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Site-directed saturation mutagenesis of polyhydroxylalkanoate synthase for efficient microbial production of poly[(*R*)-2-hydroxybutyrate]

Chiaki Hori,[1,2] Kenta Oishi,[1] Ken'ichiro Matsumoto,[1,2,*] Seiichi Taguchi,[1,2,§] and Toshihiko Ooi[1,2]

Division of Applied Chemistry, Graduate School of Engineering, Hokkaido University, N13-W8, Kita-ku, Sapporo 060-8628, Japan[1] *and CREST-JST, 4-1-8 Honcho, Kawaguchi, Saitama 332-0012, Japan*[2]

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In our previous study, artificial polyhydroxyalkanoate (PHA) poly[(*R*)-2-hydroxybutyrate] [P(2HB)] was successfully biosynthesized from racemic 2HB in recombinant *Escherichia coli* using an engineered PHA synthase, PhaC1$_{Ps}$(S325T/Q481K). Although P(2HB) has promising material properties, the low level of polymer production was a drawback. In this study, we performed directed evolution of PhaC1$_{Ps}$ towards enhanced P(2HB) accumulation in *E. coli* by site-directed dual saturation mutagenesis at the positions 477 and 481, which was known for their potential in enhancing natural PHA accumulation. By using a screening on agar plates with Nile red, eight colonies were isolated which produced a greater amount of P(2HB) compared to a colony expressing the parent enzyme PhaC1$_{Ps}$(S325T/Q481K). Among them, the cells expressing PhaC1$_{Ps}$(S325T/S477R/Q481G) [ST/SR/QG] accumulated polymer at the highest level (up to 2.9-fold). As seen in PhaC1$_{Ps}$(ST/SR/QG), glycine and basic amino acid residues (K or R) were frequently found at the two positions of the select mutated enzymes. The enzymatic activity of PhaC1$_{Ps}$(ST/SR/QG) toward 2HB-CoA was approximately 3-fold higher than that of the parent enzyme. Additionally, expression levels of the select mutated enzymes were lower than the parent. These results indicated that PhaC1$_{Ps}$ mutagenesis at the positions 477 and 481 increased specific activity toward 2HB-CoA and it could result in the enhanced production of P(2HB).

[**Key words:** Chiral polymer; P2HB; Bioplastic; Polyhydroxybutyrate; Directed evolution; Lactate-polymerizing enzyme]

Bacterial polyhydroxyalkanoate (PHA) synthase is a key enzyme in PHA production and typically polymerizes (*R*)-3-hydroxyacyl-CoAs (3HA-CoA) into PHA polymer chains (1). In our previous study, evolutionarily engineered PHA synthase that was from *Pseudomonas* sp. 61–3 with the pairwise mutations S325T and Q481K (PhaC1$_{Ps}$ST/QK) (2) was found to incorporate the artificial substrate of (*R*)-lactate (LA) into its polymer chain (3). In addition, PhaC1$_{Ps}$(ST/QK) exhibited a broad substrate specificity toward various 2-hydroxyalkanoate (2HA) monomers, such as glycolate (4) and 2-hydroxybutyrate (2HB) (4–6). Using PhaC1$_{Ps}$(ST/QK) and homologous enzymes, a variety of 2HA-containing PHAs have now been synthesized (7).

Enantiopure poly[(*R*)-2-hydroxybutyrate] [P(2HB)] was successfully produced from racemic 2HB by expressing PhaC1$_{Ps}$(ST/QK) in recombinant *Escherichia coli* (Fig. 1) (6). Biological P[(*R*)-2HB] production has two major advantages over its chemical synthesis in that a relatively inexpensive racemic 2HB precursor can be used, and that the obtained polymer possesses a higher molecular weight (8). The efficient production of P(2HB) facilitated the characterization of its material properties. It was eventually demonstrated that the P(2HB) polymer possessed a high degree of flexibility and transparency (6). P(2HB) is characterized by its structural analogy to polylactic acid (PLA), one of the widespread biobased polyesters. Tsuji et al. (9) demonstrated that PLAs and P(2HB)s with opposite stereochemistry formed a hetero-stereocomplex as well as homostereocomplex of P[(*R*)-2HB] and P[(*S*)-2HB]. In addition, their copolymer P[(*R*)-LA-*co*-(*R*)-2HB] exhibited cocrystallization behavior over a wide range of monomer compositions (10).

A drawback of the bacterial P(2HB) production system is the low polymer content (~15 wt%) compared to typical natural PHA, poly[(*R*)-3-hydroxybutyrate] [P(3HB), ~60 wt%] (6,11–13). To address this problem, the directed evolution of PhaC1$_{Ps}$(ST/QK) towards efficient P(2HB) production was utilized. It was previously demonstrated that amino acid substitutions of PhaC1$_{Ps}$ at four positions (E130, S325, S477 and Q481) influenced the activity and substrate specificities of this enzyme (2,14,15). In addition, the double saturation mutagenesis at positions 477 and 481 followed by an *in vivo* screening created a variety of mutated PhaC1$_{Ps}$ that increased P(3HB) accumulation (12). Therefore, in the present study, we applied this strategy to P(2HB) production. This is the first report on directed evolution of PHA synthase using the artificial polymer accumulation as a screening criterion. As a result, we successfully obtained several mutated enzymes that can increase P(2HB) accumulation in *E. coli*.

* Corresponding author at: Division of Applied Chemistry, Graduate School of Engineering, Hokkaido University, N13-W8, Kita-ku, Sapporo 060-8628, Japan. Tel./fax: +81 11 7066610.
E-mail address: mken@eng.hokudai.ac.jp (K. Matsumoto).
§ Present address: Department of Chemistry for Life Sciences and Agriculture, Faculty of Life Sciences, Tokyo University of Agriculture, 1-1-1 Sakuragaoka, Setagaya-ku, Tokyo 156–8502, Japan.

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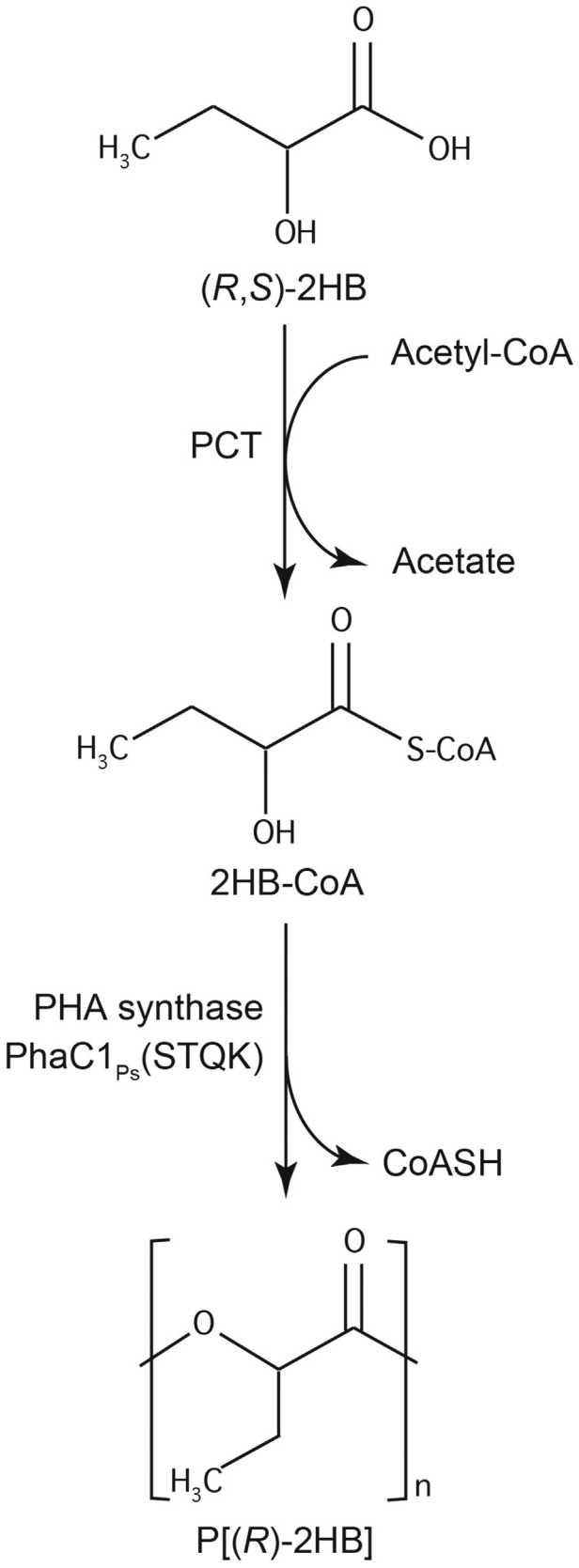

FIG. 1. Synthetic pathway of P(2HB) production by PHA synthase in *Escherichia coli*. PCT, propionyl-CoA transferase.

MATERIALS AND METHODS

Site-directed mutagenesis The S325T/S477X/Q481X mutated PHA synthase genes from *Pseudomonas* sp. 61−3 were constructed by overlap extension PCR using pTV118NpctC1ST/QK (3) as a template and the following primers; 5′-TCGAATTCGTGCTGTCCAGC**NNN**GGGCATATC**NNN**AGCATTCTGA-3′, 5′-TCGAATTCG TGCTGTCCAGCAGTGGGCATATCCAGAGCATTCTGA-3′, 5′-CGACTTACATCGATGCG CTCAAAG-3′ and 5′-GGAACCTGCAGAGATCCAAC-3′. The restriction sites are underlined. The amplified *Cla*I/*Pst*I fragment was inserted into *Cla*I/*Pst*I sites in pTV118NpctC1STQK to yield pTV118NpctC1(ST/QX/KX).

Screening on agar plates with nile red The plate screening was performed as described previously (16). In brief, *E. coli* JM109 harboring pTV118NpctC1(ST/QX/KX) was grown on a lysogeny broth (LB) agar plate containing 100 mg/L ampicillin, 2 wt% glucose, 1 wt% sodium 2HB and 0.5 mg/L Nile red for 14 h at 30°C. Nile red plate screening was performed against approximately 5000 individual colonies. Colonies with strong fluorescence were chosen and inoculated into liquid culture to determine P(2HB) production by gas chromatography analysis.

Analysis of the polymer productivity of the highly active mutants *E. coli* BW25113 cells harboring the selected pTV118NpctC1(ST/QX/KX) were grown on 2 mL LB medium containing 100 mg/L ampicillin for 14 h at 30°C. Then 1 mL culture medium was inoculated with 100 mL LB medium containing 100 mg/L ampicillin, 2 wt% glucose, 1 wt% sodium 2HB and 0.5 mg/L Nile red, then cultivated for 48 h at 30°C. P(2HB) content in the lyophilized cells from 1.5 mL of the culture medium was determined by using gas chromatography analysis, as described previously (2).

***In vitro* enzymatic assay** (*R*)-2-Hydroxybutyryl-CoA (2HB-CoA) was synthesized via the CoA transfer reaction using propionyl-CoA transferase (PCT) from *Megasphaera elsdenii*. The reaction mixture containing 1 mM acetyl-CoA, 0.25 wt% sodium 2HB and 0.1 mg/mL purified His-tagged-PCT in 100 mM Tris-HCl (pH 7.6) was incubated at 30°C for 30 min. The obtained 2HB-CoA was purified by using HPLC equipped with a reversed-phase column (TSKgelODS-80Ts, Tosoh Bioscience, Tokyo, Japan). Recombinant *E. coli* BW25113 strains harboring pTV118NpctC1(ST/QX/KX) were grown on LB medium containing 100 μg/ml ampicillin, 2 wt% glucose and 1 wt% sodium 2HB at 30°C for 12 h. Cells were harvested and rinsed with chilled deionized water, then resuspended in 200 mL of chilled 25 mM Tris-HCl buffer (pH 7.4). Subsequently, cells were disrupted by sonication (UD-200, Tomy Seiko Co., Ltd., Tokyo, Japan) on ice for 10 s 5 times. Cell debris was removed by centrifugation (12,000 × *g*, 10 min, 4°C), and the cell-free supernatant was used for further analysis. For PhaC activity assay, 0.225 mM 2HB-CoA, 0.05 % Hecameg and 0.25 mg/mL crude protein were mixed in 12.5 mM ammonium acetate (pH 7.0), then incubated at 30°C for 0, 5, 10, 20 and 30 min, respectively. The reaction was quenched by adding the same volume of 1 % trichloroacetic acid. The released CoA in the supernatant was quantified based on the absorbance at 412 nm (V-730 spectrophotometer, JASCO, Tokyo, Japan) using the thiol detection reagent 5-(3-carboxy-4-nitrophenyl)-disulfanyl-2-nitrobenzoic acid (DTNB), as described (2). One unit of enzyme is defined by the amount required for release of 1 μmol CoA per minute.

Immunoblot analysis The expression level of the PhaC1$_{Ps}$ mutants in recombinant *E. coli* BW25113 were measured by immunoblot analysis using rabbit antiserum, as described previously (2,17). The crude extract of *E. coli* expressing PhaC1$_{Ps}$(ST/QX/KX) mutants was prepared as described above. The protein concentration was measured by the Bradford protein assay. Five micrograms of protein was subjected to SDS-PAGE. The target protein, PhaC1$_{Ps}$, was immunolabeled using an Amersham ECL Select Western Blotting Detection Reagents kit (GE Healthcare Life Sciences, Marlborough, MA, USA), and the chemiluminescence was visualized using ChemiDoc XRS +(Bio-Rad, Hercules, CA, USA).

Molecular weight analysis of P(2HB) The harvested cells which accumulated P(2HB) were lyophilized and the polymer was extracted with chloroform for 72 h at room temperature, and precipitated by adding a 10-fold volume of methanol. The molecular weight of the purified polymer was determined by gel permeation chromatography (GPC) using commercial polystyrenes as the standards (18).

Protein structure modeling of PhaC1$_{Ps}$(STQK) The amino acid sequences of PhaC1$_{Ps}$(STQK) was subjected to a protein structure homology-modeling server SWISS-MODEL (https://swissmodel.expasy.org) by using the structure of the catalytic domain of the class I polyhydroxybutyrate synthase from *Cupriavidus necator* (PDB number, 5t6o) as the template.

RESULTS

Nile red plate screening of mutated PhaC1$_{Ps}$ for enhanced P(2HB) production The selected eight colonies were subjected to the liquid culture for determining P(2HB) production. They

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