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Alcoholytic Cleavage of Polyhydroxyalkanoate Chains by Class IV Synthases Induced by Endogenous and Exogenous Ethanol

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Polyhydroxyalkanoate (PHA)-producing *Bacillus* strains express class IV PHA synthase, which is composed of the subunits PhaR and PhaC. Recombinant *Escherichia coli* expressing PHA synthase from *Bacillus cereus* strain YB-4 ($PhaRC_{YB-4}$) showed an unusual reduction of the molecular weight of PHA produced during the stationary phase of growth. Nuclear magnetic resonance analysis of the low-molecular-weight PHA revealed that its carboxy end structure was capped by ethanol, suggesting that the molecular weight reduction was the result of alcoholytic cleavage of PHA chains by $PhaRC_{YB-4}$ induced by endogenous ethanol. This scission reaction was also induced by exogenous ethanol in both *in vivo* and *in vitro* assays. In addition, $PhaRC_{YB-4}$ was observed to have alcoholysis activity for PHA chains synthesized by other synthases. The PHA synthase from *Bacillus megaterium* ($PhaRC_{Bm}$) from another subgroup of class IV synthases was also assayed and was shown to have weak alcoholysis activity for PHA chains. These results suggest that class IV synthases may commonly share alcoholysis activity as an inherent feature.

Polyhydroxyalkanoates (PHAs) are a category of aliphatic polyesters synthesized by a wide variety of bacteria as an intracellular carbon and energy storage material in response to various environmental conditions. Recently, PHAs have attracted industrial attention because of their potential use as biodegradable and biocompatible thermoplastics (1). The biosynthesis of poly[(*R*)-3-hydroxybutyrate] [P(3HB)], the most commonly found natural PHA, has been well studied in *Ralstonia eutropha* and requires only three enzymes from acetyl coenzyme A (acetyl-CoA): 3-ketothiolase (PhaA), encoded by the *phaA* gene; acetoacetyl-CoA reductase (PhaB), encoded by the *phaB* gene; and PHA synthase (PhaC), encoded by the *phaC* gene (2). The coexpression of these three enzymes enables P(3HB)-negative bacteria like *Escherichia coli* to accumulate P(3HB) (3).

PHA synthases are grouped into four classes (classes I to IV) on the basis of subunit composition and substrate specificity (4). Class I synthases contain a single subunit, PhaC, and catalyze the polymerization of short-chain-length monomers (C_3 to C_5); the synthase from *R. eutropha* ($PhaC_{Re}$) is an example of this class. Class II synthases, as represented by the synthase from *Pseudomonas putida*, also contain a single PhaC subunit but catalyze the polymerization of medium-chain-length monomers (C_6 to C_{14}). Class III synthases, as represented by the synthase from *Allochromatium vinosum*, contain two heterologous subunits, PhaE and PhaC, and catalyze the polymerization of short-chain-length monomers. Class IV synthases, a recently identified class of synthases, contain two heterologous subunits, PhaR and PhaC, and catalyze the polymerization of short-chain-length monomers (C_3 to C_5). On the basis of phylogenetic analysis (see Fig. S1 in the supplemental material), the PhaC subunits in class IV synthases can be further classified into two subgroups: the *Bacillus megaterium* subgroup and the *Bacillus cereus* subgroup (5–7). These PhaC subunits recognize PhaR subunits from different subgroups (7).

P(3HB) produced by *E. coli* expressing class I to IV synthases exhibits a variety of molecular weights, depending on the characteristics of the synthase expressed (8). It is noteworthy that the synthase from *Delftia acidovorans* ($PhaC_{Da}$), a class I synthase, is capable of synthesizing high-molecular-weight P(3HB) in *E. coli* (9).

In previous studies (7, 10), it was shown that *B. cereus* strain YB-4 isolated from soil expresses a class IV PHA synthase consisting of two heterologous subunits, $PhaR_{YB-4}$ (18.5 kDa) and $PhaC_{YB-4}$ (41.7 kDa). Recombinant *E. coli* expressing $PhaRC_{YB-4}$ showed an unusual decrease in the molecular weight of P(3HB) synthesized during cultivation, especially in the stationary phase of growth. In addition, kinetic analysis indicated that the decrease in molecular weight is the result of random scission of the polymer chain (7). The same phenomenon was observed with *E. coli* expressing the class IV synthase from *Bacillus* sp. strain INT005 ($PhaRC_{Bsp}$) (11). On the other hand, *E. coli* expressing PHA synthase from *B. megaterium* ($PhaRC_{Bm}$), another class IV synthase, did not produce P(3HB) with such a low molecular weight (7). A subunit recombination study of PhaR and PhaC from *B. cereus* YB-4 and *B. megaterium* revealed that the $PhaC_{YB-4}$ subunit is responsible for the scission activity. However, unlike *E. coli*, the PHA-negative mutant *R. eutropha* $PHB^{-}4$ produced high-molecular-weight P(3HB) even when $PhaRC_{YB-4}$ was expressed (12). From these observations, we hypothesized that there might be regulatory mechanisms governing the P(3HB) scission activity of $PhaC_{YB-4}$.

The aim of this study was to investigate the mechanism governing the P(3HB) scission activity of $PhaRC_{YB-4}$. To this end, an *in vivo* scission assay was carried out, using *E. coli* JM109 as the

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TABLE 1 Bacterial strains and plasmids used in the study

Strain or plasmid	Relevant characteristics	Reference or source
Strains		
E. coli JM109	*recA1 endA1 gyrA96 thi-1 hsdR17*(r_K^- m_K^+) *e14*$^-$ (*mcrA*) *supE44 relA1* Δ(*lac-proAB*)/F′ [*traD36 proAB*$^+$ *lacI*^{q} *lacZ*ΔM15]	TaKaRa Bio
E. coli BL21(DE3)	F$^-$ *ompT hsdS*$_B$(r_B^- m_B^-) *gal dcm*	Novagen
Plasmids		
pBBR1MCS-2	Broad-host-range vector, Km^r	13
pBBR1-*phaRC*$_{YB-4}$*AB*	pBBR1MCS-2 derivative; *pha*$_{Re}$ promoter, *phaRC*$_{YB-4}$ from *B. cereus* YB-4, and *phaAB*$_{Re}$ from *R. eutropha*	12
pJRD215	Broad-host-range vector, Km^r	14
pJRD-*phaC*$_{Da}$*AB*	pJRD215 derivative; *pha*$_{Re}$ promoter, *phaC*$_{Da}$ from *D. acidovorans* DS-17, and *phaAB*$_{Re}$ from *R. eutropha* H16	This study
pGEM″ABex	pGEM-T derivative, *pha*$_{Re}$ promoter, *phaAB*$_{Re}$ from *R. eutropha* H16, Ap^r	15
pGEM-*phaC*$_{Da}$*AB*	pGEM″ABex derivative; *phaC*$_{Da}$ from *D. acidovorans* DS-17 (identical to pGEM″ABexCaC)	16
pGEM-*phaC*$_{Re}$*AB*	pGEM″ABex derivative; *phaC*$_{Re}$ from *R. etutropha* H16	17
pGEM- *phaRC*$_{YB-4}$*AB*	pGEM″ABex derivative; *phaRC*$_{YB-4}$ from *B. cereus* YB-4	7
pGEM-*phaRC*$_{Bm}$*AB*	pGEM″ABex derivative; *phaRC*$_{Bm}$ from *B. megaterium* NBRC15308^T	7
pGEM-*phaR*$_{YB-4}$*AB*	pGEM″ABex derivative; *phaR*$_{YB-4}$ from *B. cereus* YB-4	7
pGEM-*phaC*$_{YB-4}$*AB*	pGEM″ABex derivative; *phaC*$_{YB-4}$ from *B. cereus* YB-4	7
pGEM-*phaRC*$_{YB-4}$	pGEM-T derivative; *pha*$_{Re}$ promoter, *phaRC*$_{YB-4}$ from *B. cereus* YB-4	This study
pET15b	T7 promoter, His tag fusion protein	Novagen
pET15b-*phaR*$_{YB-4}$	pET15b derivative; *phaR*$_{YB-4}$ from *B. cereus* YB-4	This study
pET15b-*phaC*$_{YB-4}$	pET15b derivative; *phaC*$_{YB-4}$ from *B. cereus* YB-4	This study

host strain, by expressing PhaRC$_{YB-4}$ from high- or low-copy-number plasmids. The results indicate that PhaRC$_{YB-4}$ has alcoholytic cleavage activity for P(3HB) chains in the presence of both endogenous and exogenous ethanol. Moreover, the results from coexpression of PhaRC$_{YB-4}$ with PhaC$_{Da}$ [PhaC$_{Da}$ produces a high-molecular-weight P(3HB) used as the scissile substrate in this experiment] indicate that PhaRC$_{YB-4}$ is also able to cleave P(3HB) polymerized by another synthase. In addition, the alcoholytic cleavage activity of PhaRC$_{YB-4}$ was assayed *in vitro*. This is the first study to document alcoholysis activity in PHA synthases.

MATERIALS AND METHODS

Bacterial strains, plasmids, and culture media. The bacterial strains and plasmids used in this study are listed in Table 1. *E. coli* JM109 was used as a host strain for P(3HB) biosynthesis throughout the study. *E. coli* BL21(DE3) was used to produce His-tagged PhaR$_{YB-4}$ and PhaC$_{YB-4}$ from the expression plasmids pET15b-*phaR*$_{YB-4}$ and pET15b-*phaC*$_{YB-4}$, respectively. For preculturing, the recombinant bacteria were grown in lysogeny broth (LB) medium (10 g/liter tryptone, 5 g/liter yeast extract, 10 g/liter NaCl). For maintenance of the plasmid within the cell, ampicillin (100 mg/liter) and/or kanamycin (50 mg/liter) was added to the medium as appropriate.

Plasmid construction. To express PhaC$_{Da}$, the plasmid pJRD-*phaC*$_{Da}$*AB* was constructed as follows. Plasmid pGEM-*phaC*$_{Da}$*AB* (16) was digested with BamHI, and a DNA fragment containing the *pha*$_{Re}$ promoter, the *phaC*$_{Da}$ gene, the *phaAB*$_{Re}$ genes, and the *rrnB* T1T2 terminator was introduced into the same site in pJRD215 (14).

To remove the *phaAB*$_{Re}$ genes from pGEM-*phaRC*$_{YB-4}$*AB*, the plasmid was digested with SalI and NruI and self-ligated using a BKL kit (TaKaRa Bio Inc., Otsu, Japan) to yield pGEM-*phaRC*$_{YB-4}$.

For construction of pET15b-*phaR*$_{YB-4}$, the *phaR*$_{YB-4}$ gene was amplified by PCR using genomic DNA as the template. The following PCR primers were used: forward primer 5′-AGG TCA TAT GAT TGA TCA AAA ATT CGA TCC-3′ and reverse primer 5′-CAA GCG GGA TCC TCA CTT TTT ATT TTC TGG-3′ (underlined sequences indicate the NdeI and BamHI sites, respectively). The PCR product was digested with NdeI and BamHI and inserted into the same site in pET15b. For construction of pET15b-*phaC*$_{YB-4}$, the *phaC*$_{YB-4}$ gene was amplified by PCR using genomic DNA as the template. The following PCR primers were used: forward primer 5′-TAG AAA GGA TCC TAC ATT CGC AAC AGA ATG-3′ and reverse primer 5′-TTG GAT CCT TTA TTT TTA ATT AGA ACG CTC-3′ (underlined sequences indicate the BamHI sites). The PCR product was digested with BamHI and inserted into the same site in pET15b under T7 promoter control.

P(3HB) synthesis and isolation. Recombinant *E. coli* JM109 was cultivated in 500-ml shake flasks containing 100 ml LB medium with glucose (20 g/liter) on a reciprocal shaker (130 rpm) at 37°C for 12 to 72 h. After cultivation, cells were harvested, washed once with deionized water, and lyophilized. The polymers that had accumulated in the cells were extracted with chloroform for 72 h at room temperature and then purified with methanol. For ethanol supplementation, ethanol (Kanto Chemical, Tokyo, Japan) or [1-^{13}C]ethanol (SI Science, Saitama, Japan) was used.

GC and GPC. The P(3HB) content of the dried cells was determined by gas chromatography (GC). Samples for GC analysis were prepared by methanolysis using 15% (vol/vol) sulfuric acid (18). The number average molecular weight (M_n) and the weight average molecular weight (M_w) of P(3HB) synthesized by recombinant strains were determined by gel permeation chromatography (GPC). GPC measurements were performed at 40°C, using a Shimadzu 10A GPC system equipped with two Shodex K-806 M joint columns. Chloroform was used as the eluent at a flow rate of 0.8 ml/min. Samples for GPC analysis were prepared at a concentration of 1.0 mg/ml and passed through a 0.45-μm-pore-size filter. Molecular weights were determined using a standard curve calibrated with low-polydispersity polystyrenes.

NMR analysis. The end-group structures of isolated P(3HB)s were analyzed by NMR spectroscopy. Each polymer (30 mg) was dissolved in $CDCl_3$ (0.7 ml) and subjected to ^{1}H and ^{13}C nuclear magnetic resonance (NMR) analysis. NMR spectra were recorded using a JEOL LA500 spectrometer. For ^{1}H NMR analysis, data were collected with a 7.2-ms pulse width (90° pulse angle), a 5-s pulse repetition, a 6,000-Hz spectrum width, and 16,000 data points and at 45°C. For ^{13}C NMR analysis, data were collected with a 6.1-ms pulse width (90° pulse angle), a 5-s pulse repetition, a 27,000-Hz spectrum width, and 33,000 data points and at 45°C. Tetramethylsilane (Me_4Si) was used as an internal chemical shift standard.

PHA synthase activity assay. Cells were harvested, washed with deionized water, and stored at −80°C until the activity assay; it has been reported that cells can be stored under this condition without any detectable loss of activity (19). Cells were then resuspended in 50 mM potassium phosphate (KP_i; pH 7.0), disrupted by sonication for 3 min, and centrifuged at 1,500 × *g* for 5 min at 4°C to obtain cell lysates containing PHA synthase bound to PHA granules. Protein concentrations of crude extracts were determined using a Quant-iT protein assay kit (Invitrogen, San Diego, CA).

Synthase activity in crude extracts was determined using the 5,5′-dithiobis-(2-nitrobenzoic acid) (DTNB) method (20). An assay mixture containing 50 mM KP_i (pH 7.0) and 0.6 mM (*R*)-3-hydroxybutyryl-CoA [(*R*)-3HB-CoA] was preincubated at 30°C for 2 min, and then the reaction was started by the addition of soluble extract. The mixture volume was 800 μl in total. After a 1-min incubation at 30°C, 100 μl of the reaction mixture was mixed with 100 μl of trichloroacetic acid (5%, wt/vol) to stop the reaction. This mixture was then centrifuged at 12,000 × *g* for 10 min at 4°C, and 150 μl of its supernatant was mixed with 750 μl of 1 mM DTNB in 500 mM KP_i (pH 7.2). After incubation for 2 min at room temperature, the absorbance at 412 nm was measured. One unit was defined as the activity for production of 1 μmol of 2-nitrobenzoic acid (TNB) anion (corresponding to released CoA having a free thiol group) per min ($\varepsilon_{412} = 14.5 \times 10^3\ M^{-1} \cdot cm^{-1}$). The (*R*)-3HB-CoA used here was chemically synthesized as described previously (20).

Ethanol assay. The ethanol concentration in the culture liquid was measured by the enzymatic method using an F-kit (Roche Diagnostics, Basel, Switzerland).

Western blot analysis. For Western blot analysis of the $PhaC_{YB\text{-}4}$ subunit, bacterial cells were diluted to an optical density at 600 nm of 1.0 and subjected to sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) by the standard procedure. Separated proteins were transferred to a polyvinylidene difluoride membrane using a Bio-Rad Trans-Blot SD semidry transfer cell. Detection of $PhaC_{YB\text{-}4}$ was carried out with a rabbit antiserum specific for a C-terminal oligopeptide (N-ALLDHISS TDKQYVC-C) in $PhaC_{YB\text{-}4}$. Protein bands were visualized using goat anti-rabbit IgG conjugated to horseradish peroxidase (Toyobo, Osaka, Japan) as a secondary antibody.

Enzyme purification. His-tagged $PhaR_{YB\text{-}4}$ and $PhaC_{YB\text{-}4}$ were individually produced by *E. coli* BL21(DE3) harboring pET15b-*phaR*$_{YB\text{-}4}$ and pET15b-*phaC*$_{YB\text{-}4}$, respectively, as follows. Cells were cultivated at 30°C for 2.5 h in LB medium, and then isopropyl β-D-1-thiogalactopyranoside (IPTG; Wako Pure Chemical Industries, Osaka, Japan) was added to a final concentration of 0.1 mM. After 3 to 5 h of cultivation at 20°C, cells were harvested and stored at −80°C prior to use.

His-tagged proteins were purified using an AKTA system equipped with a HisTrap HP column (GE Healthcare, Little Chalfont, United Kingdom), as described previously (21). Aliquots of purified enzymes were frozen in liquid nitrogen and stored at −80°C. Enzyme concentrations were determined using a Quant-iT protein assay kit (Invitrogen, Carlsbad, CA). Protein purity was confirmed by SDS-PAGE by the standard procedure.

P(3HB) formation *in vitro*. *In vitro* P(3HB) polymerization was performed in a total volume of 12 ml with 100 mM KP_i (pH 7.0) containing 5 mM (*R*)-3HB-CoA and 0.27 U/ml of $PhaRC_{YB\text{-}4}$ (36 nmol) for 3 min at 30°C. The reaction mixture was divided into three equal samples: A, B, and C. These were centrifuged at 15,000 × *g* for 5 min at 4°C to obtain a precipitate containing P(3HB) and synthase. The precipitate from sample A was dried and purified using chloroform and methanol without further incubation. The precipitates from samples B and C were resuspended in 4 ml of 100 mM KP_i (pH 7.0) without and with ethanol (2.6 g/liter), respectively, and incubated at 30°C for 24 h. After that, P(3HB) was collected by centrifugation again, dried, and purified using chloroform and methanol. The molecular weight of the collected P(3HB) was determined by GPC as described above. The conversion ratio of (*R*)-3HB-CoA was calculated by determining the (*R*)-3HB-CoA concentration in the supernatant by high-pressure liquid chromatography (22).

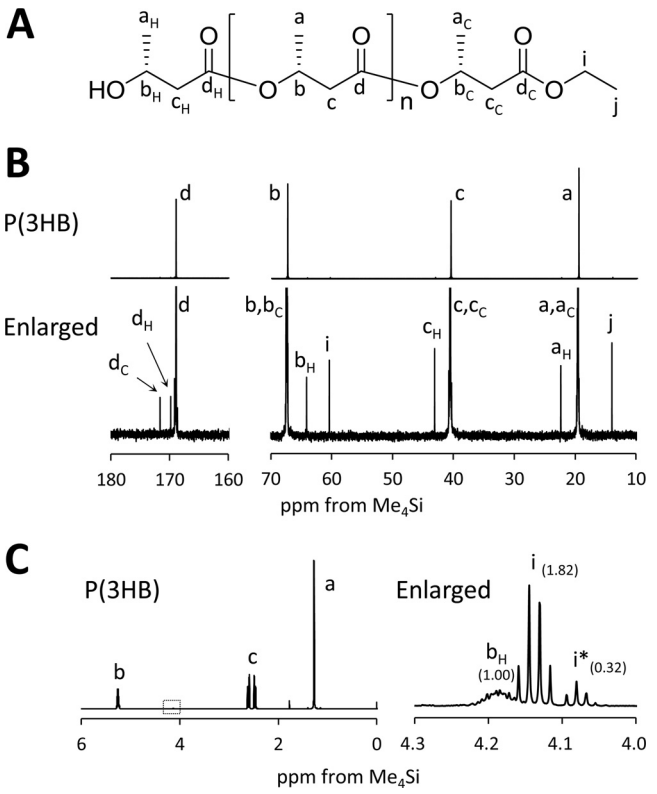

FIG 1 (A) Chemical structure of ethanol-capped P(3HB). (B) Whole and enlarged 125-MHz ^{13}C NMR spectra of low-molecular-weight P(3HB) synthesized by *E. coli* JM109 harboring pGEM-*phaRC*$_{YB\text{-}4}$*AB* ($M_n = 20 \times 10^3$; $M_w/M_n = 2.1$) cultured for 72 h at 37°C. (C) Whole and enlarged 500-MHz ^1H NMR spectra of the low-molecular-weight P(3HB). i*, the methylene resonance for alcohols longer than ethanol. The values in parentheses show the intensity ratio of each peak.

RESULTS

End-group structure analysis of low-molecular-weight P(3HB). As reported previously (7), recombinant *E. coli* JM109 expressing $PhaRC_{YB\text{-}4}$ showed an unusual reduction in the molecular weight of P(3HB) synthesized during the stationary phase of growth. A kinetic study suggested that the decrease in molecular weight was the result of random scission of the P(3HB) chain (7). In this study, to gain deeper insight into this mechanism, the end-group structures of low-molecular-weight P(3HB) were characterized by NMR spectroscopy. If the molecular weight of P(3HB) was reduced by hydrolysis, it would be expected that a free carboxy group would be detected as the end structure of the low-molecular-weight P(3HB). Low-molecular-weight P(3HB) ($M_n = 20 \times 10^3$, polydispersity index = 2.1) extracted from *E. coli* JM109(pGEM-*phaRC*$_{YB\text{-}4}$*AB*) cultured in 500-ml shake flasks containing 100 ml LB medium plus glucose (20 g/liter) at 37°C for 72 h was analyzed by ^{13}C NMR. Signals derived from the terminal structure were detected as shown in Fig. 1B. Using authentic ethyl 3-hydroxybutyrate (ethyl 3HB) as a reference compound, the results suggest that the carboxy end was capped by ethanol. The ^1H NMR analysis also suggested the existence of an ethanol-capped end (signal i) together with a minor end capped by a longer alco-

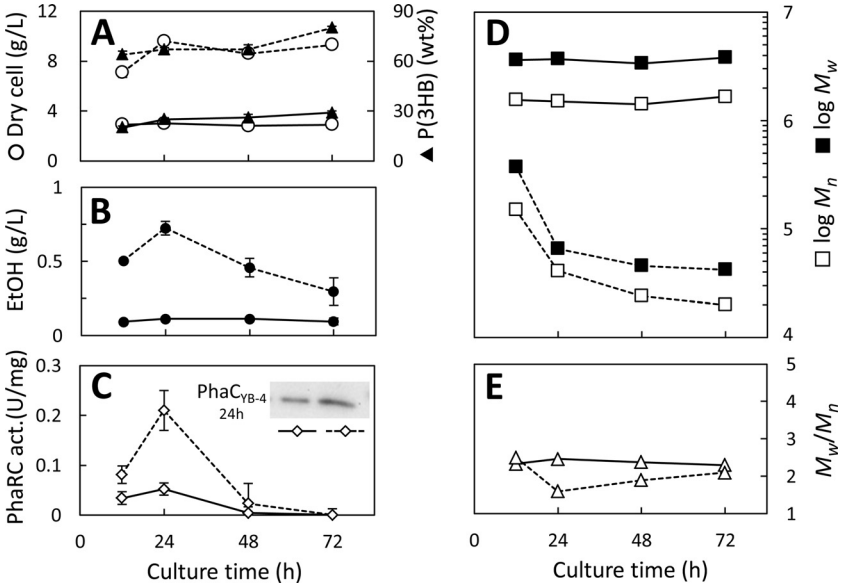

FIG 2 P(3HB) synthesis by *E. coli* JM109 harboring the high-copy-number plasmid pGEM-*phaRC*$_{YB-4}$*AB* (dashed line) and the low-copy-number plasmid pBBR1-*phaRC*$_{YB-4}$*AB* (solid line). (A) Cell dry weight (open circles) and P(3HB) content (filled triangles); (B) ethanol (EtOH) concentration in the broth; (C) synthase activity (act.) and Western blot analysis of PhaC$_{YB-4}$ after 24 h of cultivation; (D) M_n and M_w; (E) M_w/M_n. Cells were cultivated in 500-ml shake flasks containing 100 ml LB medium plus glucose (20 g/liter) at 37°C. Each experiment was carried out in triplicate.

hol (signal i*), as shown in Fig. 1C. The molar ratio of the alcohol-capped end to all ends estimated from the peak intensities of signals i plus i* and signal b_H, respectively, was almost 1:1. If the molecular weight had been reduced by hydrolysis, signals i and i* would be undetectable or relatively smaller than signal b_H. Therefore, these results strongly suggest that the molecular weight was reduced not by hydrolytic cleavage but by alcoholytic cleavage. Because *E. coli* likely produces ethanol under anaerobic and microaerophilic culture conditions (23), host-produced ethanol might have a considerable influence on the degree of alcoholytic cleavage of the P(3HB) chain.

Monitoring of ethanol concentration during cultivation. To understand the relationship between host-produced ethanol and P(3HB) molecular weight, the ethanol concentration in the culture liquid was monitored during the cultivation of strains harboring the high-copy-number plasmid pGEM-*phaRC*$_{YB-4}$*AB* and the low-copy-number plasmid pBBR1-*phaRC*$_{YB-4}$*AB*, which are produced at from 300 to 400 copies per cell (24) and approximately 30 to 40 copies per cell (25), respectively. Each recombinant strain was cultivated in LB medium containing glucose (20 g/liter) at 37°C. The culture results are shown in Fig. 2.

In the case of the strain harboring pGEM-*phaRC*$_{YB-4}$*AB* (high copy number), ethanol could be detected in the culture liquid and reached a maximum concentration of 0.72 g/liter after 24 h. This culture eventually grew to over 9 g/liter, with P(3HB) being present at 80% by weight (80 wt%) of its cell dry weight. The molecular weight of the P(3HB) synthesized by this strain was relatively high after 12 h of cultivation, and the M_n was equal to 150×10^3. However, the M_n of P(3HB) produced after 72 h of cultivation decreased to 24×10^3, which is consistent with the observation from our previous study (7).

In contrast, the strain harboring pBBR1-*phaRC*$_{YB-4}$*AB* (low copy number) produced less ethanol (0.11 g/liter after 24 h) than the strain harboring pGEM-*phaRC*$_{YB-4}$*AB*. The strain harboring pBBR1-*phaRC*$_{YB-4}$*AB* grew to nearly 3 g/liter of cell dry weight by 12 h of cultivation but did not grow more after that. The P(3HB) accumulation in this strain at 72 h was 29 wt%, which is significantly lower than that from the strain harboring pGEM-*phaRC*$_{YB-4}$*AB* (80 wt%). Notably, the M_ns at 12 h and 72 h were equally high, at $1{,}600 \times 10^3$ and $1{,}700 \times 10^3$, respectively. The different behavior of this strain with respect to the molecular weight change might be attributed to its level of ethanol production.

The PHA synthase activity in these strains was assayed using cell extracts containing synthases bound to P(3HB) granules. The results are shown in Fig. 2C. Similar patterns of change in synthase activity could be observed for these two strains, but their maximum values were quite different. For each strain, the highest level of synthase activity, 0.21 ± 0.04 U/mg protein and 0.052 ± 0.015 U/mg protein for pGEM-*phaRC*$_{YB-4}$*AB* and pBBR1-*phaRC*$_{YB-4}$*AB*, respectively, was attained after 24 h of cultivation. Western blot analysis revealed that synthase (PhaC$_{YB-4}$ subunit) concentrations in cells cultured for 24 h in the two strains were also different, corresponding to their respective levels of activity (Fig. 2C, inset). The results suggest that the plasmid copy number affected synthase expression, thereby affecting P(3HB) and ethanol production and the molecular weight of P(3HB).

Ethanol supplementation to induce alcoholytic cleavage. The strain expressing the low-copy-number plasmid pBBR1-*phaRC*$_{YB-4}$*AB* did not produce either low-molecular-weight P(3HB) or significant amounts of ethanol (Fig. 2B and D). Using this transformant, we investigated whether alcoholytic cleavage of P(3HB) could be induced by adding ethanol to the culture medium. To avoid reducing the P(3HB) molecular weight during its biosynthesis via the chain transfer (CT) reaction, which terminates PHA chain elongation by transferring PHA chains from PhaC to a hydroxy compound (26), two-stage cultivation (Fig. 3A) was carried out as follows. For the first stage, cells were cultured in LB medium containing glucose (20 g/liter) for 24 h, to

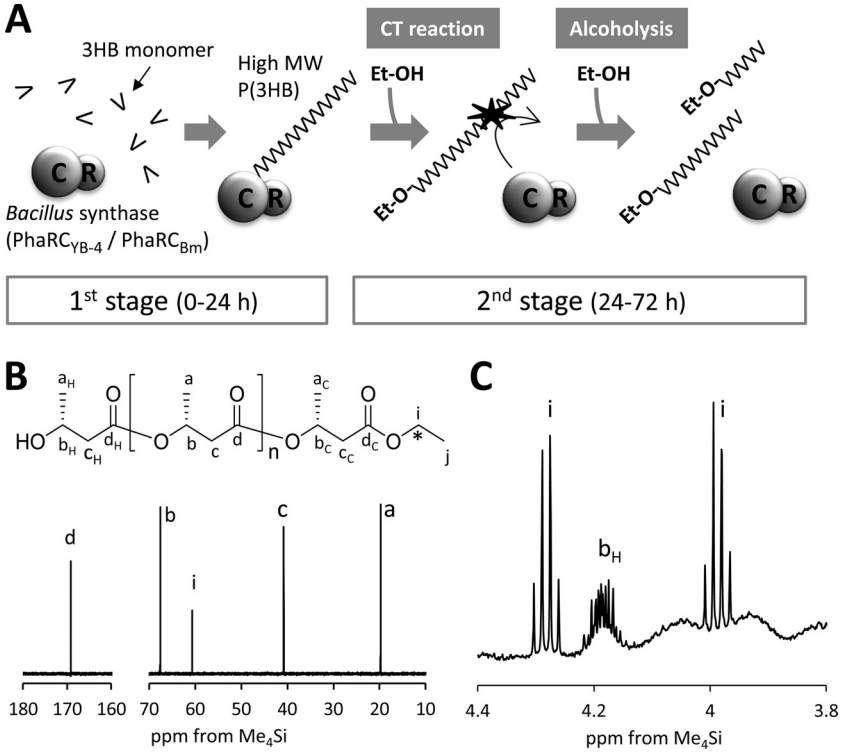

FIG 3 (A) Schematic of the two-stage culture of *E. coli* JM109 harboring pBBR1-*phaRC*$_{YB-4}$*AB* or pGEM-*phaRC*$_{Bm}$*AB*. In the first stage, cells were cultured in LB medium containing glucose (20 g/liter) at 37°C for 24 h for the production of high-molecular-weight (High MW) P(3HB). In the second stage, the cells were transferred into glucose-free M9 medium with or without ethanol (Et-OH) supplementation (4 g/liter) and incubated at 37°C for a further 48 h. Each experiment was carried out in triplicate. (B, C) NMR spectra of P(3HB) synthesized by *E. coli* JM109 harboring pBBR1-*phaRC*$_{YB-4}$*AB* with [1-^{13}C]ethanol supplementation (4 g/liter) during the second stage of culture. Asterisk in the chemical structure, ^{13}C-labeled carbon. (B) The 125-MHz ^{13}C NMR spectrum. (C) Enlarged 3.8- to 4.4-ppm region of the 500-MHz ^{1}H NMR spectrum.

allow the accumulation of high-molecular-weight P(3HB). For the second stage, the cells were transferred into glucose-free M9 medium with or without ethanol supplementation (4 g/liter) and incubated for a further 48 h. The results of the two-stage cultivation procedure are shown in Table 2. The M_n of the culture without ethanol supplementation was 1,140 × 10^3, which is almost unchanged from that during the second stage, although the M_n with ethanol supplementation was as low as 42 × 10^3. The number of P(3HB) chains increased 36.1-fold in the second stage by the presence of ethanol. From these results, it is obvious that ethanol works as a trigger for a dramatic reduction of the P(3HB) M_n.

The same experiment was conducted using 4 g/liter [1-^{13}C]-ethanol, resulting in the production of low-molecular-weight P(3HB). The ^{13}C NMR spectrum of the P(3HB) synthesized in this experiment is shown in Fig. 3B. In this figure, the signal i at 60.6 ppm arising from the end-capped ethanol was enhanced (relative to signals a, b, c, and d arising from main-chain 3HB unit) compared to that seen in Fig. 1B, due to ^{13}C enrichment. Additionally,

TABLE 2 Effect of ethanol on P(3HB) molecular weight produced by *E. coli* JM109 expressing PhaRC$_{YB-4}$ (pBBR1-*phaRC*$_{YB-4}$*AB*) and PhaRC$_{Bm}$ (pGEM-*phaRC*$_{Bm}$*AB*) in two-stage culture[a]

Expressed synthase[b]	Culture conditions[c]		Time of harvest (h)	Cell dry wt (g/liter)	P(3HB) content (wt%)	Mol wt		Relative chain no.
	First stage (0–24 h)	Second stage (24–72 h)				M_n (10^3)	M_w/M_n	
PhaRC$_{YB-4}$	LB + glucose		24	3.3 ± 0.0	27 ± 0	1,250 ± 106	1.6	1.0
	LB + glucose	M9	72	2.7 ± 0.0	31 ± 2	1,140 ± 61	2.8	1.0
	LB + glucose	M9 + ethanol	72	3.0 ± 0.0	36 ± 4	42 ± 2	11.1	36.1
PhaRC$_{Bm}$	LB + glucose		24	4.2 ± 0.0	23 ± 1	1,050 ± 68	1.9	1.0
	LB + glucose	M9	72	3.0 ± 0.0	33 ± 1	1,060 ± 72	2.0	1.0
	LB + glucose	M9 + ethanol	72	3.0 ± 0.0	35 ± 3	270 ± 28	4.8	4.2

[a] Results are means ± standard deviations from three separate experiments.
[b] pBBR1-*phaRC*$_{YB-4}$*AB* and pGEM-*phaRC*$_{Bm}$*AB* are low- and high-copy-number plasmids, respectively.
[c] Cells were cultured in LB medium containing glucose (20 g/liter) for 24 h at 37°C and then incubated in glucose-free M9 medium with or without supplementation of ethanol (4 g/liter) for a further 48 h at 37°C.

TABLE 3 P(3HB) production by various recombinant *E. coli* JM109 strains in shake flasks[a]

Expressed synthase(s)	Cell dry wt (g/liter)	P(3HB) content (wt%)	Ethanol concn (g/liter) at:		Mol wt	
			24 h	72 h	M_n (10^3)	M_w/M_n
$PhaC_{Da}$	9.4 ± 0.1	71 ± 2	0.30 ± 0.06	0.09 ± 0.01	1,440 ± 15	1.9
$PhaC_{Da}$, $PhaR_{YB-4}$	8.7 ± 0.1	67 ± 5	0.47 ± 0.03	0.28 ± 0.00	1,180 ± 70	2.0
$PhaC_{Da}$, $PhaC_{YB-4}$	9.3 ± 0.1	67 ± 2	0.41 ± 0.04	0.19 ± 0.01	1,200 ± 72	1.8
$PhaC_{Da}$, $PhaRC_{YB-4}$	8.6 ± 0.1	73 ± 5	0.50 ± 0.03	0.30 ± 0.01	14 ± 1	2.1
$PhaRC_{YB-4}$	9.3 ± 0.1	80 ± 1	0.72 ± 0.05	0.24 ± 0.01	24 ± 1	1.6

[a] Cells were cultivated in LB medium containing glucose (20 g/liter) at 37°C for 72 h. To express $PhaR_{YB-4}$ and/or $PhaC_{YB-4}$, derivatives of pGEM (a high-copy-number plasmid) were used. For expression of $PhaC_{Da}$, pJRD-*phaC*$_{Da}$*AB* (carrying *phaC*$_{Da}$, *phaA*$_{Re}$, and *phaB*$_{Re}$) was employed. Results are the averages ± standard deviations from three separate experiments.

in the ^{1}H NMR spectrum, split methylene resonance arising from the coupling of ^{1}H atoms to an adjoining ^{13}C atom at the ethyl ester end was observed at 4.0 ppm and 4.3 ppm (Fig. 3C). These results strongly suggest that exogenous ethanol was incorporated into the carboxy end of P(3HB). On the basis of these results, it can be concluded that the reduction in P(3HB) molecular weight is caused by alcoholytic cleavage of the P(3HB) chain.

In addition, the same ethanol supplementation experiment was conducted for cultures of *E. coli* expressing $PhaRC_{Bm}$. The result from this experiment is also shown in Table 2. Unexpectedly, we found that expression of $PhaRC_{Bm}$ leads to a reduction in P(3HB) molecular weight and an increase in P(3HB) chain number, although this result had not been seen in our previous study (7). This result suggests that $PhaRC_{Bm}$ expresses weak cleavage activity with P(3HB) chains if ethanol is present.

***In vivo* scission assay using $PhaRC_{YB-4}$ and $PhaC_{Da}$.** To investigate whether $PhaRC_{YB-4}$ or its subunit could affect P(3HB) chains that had been polymerized by another PHA synthase *in vivo*, we coexpressed the synthase from *B. cereus* YB-4 ($PhaR_{YB-4}$ and/or $PhaC_{YB-4}$) and the synthase from *Delftia acidovorans* ($PhaC_{Da}$) in *E. coli* JM109 (Fig. 4A). $PhaC_{Da}$ can synthesize high-molecular-weight P(3HB) ($M_n > 1{,}000 \times 10^3$) (8, 9); thus, changes in the high-molecular-weight fraction of P(3HB) were monitored by GPC as an indicator for the scission reaction.

Table 3 shows the results from the coexpression cultures. These cultures showed similar cell growth and P(3HB) accumulation, at 8.6 to 9.4 g/liter and 67 to 73%, respectively. In addition, these cultures produced approximately 0.2 g/liter ethanol by 72 h of cultivation. However, their behavior with respect to the P(3HB) molecular weight was quite different. By 24 h, the culture expressing $PhaC_{Da}$ alone produced P(3HB) with an M_n as high as $1{,}440 \times 10^3$, even though 0.3 g/liter ethanol was produced. Moreover, cultures coexpressing $PhaC_{Da}$ and $PhaR_{YB-4}$ or $PhaC_{YB-4}$ produced high-molecular-weight P(3HB), with M_ns of $1{,}180 \times 10^3$ and $1{,}200 \times 10^3$, respectively. On the other hand, the culture coexpressing $PhaC_{Da}$ and $PhaRC_{YB-4}$ produced low-molecular-weight P(3HB), with an M_n of 14×10^3. This value is much lower than that produced by the culture expressing $PhaC_{Da}$ alone. As shown in Fig. 4B, the molecular weight of P(3HB) produced by the strain coexpressing $PhaC_{Da}$ and $PhaRC_{YB-4}$ continued to decrease even after 24 h of cultivation, by which point P(3HB) synthesis would have been finished.

Figure 5A shows the molecular weight distribution of P(3HB) produced after 12 h of cultivation by the strain expressing $PhaC_{Da}$. The M_n of this sample was 939×10^3, and the distribution of the molecular weight was unimodal. Figure 5B shows the molecular weight distribution of P(3HB) produced by a strain coexpressing $PhaC_{Da}$ and $PhaRC_{YB-4}$ and demonstrates that there were remarkable changes in the molecular weight over the course of the cultivation period. The peak top of the curve shifted to the low-molecular-weight side, and the M_n decreased from 150×10^3 at 12 h to 14×10^3 at 72 h. In addition, there was a small shoulder in the high-molecular-weight side of the curve for the polymer at 12 h, which corresponds to the molecular weight of $PhaC_{Da}$-synthesized P(3HB). This shoulder was diminished with increasing culture time, and the whole peak was shifted to the low-molecular-weight side. These observations suggest that the high-molecular-weight P(3HB) synthesized by $PhaC_{Da}$ can be broken down by $PhaRC_{YB-4}$.

Induction of alcoholysis activity of $PhaRC_{YB-4}$ *in vitro*. To demonstrate the alcoholysis activity of $PhaRC_{YB-4}$ directly, an *in vitro* assay using purified $PhaRC_{YB-4}$ and chemically synthesized (*R*)-3HB-CoA was performed. *In vitro* P(3HB) polymerization was allowed to progress for 3 min at 30°C. Then, the reaction mixture was divided into three samples, A, B, and C, by volume. These samples were then centrifuged to collect the precipitate containing P(3HB) and $PhaRC_{YB-4}$. The precipitate from sample A was dried without further incubation. The precipitates from

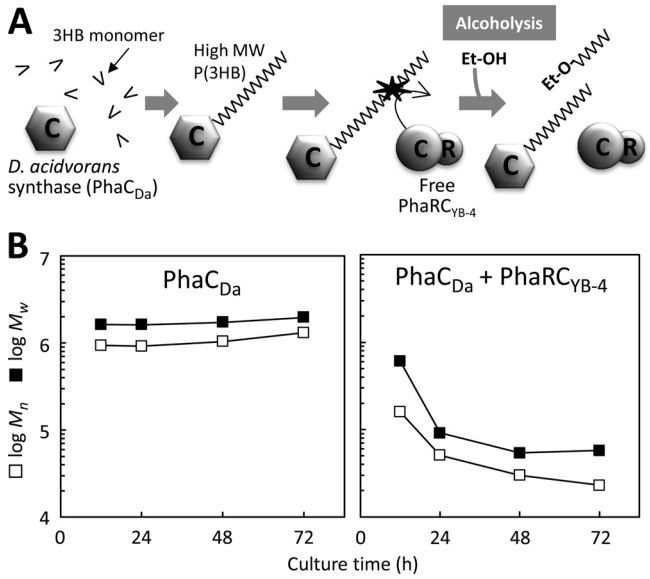

FIG 4 (A) Schematic of the *in vivo* scission assay using *E. coli* JM109 as the host strain expressing $PhaRC_{YB-4}$ (pGEM-*phaRC*$_{YB-4}$) together with $PhaC_{Da}$ from pJRD-*phaC*$_{Da}$*AB*, which provides the high-molecular-weight P(3HB) that serves as the scissile substrate in the cell. (B) Molecular weight changes of P(3HB) during cultivation in the *in vivo* assay.

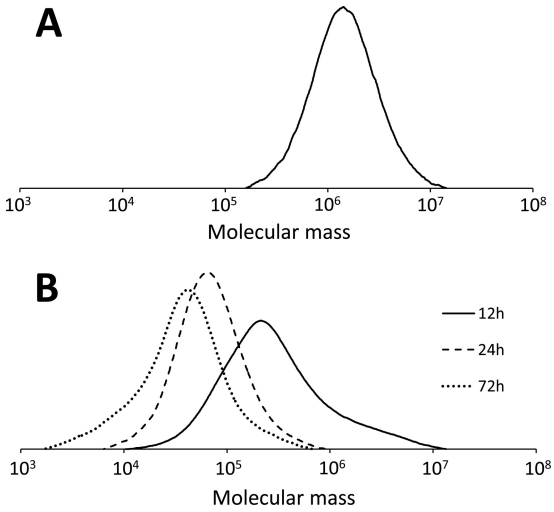

FIG 5 Molecular weight distributions of P(3HB) extracted from *E. coli* JM109 expressing $PhaC_{Da}$ cultured for 12 h (A) and *E. coli* JM109 expressing $PhaC_{Da}$ and $PhaRC_{YB-4}$ cultured for 12, 24, and 72 h (B). For expression of $PhaC_{Da}$ and $PhaRC_{YB-4}$, pJRD-*phaC*$_{Da}$*AB* (carrying *phaC*$_{Da}$, *phaA*$_{Re}$, and *phaB*$_{Re}$) and pGEM-*phaRC*$_{YB-4}$ (carrying *phaRC*$_{YB-4}$), respectively, were used. Cells were cultivated in LB medium containing glucose (20 g/liter) at 37°C.

samples B and C were suspended in phosphate buffer (pH 7.0) without and with ethanol (2.6 g/liter), respectively, and incubated at 30°C for 24 h. These P(3HB) samples were then collected for molecular weight analysis.

Table 4 shows the results of the *in vitro* assay. The M_n of P(3HB) from the initial state of incubation (sample A) was 180×10^3. This value is close to the theoretical M_n (226×10^3) estimated from the (*R*)-3HB-CoA conversion ratio (55%) and the concentration of $PhaRC_{YB-4}$ (36 nmol), using the following equation: absolute $M_n = 0.7 \times M_{n(GPC)}$, where $M_{n(GPC)}$ is the M_n determined by GPC. In this calculation, it is assumed that the PhaRC heterotetramer forms an active site similar to that seen in a class III synthase (27). After 24 h of incubation, the M_n of P(3HB) incubated without ethanol (sample B) was almost unchanged from that of sample A. On the other hand, the M_n of P(3HB) incubated with ethanol (sample C, $M_n = 17 \times 10^3$) was significantly lower than the M_ns of the other two, indicating that the P(3HB) chain was degraded via alcoholysis catalyzed by $PhaRC_{YB-4}$.

DISCUSSION

In earlier studies, it had been demonstrated that the molecular weight of PHA decreases with an increase in the synthase concentration in cells during PHA biosynthesis (28, 29). This experimental rule can be attributed to the monomer/synthase ratio, which is a well-known factor determining polymer molecular weight from organocatalytic living polymerization and is sometimes applicable to PHA biosynthesis. However, the results in this study cannot be explained by simple synthase amount-molecular weight relationships, because the molecular weight was observed to decrease even after polymer synthesis had finished. As an alternative mechanism to explain this decrease, we propose that random scission of P(3HB) chains takes place. This idea is supported by the results from previously reported kinetic analyses of the molecular weight change (7, 11). As there are no genes for P(3HB) polymerization and depolymerization in the *E. coli* genome, it has been hypothesized that the observed decrease in molecular weight was caused by exogenous PhaRC subunits. This study aimed to investigate the mechanisms underlying the unusual molecular weight decrease seen in P(3HB) produced by *E. coli* expressing $PhaRC_{YB-4}$.

In this study, NMR analysis of the low-molecular-weight P(3HB) synthesized by $PhaRC_{YB-4}$ showed that a high proportion of the carboxy end structures are capped by ethanol (Fig. 1). This finding led to the hypothesis that the reduction in P(3HB) molecular weight results from alcoholytic cleavage of P(3HB) chains. To test this hypothesis by showing that alcoholytic cleavage can be induced by the presence of ethanol, we first looked for culture conditions that would enable *E. coli* to produce high-molecular-weight P(3HB) even when $PhaRC_{YB-4}$ was being expressed. Several culture conditions were tested, and it was found that transformation with the low-copy-number plasmid pBBR1-*phaRC*$_{YB-4}$*AB* enabled *E. coli* to produce P(3HB) without a significant reduction in molecular weight. In addition, the strain harboring the low-copy-number plasmid had only a weak ability to produce ethanol (Fig. 2B). Our previous report (30) had shown that P(3HB)-producing *E. coli* produced ethanol as the main fermentation product, while plasmid-free *E. coli* strains produced acetate, providing clues to understanding the difference in ethanol production between strains harboring low- and high-copy-number plasmids. Indeed, in this study, *E. coli* harboring low-copy-number plasmid pBBR1-*phaRC*$_{YB-4}$*AB* was observed to produce smaller amounts of P(3HB) and ethanol but a larger amount of acetate than *E. coli* harboring high-copy-number plasmid pGEM-*phaRC*$_{YB-4}$*AB*) (see Table S1 in the supplemental material).

By adding ethanol to the culture of the strain harboring the low-copy-number plasmid, we examined whether alcoholytic cleavage of P(3HB) could be induced. The result clearly demonstrates that ethanol works as a trigger for the dramatic reduction of the P(3HB) molecular weight (Table 2). However, the concentration of exogenous ethanol required to induce the efficient reduction of the molecular weight of P(3HB) (4 g/liter) is much higher than that of endogenous, host-produced ethanol (0.2 to 0.8 g/liter). This may be because of differences between exogenous and endogenous ethanol; however, the concentration of intracellular ethanol might be similar under both conditions.

To obtain further evidence for the alcoholytic cleavage of the P(3HB) chain by $PhaRC_{YB-4}$, an *in vivo* scission assay system was developed by coexpressing two synthases ($PhaRC_{YB-4}$ and $PhaC_{Da}$) in *E. coli*. $PhaC_{Da}$ synthesizes high-molecular-weight P(3HB) in *E. coli*. Thus, by monitoring the change in the high-molecular-weight fraction of P(3HB), the effect of $PhaRC_{YB-4}$ on

TABLE 4 *In vitro* incubation of the P(3HB)–$PhaRC_{YB-4}$ complex in phosphate buffer with or without ethanol[a]

Sample	Incubation with additive	Incubation time (h)	Mol wt of P(3HB)		
			M_n (10^3)	M_w (10^3)	M_w/M_n
A	No incubation	0	180 ± 9	365 ± 30	2.0
B	No additive	24	193 ± 10	355 ± 16	1.8
C	Ethanol	24	17 ± 3	23 ± 7	1.4

[a] P(3HB) polymerization using purified $PhaRC_{YB-4}$ was performed for 3 min at 30°C, and the reaction mixture was divided into three equal samples, A, B, and C, by volume. Sample A represented the initial condition prior to incubation. Samples B and C were centrifuged, and the precipitates were incubated for 24 h at 30°C in fresh phosphate buffer (pH 7.0) without and with ethanol (2.6 g/liter), respectively. Results are the averages ± standard deviations from three separate experiments.

P(3HB) chains synthesized by $PhaC_{Da}$ was investigated. The result showed that the molecular weight of P(3HB) produced by a strain coexpressing the two synthases after 72 h of cultivation was as low as that produced by the strain expressing $PhaRC_{YB-4}$ alone (Table 3). This observation strongly suggests that $PhaRC_{YB-4}$ is able to cleave the chains of the high-molecular-weight P(3HB) synthesized by $PhaC_{Da}$. The *in vivo* assay also revealed that the $PhaRC_{YB-4}$ complex could induce scission of the P(3HB) chain, although the $PhaC_{YB-4}$ or $PhaR_{YB-4}$ subunit alone could not. Thus, scission activity, as well as polymerization activity, was shown to require both the $PhaC_{YB-4}$ and $PhaR_{YB-4}$ subunits.

Unlike *E. coli*, the PHA-negative mutant *R. eutropha* PHB^{-}4 produces high-molecular-weight P(3HB) when expressing $PhaRC_{YB-4}$ (12). Because the ethanol produced by this *R. eutropha* strain remains at undetectable levels (data not shown), the alcoholytic activity of $PhaRC_{YB-4}$ could not be induced.

The alcoholysis activity of $PhaRC_{YB-4}$ was directly demonstrated with an *in vitro* assay. We conducted *in vitro* synthesis of P(3HB) using purified $PhaRC_{YB-4}$ and chemically synthesized (*R*)-3HB-CoA for 3 min, followed by 24 h of incubation of P(3HB)-$PhaRC_{YB-4}$ complexes in phosphate buffer with or without ethanol. A significant decrease in the molecular weight of P(3HB) was observed only when ethanol was present. In addition, there was no detectable change in the molecular weight of P(3HB) during the incubation with ethanol under synthase-free conditions (data not shown). This observation provides convincing evidence for the alcoholysis activity of $PhaRC_{YB-4}$.

Surprisingly, weak scission activity was also observed for $PhaRC_{Bm}$ *in vivo*. This finding was unexpected, because a previous study demonstrated that $PhaRC_{Bm}$ did not have P(3HB) chain scission activity in *E. coli* (7). Later, it was found that the strain expressing $PhaRC_{Bm}$ produced less ethanol than is required for alcoholysis. Not only the PhaRC subunits from *B. cereus* YB-4 (7) and *Bacillus* sp. INT005 (11) but also the PhaRC subunit from *B. megaterium* were found to have scission activity for P(3HB) chains; therefore, class IV synthases might commonly have scission activity. As far as we have tested, the scission activities of class I synthases such as $PhaC_{Da}$ and $PhaC_{Re}$ are undetectable.

The alcoholysis activity of the PhaRC synthase was demonstrated in this study; however, a similar alcoholytic activity has been reported for lipases (31) and ester synthase/acyl-CoA:diacylglycerol acyltransferases (32). An alcoholysis activity of lipase toward triacylglycerols can be observed in the presence of excess alcohol. Recently, this reaction has attracted attention as a possible enzymatic method for biodiesel production. On the other hand, the alcoholysis activity of PhaRC could be used for the modification and functionalization of the carboxylic end of PHA. This report indicates that there are possible applications of PhaRC other than for PHA production. PhaC and lipases are members of the α/β-hydrolase fold family (2, 4); thus, the molecular mechanism underlying the alcoholic activity of these enzymes might be commonly shared.

It would be interesting if alcoholysis by $PhaRC_{YB-4}$ were involved in the regulation of PHA molecular weight in *B. cereus* YB-4. In a previous study, we observed a decrease in the PHA molecular weight when *B. cereus* YB-4 was cultured for a long time (10). Thus, $PhaRC_{YB-4}$ might be partly involved in this decrease. However, unlike *E. coli*, natural PHA producers are capable of mobilizing PHA by inherent PHA depolymerases. It is natural to think that PHA depolymerases would be mainly responsible for this decrease. The molecular weight decrease in *B. cereus* YB-4 might be associated with providing a rapid energy supply for spore and septum development (33) because low-molecular-weight PHA would be favored as a storage material to be mobilized quickly.

In summary, this study focused on the mechanisms underlying the unusual decrease in P(3HB) molecular weight observed in *E. coli* JM109 expressing $PhaRC_{YB-4}$. The low-molecular-weight P(3HB) isolated from the cells had an ethanol-capped carboxy end. Scission of P(3HB) chains was observed in the presence of ethanol in both *in vivo* and *in vitro* assays. Thus, the molecular weight was reduced not by hydrolytic cleavage but by alcoholytic cleavage via $PhaRC_{YB-4}$. This alcoholysis activity was also observed for P(3HB) chains synthesized by other synthases. Both the $PhaR_{YB-4}$ and $PhaC_{YB-4}$ subunits are essential for expression of the scission activity. Because the synthase from *B. megaterium* also exhibits scission activity, this capability might be an inherent feature of class IV synthases. The present study provides new insights into the catalytic properties of PHA synthase other than PHA polymerization.

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