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Regulation of 3-hydroxyhexanoate composition in PHBH synthesized by recombinant *Cupriavidus necator* H16 from plant oil by using butyrate as a co-substrate

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A (*R*)-3-hydroxyhexanoate (3HH) composition-regulating technology for poly (3-hydroxybutyrate-*co*-3-hydroxyhexanoate) (PHBH) production was developed using recombinant *Cupriavidus necator* H16 with butyrate as a co-substrate. A new (*R*)-3-hydroxyhexanoyl-CoA ((*R*)-3HH-CoA) synthesis pathway was designed and enhanced by replacing the PHA synthase gene (*phaC1*) of *C. necator* by the *phaC*$_{Ac}$NSDG (encoding the N149S and D171G mutant of PHA synthase from *Aeromonas caviae*) and deactivation of the *phaA* gene (encoding (β-ketothiolase) from *C. necator* H16 chromosome). The effect of butyrate as co-substrate was assessed in high-cell-density fed-batch cultures of several *C. necator* mutants, and the 3HH fraction was successfully increased by adding butyrate to the culture. Moreover, overexpression of BktB (encoding the second β-ketothiolase with broad substrate specificity) enhanced the (*R*)-3HH-CoA synthesis pathway in the *phaA* deactivated mutant of *C. necator* by promoting the condensation of acetyl-CoA and butyryl-CoA into 3-ketohexanoyl-CoA. Consequently, PHBH containing 4.2–13.0 mol % 3HH was produced from butyrate and palm kernel oil by the genetically modified *C. necator* H16 strains.

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[**Key words:** β-Ketothiolase; Butyrate; 3-Ketohexanoyl-CoA; Palm oil; Polyhydroxyalkanoate; *Ralstonia eutropha*]

Polyhydroxyalkanoates (PHAs) are polyesters synthesized by various microorganisms (1). Since most of the PHAs are produced from renewable resources such as sugars, plant oils, and carbon dioxide (CO_2), they are expected to confer environmental protection and reduce CO_2 emissions, consequently reducing global warming (2). The life cycle inventory values of energy consumption and CO_2 emissions of bio-based PHAs are much lower than those of petrochemical polymers (3). The potential environmental impacts and the biocompatibility of industrially produced bio-based PHAs have been extensively investigated (4).

The most abundant natural PHA is PHB (a homopolymer of D-3-hydroxybutyric acid ((*R*)-3HB)). The investigation of more flexible, less crystalline PHB alternatives including poly (3-hydroxybutyrate-*co*-3-hydroxyvarelate) and poly (3-hydxoxybutyrate-*co*-3-hydro xyhexanoate) (PHBH) (5–7) has been a research priority, since the high crystallinity, hardness, and brittleness of PHB limit its practical applicability. The flexibility depends on the rate of constituents in these copolymers. For instance, the elongation at break increases from 5 % to 850 % by increasing the 3HH fraction from 0 to 17 mol % (7), indicating that regulation of the 3HH composition is essential for extending the applicability of PHBH. Efficient production of PHBH with a 3HH fraction exceeding 10 mol % is an expected approach to replace conventional plastics such as polypropylene, polystyrene, and polyethylene (5).

A promising bacterium for PHBH biosynthesis is recombinant *Cupriavidus necator* (formerly *Ralstonia eutropha*) H16, which produces high levels of PHB (8–17). To increase the 3HH composition, recombinant *C. necator* strains lacking the *phaB* genes (*phaB1*, *B2*, and *B3*) have been constructed. Since these mutant strains produce PHBH at low levels, deletion of *phaB* is counterproductive for industrial-scale PHBH biosynthesis from an economic perspective (18,19). On the other hand, Tsuge et al. (20) successfully modified the PHA synthase enzyme (PhaC$_{Ac}$NSDG) that showed enhanced incorporation of longer 3-hydroxyalkanoate units such as (*R*)-3HH-CoA into a PHA. According to Kawashima et al. (21), *C. necator* expressing PhaC$_{Ac}$NSDG and several (*R*)-specific enoyl-CoA hydratases accumulates PHBH containing 10.5 mol % 3HH from soybean oil. (*R*)-specific enoyl-CoA hydratase enhances 3HH composition by converting 2-hexenoyl-CoA to (*R*)-3HH-CoA (Fig. 1); however, this enzyme requires 2-hexenoyl-CoA substrate. When plant oils are used as the sole carbon source, 2-hexenoyl-CoA is produced only as an intermediate of the β-oxidation pathway. Therefore, an alternative strategy that increases and regulates the 3HH composition, while maintaining productivity, is required.

In this study, we engineered a PHBH-producing strain in which *phaC1* is replaced by *phaC*$_{Ac}$NSDG. However, since the 3HH fraction of the PHBH yielded from palm kernel oil (PKO) as a sole carbon source remained below our expected amount, we supplied butyrate as a precursor of 3-ketohexanoyl-CoA. This strategy should increase the 3HH content, because β-ketothiolase encoded by *bktB* of *C. necator* H16 is expected to enhance the biosynthesis of 3-ketohexanoyl-CoA as a precursor of (*R*)-3HH-CoA from acetyl-CoA

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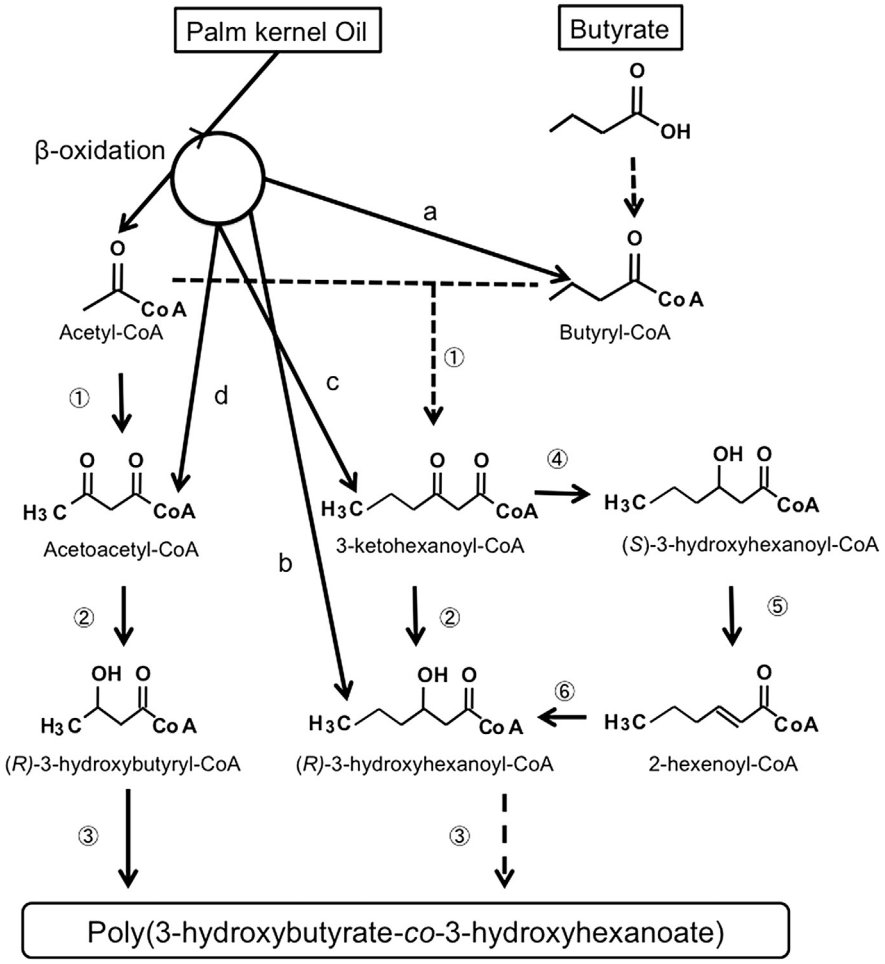

FIG. 1. Artificial metabolic pathway for PHBH biosynthesis from PKO and butyrate in AS strain. Dotted lines show the pathway enhanced in this study. 1; β-ketothiolase, 2; (*R*)-specific reductase, 3; PHA synthase, 4; hydroxyacyl-CoA dehydrogenase, 5; enoyl-CoA hydratase, 6; (*R*)-specific enoyl-CoA hydratase. a, c, d, intermediates of the β-oxidation pathway: butyryl-CoA, 3-ketohexanoyl-CoA and acetoacetyl-CoA, respectively. b, (*R*)-3HH-CoA is produced from 2-hexenoyl-CoA (an intermediate of the β-oxidation pathway) by (*R*)-specific enoyl-CoA hydratase.

and butyryl-CoA (Fig. 1) (22,23). Furthermore, we deactivated the *phaA* gene to effectively convert acetyl-CoA and butyryl-CoA to 3-ketohexanoyl-CoA, and thereby increase the 3HH monomer fraction of PHBH yielded from PKO.

MATERIALS AND METHODS

Strains and culture conditions Table 1 lists the strains, plasmids, and oligonucleotide primers used in this study. pMT5071 (24) was used for the homologous recombination of *C. necator*. *Escherichia coli* S17-1 (25) and the recombinant strains were cultivated in LB medium. The *C. necator* strains were cultured in MB medium (26). All cultures were grown at 30°C.

Construction of KNK005 To construct the KNK005 strain, the *phaC1* gene on the chromosome of *C. necator* H16 was replaced by $phaC_{Ac}$ NSDG (*Aeromonas caviae*-derived PHBH synthase mutant gene) (20,27) as follows. A pBluescript II KS(−) (Toyobo, Osaka, Japan) was treated with *Pst*I, then blunted using a DNA blunting kit (Takara Bio, Otsu, Japan) and ligated to generate the plasmid, pBlue-New, with a defective *Pst*I site. The approximately 2.4 kbp of d13 fragment containing $phaC_{Ac}$ was excised from pJRD215-EE32d13 (27) by *Eco*RI and cloned into the *Eco*RI site of pBlue-New to obtain pBlue-d13. Subsequently, the N149S and D171G fragments were amplified with primer sets 149-fw/149-rv and 171-fw/171-rv, respectively, and E2-50-derived plasmid (28) as a template. The two amplified fragments were mixed and joined by PCR; the resulting 1005 bp fragment containing a part of doubly mutated $phaC_{Ac}$ (NSDG) was digested with *Pst*I and *Xho*I, and cloned into pBlue-d13 treated with the same enzymes in a fragment interchange process to obtain pBlue-N149S/D171G. The base sequence analysis was conducted in a Perkin Elmer Applied Biosystems DNA sequencer, 310 Genetic Analyzer. The gene was confirmed as a mutant gene encoding serine in place of asparagine at the 149th amino acid site and glycine in place of aspartic acid at the 171st site of $PhaC_{Ac}$.

Next, a DNA fragment containing a structural *phaC1* gene was amplified with the primer set phaC-fw/phaC-rv and *C. necator* H16 genomic DNA as a template. The approximately 3.1 kbp obtained fragment was digested with *Bam*HI and subcloned into the vector pBluescript II KS(−) (Toyobo) to generate pBlue-phaC1. The approximately 1.9 kbp amplified fragment containing $phaC_{Ac}$NSDG mutant gene with primers nsdg-fw/nsdg-rv and pBlue-N149S/D171G as a template was digested with *Sbf*I and *Csp*45, ligated into the pBlue-phaC1 treated with the same enzymes to generate pBlue-phaC1:N149S/D171G. A plasmid pJRD215 (ATCC37533) was digested with *Xho*I and *Dra*I to isolate an approximately 1.3 kbp DNA fragment containing a kanamycin resistance gene; the fragment was then blunted and inserted into pBlue-phaC1:N149S/D171 G at the *Sal*I site (pBlue-phaC1:N149S/D171G-Km).

Subsequently, plasmid pMT5071 was digested with *Not*I to isolate an approximately 8 kbp DNA fragment containing the *SacB* gene. This fragment was inserted into pBlue-phaC1:N149S/D171G-Km at the *Not*I site to generate the plasmid for gene substitution (pBlue-phaC1:N149S/D171G-Km-sacB). *E. coli* S17-1 was transformed with the pBlue-phaC1:N149S/D171G-Km-sacB and incubated in a mixed culture with *C. necator* H16. A conjugal transfer was performed, as previously described (25). The strain containing the $phaC_{Ac}$ NSDG gene in place of *phaC1* was isolated by PCR, and their base sequences were determined in a DNA sequencer 310 Genetic Analyzer.

Construction of *C. necator* AS strain The *phaA* gene on the KNK005 chromosome was substituted by an inactivated *phaA* gene, *phaA*stop16, to enhance a synthetic pathway of (*R*)-3HH-CoA from acetyl-CoA and butyryl-CoA. For the *phaA* deactivation, approximately 1.1 kbp of fragment containing the *phaA* gene was amplified with the primer set phaa-fw/phac-rv and the KNK005 genomic DNA as a template, and cloned into the *Bam*HI site of pBluescriptII KS (Toyobo) to obtain pBlue-phaA. The inactivated *phaA* gene was amplified by LA PCR *in vitro* Mutagenesis Kit (Takara Bio) with the primer phaastop; 5′-CGCGGTCGGC**TAG**CTTGGCGGCTC-3′ and pBlue-phaA as a template to generate Blue-phaAstop16. The underlined sequence in the primer indicates the codon to replace Lys16 with STOP codon. A DNA fragment containing approximately 1.2 kbp of the kanamycin resistance gene was amplified with the primer set km-fw/km-rv

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