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Improvement of Poly(3-Hydroxybutyrate) [P(3HB)] Production in *Corynebacterium glutamicum* by Codon Optimization, Point Mutation and Gene Dosage of P(3HB) Biosynthetic Genes

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In our previous study, a system for producing poly(3-hydroxybutyrate) [P(3HB)] was established by introducing a polyhydroxyalkanoate (PHA) biosynthetic gene operon (*phaCAB*$_{Re}$) derived from *Ralstonia eutropha* into *Corynebacterium glutamicum*. In this study, two experimental strategies have been applied to improve P(3HB) production in recombinant *C. glutamicum*. One is a codon optimization of the N-terminal-coding region of the PHA synthase (PhaC$_{Re}$) gene focusing on the codon usage preference for the translation system of *C. glutamicum*. The other is the replacement of wild-type *phaC*$_{Re}$ with a modified gene encoding a mutation of Gly4Asp (G4D), which enhanced the production of PhaC$_{Re}$ and P(3HB) in *Escherichia coli*. The introduction of these engineered PHA synthase genes into *C. glutamicum* enhanced the production of PhaC$_{Re}$ and P(3HB). Interestingly, we found that these gene modifications also caused increases in the concentration of the translation products of the genes encoding monomer-supplying enzymes, β-ketothiolase (PhaA$_{Re}$) and acetoacetyl-CoA reductase (PhaB$_{Re}$). This finding prompted us to carry out a gene dosage of *phaAB*$_{Re}$ for a double plasmid system, and the highest production (52.5 wt%) of P(3HB) was finally achieved by combining the gene dosage of *phaAB*$_{Re}$ with codon optimization. The molecular weight of P(3HB) was also increased by approximately 2-fold, as was P(3HB) content. Microscopic observation revealed that the volume of the cells accumulating P(3HB) was increased by more than 4-fold compared with the non-P(3HB)-accumulating cells without filamentous morphologenesis observed in *E. coli*.

[**Key words:** *Corynebacterium glutamicum*, poly(3-hydroxybutyrate), rate-determining step, codon optimization, gene dosage]

Polyhydroxyalkanoates (PHAs) are a class of biopolyesters produced by a large number of bacteria as an energy storage material under nutrient-imbalanced conditions. PHAs have received considerable research attention because they can be produced from renewable carbon sources, such as vegetable oils, sugars and even from carbon dioxide. In addition, the polyesters are highly-biodegradable and biocompatible, which is an advantage for medical applications (1). In spite of the several advantages of PHAs compared with petroleum-derived plastics, their use is currently limited owing to the high cost of production (2).

Poly(3-hydroxybutyrate) [P(3HB)] is the most widely studied and the best characterized PHA. Most of the studies on P(3HB) accumulation by bacteria have been carried out using gram-negative bacteria (2–4). P(3HB) accumulation by gram-negative bacteria, however, has a disadvantage for medical and food conduct applications because of contamination with pyrogenic compounds referred to as endotoxin (*e.g.*, lipopolysaccharides). There have been few attempts to remove endotoxin during the preparation of PHA, but a trace amount of endotoxin always remained in the material (5, 6). To overcome this problem, studies on P(3HB) accumulation by gram-positive bacteria, such as *Bacillus* and *Streptomyces*, have come into the spotlight because these bacteria do not contain endotoxin (7).

Corynebacterium glutamicum, a gram-positive bacterium, offers several advantages over other gram-positive bacteria. This bacterium is generally regarded as a safe microorganism, and large-scale fermentation technology has been established for efficiently fermenting amino acids such as glutamate, valine and lysine. These amino acids produced by *C. glutamicum* have been used for several decades in food, feed and pharmaceutical products (8–10). The whole genome sequence of *C. glutamicum* was analyzed in 2003 (11). Therefore, this bacterium is suitable for metabolic engineering to produce endotoxin-free PHA with the coproduction of amino acids and potentially with a lower production cost.

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Previously, we established a P(3HB) production system by introducing the *Ralstonia eutropha*-derived $phaCAB_{Re}$ operon into *C. glutamicum* (12). The strong promoter used for the cell surface protein gene derived from *C. glutamicum* was functionally driven for the expression of the $phbCAB_{Re}$ operon and P(3HB) production. However, the P(3HB) content in *C. glutamicum* was still lower than those in *Escherichia coli* and *R. eutropha* (13, 14). In this study, we improved P(3HB) production in *C. glutamicum* using several genetic strategies, codon optimization, point mutation and gene dosage of the PHA biosynthetic genes. The changes in the molecular weight of the P(3HB) polymer and cell morphology of the P(3HB)-accumulating *C. glutamicum* were also investigated.

MATERIALS AND METHODS

Bacterial strains and culture conditions *E. coli* JM109 was used as the host for genetic manipulation and was grown at 37°C in Luria–Bertani (LB) medium. When necessary, ampicillin (100 μg/ml), kanamycin (50 μg/ml), or chloramphenicol (30 μg/ml) was added to the medium. *C. glutamicum* ATCC13869, kindly provided by Dr. K. Yokoyama of Ajinomoto Co., Inc., was used as the host for P(3HB) production. *C. glutamicum* was transformed by electroporation as described previously (15). Two media were used; a nutrient-rich medium (CM2G; 16) for preculture, and a minimal medium (MMTG; 16) for the main culture of *C. glutamicum* to produce P(3HB) polymer. Cells were grown at 30°C. Ampicillin (100 μg/ml), kanamycin (50 μg/ml), or chloramphenicol (5 μg/ml) was added to the preculture when needed.

Codon optimization of N-terminal coding region of $phaC_{Re}$ There is a difference in the frequency of codon usage between *R. eutropha* and *C. glutamicum*. As shown in Fig. 1, five low-usage codons in the N-terminal coding region of $phaC_{Re}$ were replaced with synonymous high-usage codons in *C. glutamicum*. A 1.1-kb *Kpn*I-*Bgl*II fragment of pPS-*phaCAB* (12) including the N-terminal coding region of $phaC_{Re}$ was subcloned into the *Kpn*I-*Bam*HI site of pUC19 to yield pUC19C. pUC19C′ was prepared using the following PCR conditions. A forward primer of 5′-GCAGCATCC ACCCAGGAAGGCAAGTCCCAA-3′ and a reverse primer of 5′-TGCGCCCTTGCCGGTTGCCATGATTTGA-3′ were used (the underlined nucleotides are the points mutated to change low-usage codons to synonymous high-usage codons). pUC19C was used as a template for PCR. The PCR product was allowed to self-ligate to yield the plasmid pUC19C′.

Construction of expression plasmids An *Eco*RV-*Mun*I fragment of pUC19C′ including the optimized N-terminal coding region of $phaC_{Re}$ was inserted into pPS-*phaCAB*, which was digested with the same restriction enzymes, to produce pPS-*C′AB*. pPS-*G4DAB* was constructed using a 4.3-kb blunt-ended *Csp*45I-*Bam*HI fragment of pGEM-*phaG4DAB* (17). The fragment was subcloned into the blunt-ended *Bst*EII-*Bam*HI site of pPSPTG1 to yield pPS-*G4DAB*.

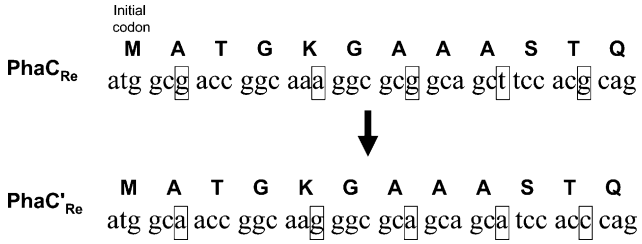

FIG. 1. Codon optimization of N-terminal coding region of PHA synthase gene ($phaC_{Re}$). Boxed letters indicate changed nucleotides for synonymous codons.

pGEM-*phaAB* was digested with *Pst*I, blunt ended by T4 DNA polymerase and digested with *Bam*HI. The 2.4-kb fragment containing P(3HB)-monomer-supplying genes (*phaAB*) was ligated into pPSPTG1, which was digested with *Bst*EII, blunt ended by T4 DNA polymerase and digested with *Bam*HI, to construct pPS-*AB*.

pVC7-*phaCAB* and pVC7-*phaAB* were constructed using 5-kb and 3.2-kb *Eco*RI-*Bam*HI fragments of pPS-*phaCAB* containing the *cspB* promoter, *phaCAB* and the *pha* terminator or the *Eco*RI-*Bam*HI fragment of pPS-*phaAB* containing the *cspB* promoter, *phaAB* and the *pha* terminator subcloned into pVC7, which was digested with the same restriction enzymes, producing both expression vectors, respectively. The plasmids used in this study are listed in Table 1.

Preparations of proteins for immunoblot analysis Recombinant *E. coli* cells were grown at 37°C in 2 ml of LB medium for 14 h. An aliquot of 17.5 μl was transferred into 1.75 ml of fresh LB medium and was further grown for 9 h at 37°C. Cells were harvested by centrifugation and whole-cell extracts of the recombinant *E. coli* were prepared by sonication (5 s, 3 times). A soluble fraction was obtained by centrifugation at 13,700×*g* for 10 min at 4°C.

The *C. glutamicum* transformants were cultivated at 30°C for 56 h on MMTG. Cells were harvested by centrifugation and then disrupted by sonication (5 s, 6 times) on ice. An insoluble fraction including P(3HB)-granule-associated PHA synthase and a soluble fraction including β-ketothiolase and acetoacetyl-CoA reductase ($PhaA_{Re}$ and $PhaB_{Re}$) were obtained from the precipitate and supernatant of the disrupted recombinant cells after centrifugation (12,000×*g*, 4°C, 10 min). Protein concentrations were determined by the method of Bradford (18) using a protein assay kit (Bio-Rad) with bovine serum albumin as a standard protein.

Immunoblot analysis Proteins were separated by sodium dodecyl sulfate-10% polyacrylamide gel electrophoresis as described by Laemmli (19). Separated proteins were then transferred to a polyvinylidene fluoride membrane using an iBlot transfer package

TABLE 1. Plasmids used in this study

Plasmid	Relevant characteristics	Source or reference
pPSPTG1	*Corynebacterium-E. coli* shuttle vector; *cspB* promoter, Km^r	16
pPS-*phaCAB*	*cspB* promoter, $phaC_{Re}$, $phaA_{Re}$, $phaB_{Re}$, Km^r	12
pPS-*AB*	*cspB* promoter, $phaA_{Re}$, $phaB_{Re}$, Km^r	This study
pPS-*C′AB*	*cspB* promoter, $phaC'_{Re}$, $phaA_{Re}$, $phaB_{Re}$, Km^r	This study
pPS-*G4DAB*	*cspB* promoter, $phaG4D_{Re}$, $phaA_{Re}$, $phaB_{Re}$, Km^r	This study
pVC7	*Corynebacterium-E. coli* shuttle vector; Cm^r	16
pVC7-*AB*	*cspB* promoter, $phaA_{Re}$, $phaB_{Re}$, Cm^r	This study
pVC7-*CAB*	*cspB* promoter, $phaC_{Re}$, $phaA_{Re}$, $phaB_{Re}$, Cm^r	This study
pUC19C	$phaC_{Re}$	This study
pUC19C′	$phaC'_{Re}$	This study