were approximately 3.0- and 2.3-fold higher than those in MF02 (C_4: 9.1 U/mg, C_6: 5.2 U/mg).

When *phaC$_{NSDG}$*-*phaJ$_{Ac}$* genes were inserted at the site, the resulting strain NSDG-P1$_{Re}$CJ accumulated P(3HB-*co*-3HHx) with 3HHx fraction of 10.5 mol % (Table 1). This was 6.6-fold larger than that in the copolymer synthesized by the strain NSDG, although the PHA production was decreased from 5.9 to 4.8 g/l.

Effects of replacement of *phaP1$_{Re}$* by *phaP$_{Ac}$* on PHA biosynthesis

phaP1$_{Re}$ on chromosome 1 was replaced by *phaP$_{Ac}$* from *A. caviae* in the recombinant strains of *R. eutropha* expressing *phaC$_{NSDG}$* (Fig. 2), in order to investigate the change of PHA biosynthesis profiles by phasin derived from the same source as PHA synthase. Interestingly, this genetic modification tended to increase 3HHx composition without serious reduction of PHA production from soybean oil in all the strains examined, as shown in Table 1. The compositional change was noticeable in the strains expressing *phaJ$_{Ac}$* and the additional *phaC$_{NSDG}$* at the downstream of *phaP*, as the 3HHx fraction in P(3HB-*co*-3HHx) significantly increased to 17.2 mol % in NSDG-P$_{Ac}$CJ compared to 10.5 mol % in NSDG-P1$_{Re}$CJ. The 3HHx compositions of the copolymers synthesized by NSDG-P$_{Ac}$J and MF02-P$_{Ac}$ (6.4–8.8 mol %) were also higher than those in the corresponding strains having native PhaP1$_{Re}$ (4.6–6.5 mol %). The effect of the phasin replacement was not remarkable in the strains not expressing *phaJ$_{Ac}$*, such as NSDG and NSDG-P1$_{Re}$C.

Molecular weights of PHAs accumulated in *R. eutropha* recombinant strains

The number-average-molecular weights (M_n) of P(3HB-*co*-3HHx) synthesized by the *phaP*-replaced strains NSDG-P$_{Ac}$ and NSDG-P$_{Ac}$J were 1.8- and 1.4-fold higher than those by the corresponding parent strains NSDG

Kawashima *et al. Microb Cell Fact* (2015) 14:187

Page 4 of 12
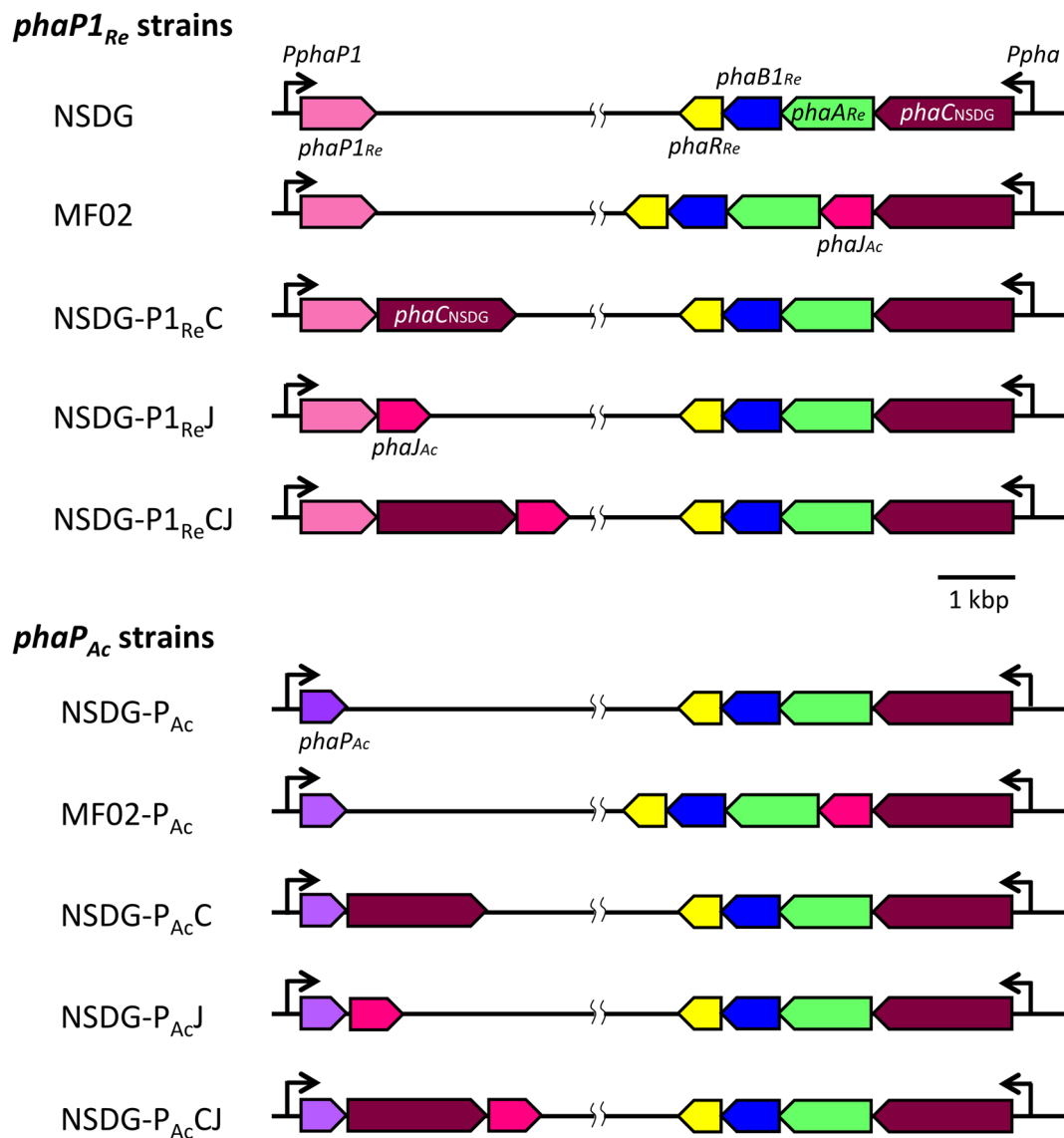
Fig. 2 Schematic diagram of genotypes of *R. eutropha* NSDG-based recombinant strains. *phaA_Re*, β-ketothiolase gene; *phaB1_Re*, NADPH-acetoacetyl-CoA reductase gene; *phaR_Re*, a gene encoding PHA-binding transcriptional repressor; *phaJ_Ac*, (*R*)-specific enoyl-CoA hydratase gene; *phaC_NSDG*, a gene encoding N143S/D171G mutant of PHA synthase from *A. caviae*; P_{phaP1} and P_{pha}, promoter regions of *phaP1_Re* and *phaCAB1_Re* in *R. eutropha*, respectively. *Re R. eutropha*, *Ac A. caviae*

and NSDG-P1_ReJ, respectively (Table 2). The results suggested that PhaP_Ac allowed PhaC_NSDG co-existed on the granule surface to synthesize longer PHA chain than PhaP1_Re. The strains NSDG-P1_ReCJ and NSDG-P_AcCJ accumulated PHA with drastically decreased M_n to approximately one-ninth of those obtained by the strains not having the second copy of *phaC_NSDG*. The values of polydispersity index of the resulting PHAs were not changed by the replacement of phasin.

Kinetic parameters of PHA synthase on the surface of PHA granules

PHA synthase and phasin were bound on the surface of PHA granules contained in a cellular insoluble fraction. The kinetic analysis of PhaC_NSDG on PHA granules was done using the insoluble fraction of *R. eutropha* to investigate the effects of the phasin replacement on the catalytic properties of PhaC_NSDG. We initially attempted the analysis for the strains NSDG and NSDG-P_Ac grown

Kawashima *et al. Microb Cell Fact* (2015) 14:187

Page 5 of 12

Table 1 PHA biosynthesis by *R. eutropha* recombinant strains from soybean oil

Strain	Dry cell mass (g/L)	PHA content (wt%)	PHA (g/L)	3HHx composition (mol %)
(*phaP1~Re~* strains)				
NSDG	6.9 ± 0.02	86 ± 4.3	5.9	1.6 ± 0.03
NSDG-P1~Re~C	6.7 ± 0.21	91 ± 0.3	6.0	1.8 ± 0.10
NSDG-P1~Re~J	6.7 ± 0.05	89 ± 1.5	6.0	6.5 ± 0.09
NSDG-P1~Re~CJ	5.7 ± 0.18	84 ± 1.3	4.8	10.5 ± 0.26
MF02	6.8 ± 0.02	88 ± 0.51	6.0	4.6 ± 0.07
(*phaP~Ac~* strains)				
NSDG-P~Ac~	7.1 ± 0.18	89 ± 1.1	6.3	2.0 ± 0.05
NSDG-P~Ac~C	6.4 ± 0.11	88 ± 1.5	5.6	2.1 ± 0.51
NSDG-P~Ac~J	7.0 ± 0.40	87 ± 1.1	6.1	8.8 ± 0.24
NSDG-P~Ac~CJ	6.3 ± 0.07	79 ± 3.3	5.0	17.2 ± 0.18
MF02-P~Ac~	7.2 ± 0.30	90 ± 1.2	6.5	6.4 ± 0.10

The cells were cultivated in an MB medium containing 1 % (v/v) soybean oil at 30 °C for 72 h (n = 3)

Table 2 Molecular weights of PHA synthesized by *R. eutropha* recombinant strains from soybean oil

Strain	PhaP	$M_n \times 10^5$	$M_w \times 10^5$	PDI
NSDG	PhaP1~Re~	7.5 ± 3.1	24.4 ± 5.23	3.56 ± 0.80
NSDG-P~Ac~	PhaP~Ac~	13.5 ± 2.3	42.3 ± 5.93	3.16 ± 0.26
NSDG-P1~Re~J	PhaP1~Re~	10.0 ± 2.1	33.8 ± 6.05	3.52 ± 0.90
NSDG-P~Ac~J	PhaP~Ac~	13.8 ± 1.2	48.1 ± 2.16	3.52 ± 0.25
NSDGP1~Re~CJ	PhaP1~Re~	1.2 ± 0.17	2.8 ± 0.25	2.28 ± 0.21
NSDG-P~Ac~CJ	PhaP~Ac~	1.5 ± 0.36	3.6 ± 1.39	2.35 ± 0.32

PHA was extracted and purified from the cells cultivated in an MB medium containing 1 % (v/v) soybean oil at 30 °C for 72 h (n = 3). M_n number-average molecular weight, M_w weight-average molecular weight, *PDI* polydispersity index (M_w/M_n)

on soybean oil, however, PHA synthase activity of the insoluble fractions was too low to determine reliable parameters. Therefore, the strains NSDG-P1~Re~CJ and NSDG-P~Ac~CJ, both harboring the second copy of *phaC-~NSDG~*, were used for the sources of the insoluble fraction.

When the polymerization activities of PhaC~NSDG~ were determined toward (*R*)-3HB-CoA (C~4~) and (*R*)-3HHx-CoA (C~6~), no lag-phase of the absorbance change was observed. The reactions followed Michaelis–Menten kinetics, and the determined kinetic parameters are shown in Table 3. PHA synthases in both the strains showed lower activity to (*R*)-3HHx-CoA than to (*R*)-3HB-CoA. Interestingly, the replacement of PhaP1~Re~ by PhaP~Ac~ led to 2.6 to 2.8-fold increase in the V_{max} values, and significantly reduced the K_m values to one-fourth to one-tenth. The C~6~/C~4~ ratios of V_{max} were almost the same as each other for the two synthase samples, while the C~6~/C~4~ ratio of K_m was markedly decreased from 1.9 with PhaP1~Re~ (NSDG-P1~Re~CJ) to 0.71 with PhaP~Ac~ (NSDG-P~Ac~CJ). These results indicated that the increase of affinity of PhaC~NSDG~ by the phasin replacement was more significant to the longer C~6~-substrate. Consequently, the C~6~/C~4~ ratio of V_{max}/K_m of the synthase in the *phaP~Ac~*-expressing strain was 3.0-fold larger than that in the strain expressing native *phaP1~Re~*, which was consistent with the increase of the 3HHx fraction in the resulting PHA. The amounts of the PhaC~NSDG~ protein in the insoluble fractions of NSDG-P1~Re~CJ and NSDG-P~Ac~CJ, determined by Western blot analysis, were similar level as shown in Fig. 3a, indicating that the differences in the kinetic parameters were not due to changes in the amount of PHA synthase on the granule surface. We also observed that (*R*)-specific enoyl-CoA hydratase activity to the C~4~- and C~6~-substrates were not different between the soluble fractions of the two strains (C~4~: 14.3–15.3 U/mg and C~6~: 1.1–1.3 U/mg, respectively). The similar amounts of PhaC~NSDG~ and activities of PhaJ~Ac~ in the two strains indicated little influence of the replacement of *phaP* on transcription driven by the upstream promoter region.

Confirmation of phasin replacement and identification of proteins in PHA granule fractions

The proteins in PHA granule fractions of the strains NSDG-P1~Re~CJ and NSDG-P~Ac~CJ, prepared by

Table 3 Kinetic parameters of PhaC~NSDG~ in insoluble fractions of *R. eturopha* recombinant strains toward (*R*)-3HB-CoA (C~4~) and (*R*)-3HHx-CoA (C~6~)

Strain (genotype)	Substrate chain length	K_m (µM)	C~6~/C~4~ ratio	V_{max} (µmol/min/ mg-insoluble[a])	C~6~/C~4~ ratio	V_{max}/K_m	C~6~/C~4~ ratio
NSDG-P1~Re~CJ	C~4~	320	1.9	0.21	0.20	6.4×10^{-4}	0.10
(*phaP1~Re~-phaC~NSDG~-phaJ~Ac~*)	C~6~	614		0.041		6.6×10^{-5}	
NSDG-P~Ac~CJ	C~4~	86	0.71	0.52	0.21	6.1×10^{-3}	0.30
(*phaP~Ac~-phaC~NSDG~-phaJ~Ac~*)	C~6~	61		0.11		1.8×10^{-3}	

The insoluble fractions were prepared from the cells cultivated in an MB medium containing 1 % (v/v) soybean oil at 30 °C for 48 h
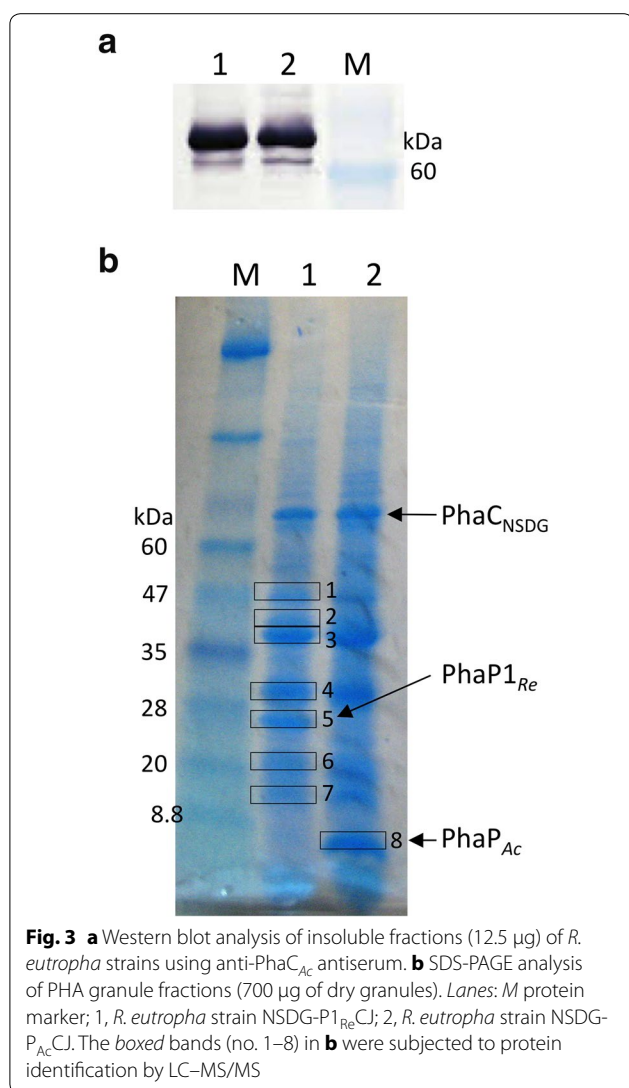
[a] Dry weight of the insoluble fraction

Kawashima *et al. Microb Cell Fact* (2015) 14:187

Page 6 of 12

Fig. 3 **a** Western blot analysis of insoluble fractions (12.5 μg) of *R. eutropha* strains using anti-PhaC$_{Ac}$ antiserum. **b** SDS-PAGE analysis of PHA granule fractions (700 μg of dry granules). *Lanes: M* protein marker; 1, *R. eutropha* strain NSDG-P1$_{Re}$CJ; 2, *R. eutropha* strain NSDG-P$_{Ac}$CJ. The *boxed* bands (no. 1–8) in **b** were subjected to protein identification by LC–MS/MS

and RNA polymerase subunit α in the no. 2 band. These proteins were considered to be the result of contamination of other cell fractions, but possibility of the presence on the surface as PGAPS could not be excluded, as observed and discussed previously [16, 31]. Although *R. eutropha* possesses many phasins [3, 24, 29, 30], only PhaP5$_{Re}$ could be detected in the band no. 7 besides PhaP1$_{Re}$ in NSDG-P1$_{Re}$CJ.

Electron microscopy

The accumulation of PHA granules within *R. eutropha* H16, NSDG, NSDG-P$_{Ac}$, NSDG-P1$_{Re}$CJ, and NSDG-P$_{Ac}$CJ grown on soybean oil were observed by transmission electron microscopy (Fig. 4). The wild strain H16 accumulated 10–16 of small PHA granules per one cell, whereas NSDG and NSDG-P$_{Ac}$ formed one to three of much larger PHA granule(s). In the cells of NSDG-P1$_{Re}$CJ and NSDG-P$_{Ac}$CJ, granules with various sizes were existed together. The size and number of PHA granules were greatly affected by the kind of PHA synthase, but not by phasin on the granule surface. The reason for these observations has been unclear yet, but the formation of less larger granules in the strain NSDG was similar to phenotype of the *phaP1$_{Re}$*-disruptant [17]. The hydrophilicity of cytosolic side of the PhaC$_{NSDG}$ protein on PHA granules may be lower than that of PhaC$_{Re}$, leading to formation of large granules with reduced surface area.

Discussion

The gene of the major phasin in *R. eutropha* H16, *phaP1$_{Re}$*, is one of the most highly expressed genes at the PHA accumulation phase [19, 20]. Barnard et al. have achieved T7 promoter-driven high level production of recombinant proteins by using the *phaP1$_{Re}$* locus as the integration site for T7 RNA polymerase [32]. We here applied the downstream of *phaP1$_{Re}$* as an insertion site for PHA biosynthesis-related gene(s) in *R. eutropha*, because the induction profile and expression level of *phaP1$_{Re}$* higher than *phaCAB1$_{Re}$* were expected to be advantage for PHA biosynthesis. When *phaC$_{NSDG}$* was solely inserted at the site in the strain NSDG, no change in P(3HB-*co*-3HHx) composition was observed, probably due to poor provision of (*R*)-3HHx-CoA through the intact channeling pathway from β-oxidation to PHA biosynthesis. Indeed, enhancement of the channeling pathway by expression of *phaJ$_{Ac}$* significantly increased 3HHx composition without impairment of PHA production, as observed previously [10, 12]. The results of the cultivation suggested that the expression level of the gene located in the *phaP1$_{Re}$* locus was higher than within *pha* operon, given the higher 3HHx composition in the resulting P(3HB-*co*-3HHx) and higher enoyl-CoA hydratase activity in NSDG-P1$_{Re}$J

density-gradient ultracentrifugation, were separated by SDS-PAGE (Fig. 3b), and the major eight protein bands were subjected to LC–MS/MS analysis for identification. The details of the results are shown in Additional file 1: Table S1. The 24-kDa protein (no. 5) in NSDG-P1$_{Re}$CJ was determined to be PhaP1$_{Re}$. In the strain NSDG-P$_{Ac}$CJ, this band was disappeared and a smaller protein (no. 8) in large abundance, not present in NSDG-P1$_{Re}$CJ, was identified to be PhaC$_{Ac}$. This result clearly indicated actual replacement of major phasin on PHA granules to PhaP$_{Ac}$ in NSDG-P$_{Ac}$CJ. The protein band no. 3 was outer membrane protein (porin), and the bands of no. 1, 2, 4, 6, and 7 contained multiple kinds of proteins including various membrane proteins, ribosomal proteins, and hypothetical proteins. Several proteins involved in important cellular functions were also detected, such as elongation factor Tu and cell division protein FtsA in the no. 1 band
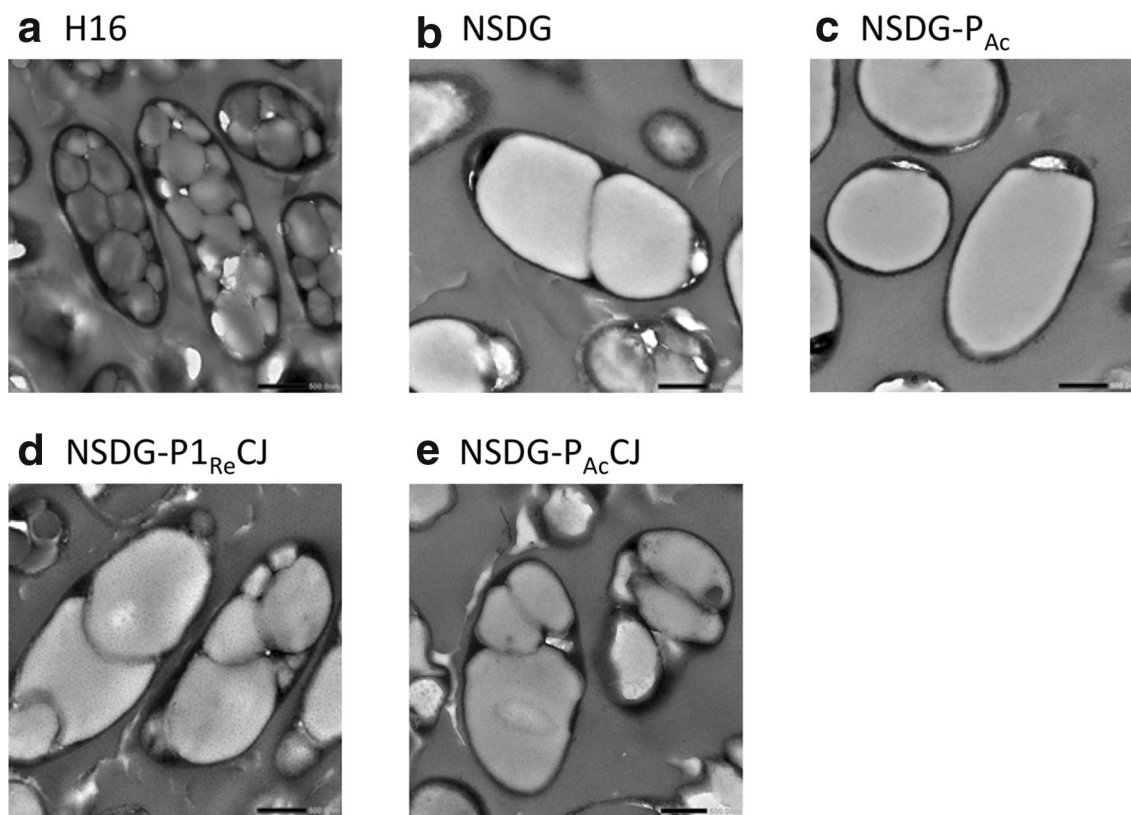
Kawashima *et al. Microb Cell Fact* (2015) 14:187

Page 7 of 12

Fig. 4 Transmission electron micrographs of ultrathin sections of *R. eutropha* strains. Magnification: ×10,000; *scale bar* 0.5 μm

(*phaP1_{Re}-phaJ_{Ac}*) than those in the strain MF02 (*phaC*-$_{NSDG}$-*phaJ_{Ac}-phaAB1_{Re}*). The insertion of the second copy of *phaC*$_{NSDG}$ at the downstream of *phaP1_{Re}* led to drastic decrease of the molecular weight of PHA by less than one-tenth. Previous studies [33–35] reported a negative correlation of intracellular PhaC activity or *phaC* expression level with P(3HB) molecular weight, and one of possible reasons for this phenomenon may be due to large number of catalytic molecules (PhaC$_{NSDG}$) relative to substrate molecules [(*R*)-3HA-CoAs]. The present study was consistent with the previous observations, and the similar effects of plasmid-borne multi copies of *phaC* on P(3HB-*co*-3HHx) biosynthesis has been also seen in recombinant *R. eutropha* grown on plant oils [11].

This study also demonstrated a novel engineering strategy for compositional regulation of P(3HB-*co*-3HHx); the phasin replacement. We previously observed that overexpression of *phaC_{Ac}* together with *phaP_{Ac}* in *A. caviae* significantly increased the 3HHx fraction in PHA produced from fatty acids when compared to overexpression of *phaC_{Ac}* alone [26]. One of possible explanation for this phenomenon was some changes of catalytic properties of PhaC$_{Ac}$ in the presence of PhaP$_{Ac}$ derived from the same source. However, such compositional change had not been

observed when *phaPC_{Ac}* were introduced in *R. eutropha* PHB^{-}4 [6]. We assumed that, even if PhaP$_{Ac}$ affected catalytic properties of PhaC$_{Ac}$, the effects might be disturbed by the presence of large amount of native phasins covering the surface of PHA granules in *R. eutropha*. The *phaP1_{Re}* encoding major phasin was thus replaced by *phaP_{Ac}* by homologous recombination, and the results demonstrated that the phasin replacement tended to increase 3HHx fraction in P(3HB-*co*-3HHx) with keeping high PHA contents on soybean oil. Further kinetic analysis of PhaC$_{NSDG}$ using native granules clarified interesting changes of the catalytic properties depending on the origin of phasin. The replacement of PhaP1$_{Re}$ by PhaP$_{Ac}$ significantly increased the catalytic efficiency of PhaC$_{NSDG}$ that had been already active form on the PHA granule surface. It has been reported that both PhaP$_{Ac}$ and PhaP1$_{Re}$ enhanced the polymerization activity of PhaC$_{Ac}$ toward (*R*)-3HB-CoA in in vitro assay using the free proteins not associated with PHA granules, where the activation by PhaP$_{Ac}$ was slightly stronger than that by PhaP1$_{Re}$ [28]. It should be noted that the enhancement of polymerization activity by the replacement of PhaP1$_{Re}$ with PhaP$_{Ac}$ on the native granules (2.5 to 2.7-fold) was much larger than that observed in the case using the free proteins (1.3-fold). In addition, the

Kawashima *et al. Microb Cell Fact* (2015) 14:187

Page 8 of 12

phasin replacement more specifically increased the affinity of PhaC$_{NSDG}$ toward the longer (R)-3HHx-CoA, as seen in the increase of C$_6$/C$_4$ ratio of the K$_m$ values (Table 3). It was feasible that the higher relative affinity of the polymerizing enzyme to the C$_6$-substrate was responsible for the larger 3HHx fraction in PHA synthesized by the strain NSDG-P$_{Ac}$CJ. Ushimaru et al. proposed that phasin helps to withdraw a PHA chain from PhaC$_{Ac}$ exhibiting lower turnover rate than PhaC1$_{Re}$, and thus prevent the chain from aggregation that potentially blocks the release site in the active dimer [28]. This idea can well explain the activation of PhaC$_{Ac}$ by phasins, but not enough to understand the great increase of the substrate affinity of PhaC$_{NSDG}$, particularly to the C$_6$-substrate, by the phasin replacement. Although the detailed mechanism has been remained to be cleared, it was supposed that phasin could interact with active PHA synthase on the granule surface. In the case of specific combination of PHA synthase and phasin such as PhaC$_{NSDG}$ and PhaP$_{Ac}$, the interaction might induce some structural change potentially affecting the affinity and specificity toward the substrates into PHA synthase (Fig. 5). The molecular weights of the accumulated PHA also tended to be increased by the phasin replacement. The higher activity of PhaC$_{NSDG}$ in the presence of PhaP$_{Ac}$ on the granule surface would lead formation of longer polymer chains than the enzyme with PhaP1$_{Re}$.

The expression of *phaJ* was very effective for increasing 3HHx composition in P(3HB-*co*-3HHx) synthesized from fatty acids and plant oils by recombinant *R. eutropha* strains [10–12]. However, we previously observed saturation of the provision of 3HHx unit by this strategy [12]. Another strategy for increasing 3HHx composition was suppression of 3HB unit formation by disruption of *phaA* encoding thiolase, although this accompanied reduction

of PHA production [10]. The advantage of the present phasin replacement strategy was further increase of 3HHx composition even in the *phaJ*-expressing strains without impairment of PHA biosynthesis. The *R. eutropha* strains applying this novel strategy can efficiently produce P(3HB-*co*-3HHx) composed of 9–17 mol % 3HHx fractions, which was suitable for practical applications with adequate flexibility [5].

Conclusion

This study demonstrated that the downstream of *phaP1$_{Re}$* on chromosome 1 of *R. eutropha* was a useful site for integration of PHA biosynthesis genes to achieve the overexpression during the PHA accumulation phase. In addition, we found that replacement of the major phasin PhaP1$_{Re}$ in recombinant *R. eutropha* by PhaP$_{Ac}$ from *A. caviae* resulted in increase of 3HHx composition of P(3HB-*co*-3HHx) without serious decrease of the productivity on soybean oil.

A general engineering strategy for alteration of PHA copolymer composition has been modification of metabolic flux of monomer-supplying pathways. The phasin replacement is a novel strategy not directly modifying metabolic pathways, thus there is no need to consider unexpected negative effects on global metabolisms. It was also shown that catalytic properties of PHA synthase were affected by phasin co-existed on the surface of PHA granules, and significance of the effects appeared to be depended on the kind of phasin. These results were interesting in understanding the role of phasin in PHA biosynthesis. In terms of the application, the use of adequate pair of PHA synthase and phasin would allow us to obtain PHA copolymers with desired composition with high productivity.
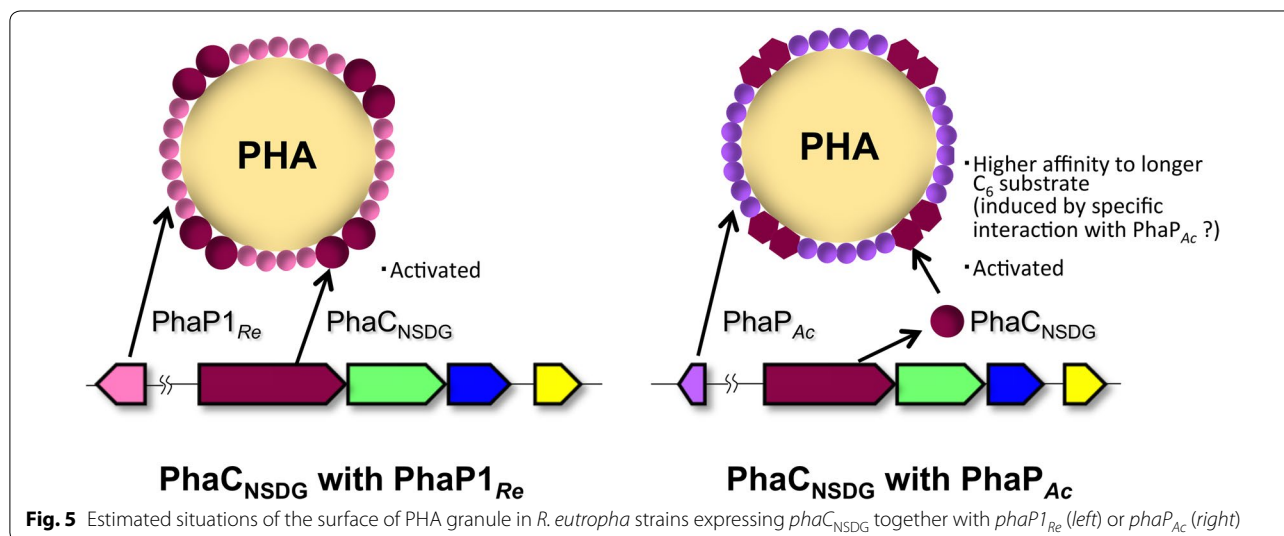
Fig. 5 Estimated situations of the surface of PHA granule in *R. eutropha* strains expressing *phaC$_{NSDG}$* together with *phaP1$_{Re}$* (*left*) or *phaP$_{Ac}$* (*right*)

Kawashima *et al. Microb Cell Fact* (2015) 14:187

Page 9 of 12

Methods

Bacterial strains and plasmids

The bacterial strains and plasmids used in this study are listed in Additional file 1: Table S2. *R. eutropha* strains were cultivated at 30 °C in a nutrient-rich (NR) medium containing 10 g of meat extract, 10 g of polypeptone, 2 g of yeast extract in 1 L of tap water. *E. coli* strains were cultivated at 37 °C in a Lysogeny broth (LB) medium. Kanamycin (100 mg/L) or ampicillin (50 mg/L) was added to the medium when necessary.

Construction of plasmids and strains

DNA manipulations were carried out according to standard procedures, and the sequences of oligonucleotide primers used for PCR are shown in Additional file 1: Table S3. KOD-Plus-ver.2 or KOD-Plus-Neo DNA polymerase and Ligation High ver.2 (Toyobo, Otsu, Japan) were used for PCR amplification and ligation, respectively.

The upstream and downstream regions flanked to *phaP1*$_{Re}$ were individually amplified by PCR from *R. eutropha* H16 genomic DNA as a template with primer sets of phaP$_{Re}$out-Fw/phaP$_{Re}$-Inv1 and phaP$_{Re}$out-Rv/phaP$_{Re}$-Inv2, respectively. The latter fragment was 5′-phosphorylated, ligated with the former, and the upstream–downstream fragment was subjected to the second PCR using phaP$_{Re}$out-Fw/phaP$_{Re}$out-Rv primers. The amplified upstream–downstream fusion was inserted into pK18mobsacB at the SmaI site, leading to construction of pK18mobsacB-ΔP1.

The vectors for replacement of *phaP1*$_{Re}$ by *phaP*$_{Ac}$, *phaP*$_{Ac}$-*phaC*$_{NSDG}$, *phaP*$_{Ac}$-*phaC*$_{NSDG}$-*phaJ*$_{Ac}$, or *phaPJ*$_{Ac}$ were constructed as follows. The coding region of *phaC*$_{NSDG}$ was amplified from pTA2-NSDG [10] by PCR using a primer set of pEE32R-C$_{Ac}$up-inv/pEE32R-C$_{Ac}$down-inv. A linear fragment not containing *phaC*$_{Ac}$ in pEE32 harboring *phaPCJ*$_{Ac}$ [6] was prepared by inverse PCR with phaP$_{Re}$-Inv1/phaP$_{Re}$-Inv2 primers, and then ligated with the *phaC*$_{NSDG}$ fragment to obtain pEE32-NSDG harboring *phaP*$_{Ac}$-*C*$_{NSDG}$-*J*$_{Ac}$. The fragments of *phaP*$_{Ac}$, *phaP*$_{Ac}$-*C*$_{NSDG}$, and *phaP*$_{Ac}$-*C*$_{NSDG}$-*J*$_{Ac}$ were amplified with pEE32-NSDG as a template and primer sets of phaP$_{Ac}$-Fw/phaP$_{Ac}$-Rv, phaP$_{Ac}$-Fw/phaC$_{NSDG}$-Rv, and phaP$_{Ac}$-Fw/phaJ$_{Ac}$-Rv, respectively. The amplified fragments were 5′-phosphorylated and then individually inserted between upstream and downstream regions of *phaP1*$_{Re}$ by ligation with an inverse PCR product obtained with pK18mobsacB-ΔP1 as a template and phaP$_{Re}$-Inv1/phaP$_{Re}$-Inv2 primers. The resulting plasmids were designated pK18mobsacB-P$_{Ac}$, pK18mobsacB-P$_{Ac}$C, and pK18mobsacB-P$_{Ac}$CJ, respectively. pK18mobsacB-P$_{Ac}$J was constructed by inverse PCR for elimination of the *phaC*$_{NSDG}$ region from pK18mobsacB-P$_{Ac}$CJ with a primer set phaP$_{Ac}$down-Inv/phaJ$_{Ac}$-Fw, followed by self-ligation after 5′-phosphorylation.

pK18mobsacB-P1$_{Re}$C, pK18mobsacB-P1$_{Re}$J and pK18mobsacB-P1$_{Re}$CJ, used for insertion of the genes from *A. caviae* at downstream of *phaP1*$_{Re}$, were constructed from pK18mobsacB-P$_{Ac}$C, pK18mobsacB-P$_{Ac}$J and pK18mobsacB-P$_{Ac}$CJ, respectively. Linear fragments not having the *phaP*$_{Ac}$ region were prepared from these plasmids by inverse PCR using phaP$_{Re}$-Inv1/phaP$_{Ac}$-down-Inv primers. The coding region of *phaP1*$_{Re}$ was amplified with *R. eutropha* H16 genome DNA and a primer set of phaP$_{Re}$-Fw/phaP$_{Re}$-Rv, and the *phaP1*$_{Re}$ fragment was ligated with the corresponding inverse PCR product with the same direction as the *phaP1* promoter.

All the pK18mobsacB-based plasmids were transferred from *E. coli* S17-1 by conjugation to *R. eutropha* strains NSDG or MF02, and the recombinant strains formed by pop-in-pop-out recombination were selected, according to the procedures reported previously [10].

PHA production by recombinant *R. eutropha* strains

PHA production by *R. eutropha* strains was carried out on a reciprocal shaker (115 strokes/min) at 30 °C in a 500 ml flask with a 100 ml of a nitrogen-limited mineral salts (MB) medium and 0.1 ml of trace-element solution [36]. Soybean oil was directly added to the medium at 1.0 % (v/v) as a sole carbon source. After the cultivation for 72 h, the cells were harvested, washed with cold 70 % ethanol, washed again with deionized water, and then lyophilized. The cellular PHA content and monomer composition were determined by gas chromatography (GC) after methanolysis of the dried cells in the preference of 15 % sulfuric acid [35]. Extraction and purification of the accumulated PHA from the dried cells, and determination of molecular weight by gel permutation chromatography (GPC) were performed as described previously [37].

Enzyme assay

Crotonyl-CoA was synthesized by condensation of crotonic anhydride (Tokyo Chemical Industry, Tokyo, Japan) with lithium salt of CoA-SH [38]. Chemical synthesis of (R)-3HB-CoA and *trans*-2-hexenoyl-CoA by a mixed-anhydride method, and enzymatic conversion of *trans*-2-hexenoyl-CoA to (R)-3HHx-CoA were performed as described previously [39], except for the use of recombinant PhaJ4a$_{Re}$ [12] as (R)-specific enoyl-CoA hydratase.

Ralstonia eutropha strains were cultivated for 48 h in an MB medium at 30 °C with 1.0 % (v/v) soybean oil as a carbon source. The grown cells were harvested, washed by Tris–HCl buffer (pH 7.5), and then disrupted by high pressure homogenization as described previously [36]. The disrupted cells were centrifuged (18,800*g*, 10 min,

Kawashima *et al. Microb Cell Fact* (2015) 14:187

Page 10 of 12

4 °C) to separate the soluble fraction and insoluble fraction containing native PHA granules. PHA synthase activity of the insoluble fraction was assayed with the reaction mixture composed 0.05–0.5 mM (*R*)-3HB-CoA or (*R*)-3HHx-CoA, 1 mM 5,5′-dithiobis (2-nitrobenzoic acid) [DTNB] in 50 mM phosphate buffer (pH 7.2), and 10 µl of the insoluble fraction. The increase in absorbance at 412 nm corresponding to release of free CoA-SH was measured spectrophotometrically at 30 °C ($\varepsilon_{412} = 14.5 \times 10^3$). Enoyl-CoA hydratase activity, including both (*S*)- and (*R*)-specific activities, in the soluble fraction was determined in the reaction mixture composed of 250 µM crotonyl-CoA or *trans*-2-hexenoyl-CoA in 50 mM Tris–HCl (pH 8.0) and 5 µl of the soluble fraction. The hydration of the enoyl-CoA substrates were monitored as decrease in absorbance at 263 nm at 30 °C ($\varepsilon_{263} = 6.7 \times 10^3$ M^{-1} cm^{-1}). (*R*)-specific enoyl-CoA hydratase activity in the soluble fraction was determined by the hydration of the enoyl-CoA substrates coupled with polymerization of the resulting (*R*)-3-hydroxyalyl-CoAs of C_4 and C_6 by using the insoluble fraction of *R. eutropha* NSDG-P$_{Ac}$C as a source of PHA synthase. The reaction mixture was composed of 0.20 mM *trans*-2-enoyl-CoA, 1 mM DTNB in 50 mM phosphate buffer (pH 8.0), 5 µl of the insoluble fraction of the strain NSDG-P$_{Ac}$C, and the increase of absorbance at 412 nm was monitored at 30 °C.

The aliquot of the insoluble fraction was lyophilized to determine the dry weight, and used to calculate specific activities. The proteins contained in 12.5 µg of the insoluble fraction were separated by sodium dodecylsulfate polyacrylamide gel electrophoresis (SDS-PAGE) with 15 % acrylamide gel, according to the standard procedure. Western blot analysis for PhaC$_{NSDG}$ was performed using a specific antiserum against PhaC$_{Ac}$ [26]. A goat anti-rabbit IgG (Fc fragment specific)-alkaline phosphatase conjugate (Calbiochem, CA, USA) was used as the secondary antibody, and 1-Step NBT/BCIP plus suppressor (Thermo Fisher Scientific, MA, USA) was used to detect the signals according to the manufacture's instruction. Protein concentration of the soluble fraction was determined by the method of Bradford with bovine serum albumin as the standard.

Transmission electron microscope

The cells of the *R. eutropha* strains cultivated in an MB medium with 1 % (v/v) soybean oil at 30 °C for 48 h were harvested, washed, and resuspended in 0.1 M phosphate buffer (pH 7.2) containing 0.1 M sucrose. The cells were fixed with 2.5 % (w/v) glutaraldehyde in 0.1 M phosphate buffer (pH 7.2) containing 0.1 M sucrose, and subsequently with 1 % (w/v) osmium tetroxide in the buffer. The fixed cells were dehydrated in a graded ethanol and embedded with epoxy resin Quetol 651 (Nissin

EM, Tokyo, Japan). The ultrathin sections (80 nm) of the embedded cells were prepared with a Leica UC7 ultramicrotome. The sections on a copper grid were stained with EM stainer (Nissin EM) followed by Reynolds's lead citrate at room temperature. The specimens were observed on a JEOL 1400Plus transmission electron microscope with an accelerating voltage of 100 kV.

Isolation of native PHA granule fractions and identification of proteins

The insoluble fractions of *R. eutropha* strains were loaded on the top of a discontinuous glycerol gradient of 2 mL of 88 % glycerol and 8 mL of 44 % glycerol in 100 mM Tris–HCl (pH 7.5). After the ultracentrifugation (210,000*g*, 40 min, 4 °C), the PHA granules at the interphase of 44–88 % glycerol were collected, following by washing with 100 mM Tris–HCl (pH 7.5). The granules resuspended in 1 mL of the buffer were subsequently loaded on a discontinuous sucrose gradient prepared with 1 mL of 1.8 M sucrose, 2.9 mL of 1.6, 1.4, and 1.2 M sucrose, and 1 mL of 1.0 M sucrose in the buffer, and then subjected to ultracentrifugation (210,000*g*, 2 h, 4 °C). The band of the PHA granules were observed at the interphase of 1.2–1.4 M sucrose for P(3HB-*co*-3HHx) composed of 10.5 mol % 3HHx (produced by the strain NSDG-P1$_{Re}$CJ), and that of 1.0–1.2 M sucrose for P(3HB-*co*-3HHx) composed of 17.2 mol % 3HHx (produced by the strain NSDG-P$_{Ac}$CJ). The bands of PHA granules were recovered, washed, and resuspended in 100 mM Tris–HCl (pH 7.5).

The PHA granule suspension containing approximately 700 µg of dried inclusion was separated by SDS-PAGE with 4–15 % gradient gel (Bio-Rad, Hercules, CA, USA). The tryptic peptides were prepared from the SDS-PAGE gel, and subjected to mass spectrometric analysis using an LC–MS system equipped with Acquity UPLC apparatus (Waters, Milford, MA, USA) and Synapt High Definition Mass Spectrometer (Waters). LC was performed with a reversed-phase C_{18} column (75 µm × 150 mm, 1.7 µm particle size) with the mobile phase of acetonitrile gradient in 1 % formic acid at the flow rate of 0.5 µl/min. The mass spectrometry was done in the positive ion mode. The MS/MS spectra were analyzed by Protein Lynx Global Server (Waters).

Additional file

Additional file 1. **Table S1**. Identification of proteins in PHA granule fractions from *R. eutropha* recombinant strains grown on soybean oil. **Table S2**. Bacterial strains and plasmids used in this study. **Table S3**. Sequences of primers used in this study.

Authors' contributions

YK carried out the experiments and data analyses, and wrote the manuscript. IO and SN participated in the design and coordination of the study. TF

Kawashima *et al. Microb Cell Fact* (2015) 14:187

Page 11 of 12

designed the experiments and prepared the manuscript. All authors read and approved the final manuscript.

References
1. Taguchi S, Iwata T, Abe H, Doi Y. Poly(hydroxyalkanoate)s. In: Matyjaszewski K, Möller M, editors. Polymer science: a comprehensive reference. Amsterdam: Elsevier;2012. p. 157–82.
2. Keshavarz T, Roy I. Polyhydroxyalkanoates: bioplastics with a green agenda. Curr Opin Microbiol. 2010;13:321–6.
3. Pohlmann A, Fricke WF, Reinecke F, Kusian B, Liesegang H, Cramm R, Eitinger T, Ewering C, Potter M, Schwartz E, Strittmatter A, Voss I, Gottschalk G, Steinbüchel A, Friedrich B, Bowien B. Genome sequence of the bioplastic-producing "Knallgas" bacterium *Ralstonia eutropha* H16. Nat Biotechnol. 2006;24:1257–62.
4. Reinecke F, Steinbüchel A. *Ralstonia eutropha* strain H16 as model organism for PHA metabolism and for biotechnological production of technically interesting biopolymers. J Mol Microbiol Biotechnol. 2008;16:91–108.
5. Doi Y, Kitamura S, Abe H. Microbial synthesis and characterization of poly(3-hydroxybutyrate-*co*-3-hydroxyhexanoate). Macromolecules. 1995;28:4822–8.
6. Fukui T, Doi Y. Cloning and analysis of the poly (3-hydroxybutyrate-*co*-3-hydroxyhexanoate) biosynthesis genes of *Aeromonas caviae*. J Bacteriol. 1997;179:4821–30.
7. Fukui T, Shiomi N, Doi Y. Expression and characterization of (*R*)-specific enoyl coenzyme a hydratase involved in polyhydroxyalkanoate biosynthesis by *Aeromonas caviae*. J Bacteriol. 1998;180:667–73.
8. Tsuge T, Watanabe S, Shimada D, Abe H, Doi Y, Taguchi S. Combination of N149S and D171G mutations in *Aeromonas caviae* polyhydroxyalkanoate synthase and impact on polyhydroxyalkanoate biosynthesis. FEMS Microbiol Lett. 2007;277:217–22.
9. Mifune J, Nakamura S, Fukui T. Targeted engineering of *Cupriavidus necator* chromosome for biosynthesis of poly (3-hydroxybutyrate-*co*-3-hydroxyhexanoate) from vegetable oil. Can J Chem. 2008;86:621–7.
10. Mifune J, Nakamura S, Fukui T. Engineering of *pha* operon on *Cupriavidus necator* chromosome for efficient biosynthesis of poly(3-hydroxybutyrate-*co*-3-hydroxyhexanoate) from vegetable oil. Polym Degrad Stab. 2010;95:1305–12.
11. Budde CF, Riedel SL, Willis LB, Rha C, Sinskey AJ. Production of poly(3-hydroxybutyrate-*co*-3-hydroxyhexanoate) from plant oil by engineered *Ralstonia eutropha* strains. Appl Environ Microbiol. 2011;77:2847–54.
12. Kawashima Y, Cheng W, Mifune J, Orita I, Nakamura S, Fukui T. Characterization and functional analyses of *R*-specific enoyl coenzyme A hydratases in polyhydroxyalkanoate-producing *Ralstonia eutropha*. Appl Environ Microbiol. 2012;78:493–502.
13. Insomphun C, Mifune J, Orita I, Numata K, Nakamura S, Fukui T. Modification of β-oxidation pathway in *Ralstonia eutropha* for production of poly(3-hydroxybutyrate-*co*-3-hydroxyhexanoate) from soybean oil. J Biosci Bioeng. 2014;117:184–90.
14. Insomphun C, Xie H, Mifune J, Kawashima Y, Orita I, Nakamura S, Fukui T. Improved artificial pathway for biosynthesis of poly(3-hydroxybutyrate-*co*-3-hydroxyhexanoate) with high C_6-monomer composition from fructose in *Ralstonia eutropha*. Metab Eng. 2015;27:38–45.
15. Pötter M, Steinbüchel A. Poly(3-hydroxybutyrate) granule-associated proteins: impacts on poly(3-hydroxybutyrate) synthesis and degradation. Biomacromolecules. 2005;6:552–60.
16. Jendrossek D, Pfeiffer D. New insights in the formation of polyhydroxyalkanoate granules (carbonosomes) and novel functions of poly(3-hydroxybutyrate). Environ Microbiol. 2014;16:2357–73.
17. Wieczorek R, Pries A, Steinbüchel A, Mayer F. Analysis of a 24-kilodalton protein associated with the polyhydroxyalkanoic acid granules in *Alcaligenes eutrophus*. J Bacteriol. 1995;177:2425–35.
18. Wieczorek R, Steinbbchel A, Schmidt B. Occurrence of polyhydroxyalkanoic acid granule-associated proteins related to the *Alcaligenes eutrophus* H16 GA24 protein in other bacteria. FEMS Microbiol Lett. 1996;135:23–30.
19. Brigham CJ, Budde CF, Holder JW, Zeng Q, Mahan AE, Rha C, Sinskey AJ. Elucidation of β-oxidation pathways in *Ralstonia eutropha* H16 by examination of global gene expression. J Bacteriol. 2010;192:5454–64.
20. Shimizu R, Chou K, Orita I, Suzuki Y, Nakamura S, Fukui T. Detection of phase-dependent transcriptomic changes and Rubisco-mediated CO_2 fixation into poly (3-hydroxybutyrate) under heterotrophic condition in *Ralstonia eutropha* H16 based on RNA-seq and gene deletion analyses. BMC Microbiol. 2013;13:169–83.
21. Pötter M, Madkour MH, Mayer F, Steinbüchel A. Regulation of phasin expression and polyhydroxyalkanoate (PHA) granule formation in *Ralstonia eutropha* H16. Microbiology. 2002;148:2413–26.
22. Tian J, He A, Lawrence AG, Liu P, Watson N, Sinskey AJ, Stubbe J. Analysis of transient polyhydroxybutyrate production in *Wautersia eutropha* H16 by quantitative western analysis and transmission electron microscopy. J Bacteriol. 2005;187:3825–32.
23. York GM, Stubbe J, Sinskey AJ. New insight into the role of the PhaP phasin of *Ralstonia eutropha* in promoting synthesis of polyhydroxybutyrate. J Bacteriol. 2001;183:2394–7.
24. Pfeiffer D, Jendrossek D. Interaction between poly(3-hydroxybutyrate) granule-associated proteins as revealed by two-hybrid analysis and identification of a new phasin in *Ralstonia eutropha* H16. Microbiology. 2011;157:2795–807.
25. Cho M, Brigham CJ, Sinskey AJ, Stubbe J. Purification of polyhydroxybutyrate synthase from its native organism, *Ralstonia eutropha*: implications for the initiation and elongation of polymer formation in vivo. Biochemistry. 2012;51:2276–88.
26. Fukui T, Kichise T, Iwata T, Doi Y. Characterization of 13 kDa granule-associated protein in *Aeromonas caviae* and biosynthesis of polyhydroxyalkanoates with altered molar composition by recombinant bacteria. Biomacromolecules. 2001;2:148–53.
27. Pfeiffer D, Jendrossek D. PhaM is the physiological activator of poly(3-hydroxybutyrate) (PHB) synthase (PhaC1) in *Ralstonia eutropha*. Appl Environ Microbiol. 2014;80:555–63.
28. Ushimaru K, Motoda Y, Numata K, Tsuge T. Phasin proteins activate *Aeromonas caviae* polyhydroxyalkanoate (PHA) synthase but not *Ralstonia eutropha* PHA synthase. Appl Environ Microbiol. 2014;80:2867–73.
29. Pötter M, Müller H, Reinecke F, Wieczorek R, Fricke F, Bowien B, Friedrich B, Steinbüchel A. The complex structure of polyhydroxybutyrate (PHB) granules: four orthologous and paralogous phasins occur in *Ralstonia eutropha*. Microbiology. 2004;150:2301–11.
30. Pfeiffer D, Jendrossek D. Localization of poly(3-hydroxybutyrate) (PHB) granule-associated proteins during PHB granule formation and identification of two new phasins, PhaP6 and PhaP7, in *Ralstonia eutropha* H16. J Bacteriol. 2012;194:5909–21.
31. Sznajder A, Pfeiffer D, Jendrossek D. Comparative proteome analysis reveals four novel polyhydroxybutyrate (PHB) granule-associated proteins in *Ralstonia eutropha* H16. Appl Environ Microbiol. 2015;81:1847–58.
32. Barnard GC, Henderson GE, Srinivasan S, Gerngross TU. High level recombinant protein expression in *Ralstonia eutropha* using T7 RNA polymerase based amplification. Protein Expr Purif. 2004;38:264–71.
33. Kahar P, Tsuge T, Taguchi K, Doi Y. High yield production of polyhydroxyalkanoates from soybean oil by *Ralstonia eutropha* and its recombinant strain. Polym Degrad Stab. 2004;83:79–86.
34. Hiroe A, Tsuge K, Nomura CT, Itaya M, Tsuge T. Rearrangement of gene order in the *phaCAB* operon leads to effective production of

Kawashima *et al. Microb Cell Fact* (2015) 14:187

Page 12 of 12

ultrahigh-molecular-weight poly[(*R*)-3-hydroxybutyrate] in genetically engineered *Escherichia coli*. Appl Environ Microbiol. 2012;78:3177–84.

35. Sim SJ, Snell KD, Kim BW, Rha CK, Sinskey AJ. Increased poly-β-hydroxybutyrate (PHB) chain length by the modulation of PHA synthase activity in recombinant *Escherichia coli*. Biotechnol Lett. 2001;23:2057–61.

36. Kato M, Bao HJ, Kang C-K, Fukui T, Doi Y. Production of a novel copolyester of 3-hydroxybutyric acid and medium-chain-length 3-hydroxyalkanoic acids by *Pseudomonas* sp. 61-3 from sugars. Appl Microbiol Biotechnol. 1996;45:363–70.

37. Fukui T, Mukoyama M, Orita I, Nakamura S. Enhancement of glycerol utilization ability of *Ralstonia eutropha* H16 for production of polyhydroxyalkanoates. Appl Microbiol Biotechnol. 2014;98:7559–68.

38. Simon EJ, Shemin D. The preparation of *S*-succinyl coenzyme A. J Am Chem Soc. 1953;75:2520.

39. Fukui T, Chou K, Harada K, Orita I, Nakayama Y, Bamba T, Nakamura S, Fukusaki E. Metabolite profiles of polyhydroxyalkanoate-producing *Ralstonia eutropha* H16. Metabolomics. 2013;10:190–202.