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Development of polyhydroxybutyrate biosynthesis in *Bacillus subtilis* with combination of PHB-associated genes derived from *Ralstonia eutropha* and *Bacillus megaterium*

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ABSTRACT

Polyhydroxybutyrate (PHB) is a biodegradable polymer synthesized by bacteria for carbon storage in response to environmental stress. *B. subtilis* is generally recognized as a safe Gram-positive bacterium and is widely used for industrial-scale production of proteins and chemicals. Owing to lack of toxic lipopolysaccharides, *B. subtilis* is a superior alternative to the Gram-negative bacteria presently used for PHB production. In this study, we introduced PHB synthesis genes from *R. eutropha* and *B. megaterium* into *B. subtilis* for PHB production. The highest yield of PHB was achieved by coexpression of *phaA* from *R. eutropha* and *phaR*, *phaB*, and *phaC* from *B. megaterium* (up to 13.02% ± 1.67% of total cell dry weight in LB medium containing 1% glucose). The results indicate that *B. subtilis* is potentially used as a safe vehicle for producing PHB.

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1. Introduction

Biodegradable plastics are biopolymers that are processed to assume the features of plastic. A plastic-like product that can be buried in soil and broken down by microorganisms can reduce the harmful environmental impact of conventional plastics [1]. Biodegradable materials also have the advantages of biocompatibility and bioadsorption, which are important for the development of products and devices for medical applications. Polyhydroxyalkanoates (PHAs), which are natural biopolymers currently being developed into a type of biodegradable plastic [2], accumulate as esters in microorganisms [3]. PHA yield is affected by environmental stress in native strains [4]; microorganisms will synthesize PHA for energy storage in nitrogen-limited but carbon-rich environments [2,3,5]. When the carbon source is depleted, microorganisms break down these PHAs for growth via specific enzymes [6]. Polyhydroxybutyrate (PHB), one of the most common types of PHA, is similar to polypropylene, has a short chain length, and is stored as an insoluble droplet in the cytoplasm [2,3].

PHB biosynthesis involves three steps: β-ketoacyl-CoA thiolase (encoded by *phaA*) is first condensed into two molecules of acetyl-CoA to generate acetoacetyl-CoA; this is then reduced to (*R*)-3-hydroxybutyryl-CoA by the nicotinamide adenine dinucleotide phosphate (NADPH)-dependent enzyme acetoacetyl-CoA reductase (encoded by *phaB*). The final step is the polymerization of (*R*)-3-hydroxybutyryl-CoA to PHB by PHB synthase (encoded by *phaC*) [7–10]. This mechanism has been extensively studied in *R. eutropha* [11–13]. In the *R. eutropha* genome, the *phaA* and *phaB* genes are found in a single operon but *phaC* is in different location. In *B. megaterium*, the first strain in which PHA production was investigated, the phaRBC operon contains *phaB*, *phaC*, and *phaR*; the latter two are essential for PHA synthase activity, with PhaR acting as an activator of PhaC [14].

Although *Escherichia coli* can be induced to synthesize recombinant PHB [15,16], the outer membrane of *E. coli* contains endotoxins, such as lipopolysaccharide, that strongly stimulate the immune response [17], thereby limiting potential applications and increasing the cost of PHB materials if dangerous contaminants need to be removed during production. These limitations can be overcome by using *B. subtilis*, a generally recognized as safe (GRAS) strain that is used in industrial production of enzymes, amino acids, recombinant proteins, and chemicals in food, beverage, and detergents [18–23].

Previous studies have used *B. subtilis* as host for production of PHA by cloning *phaC1* and *phaAB* genes from *Pseudomonas aeruginosa* and *R. eutropha* into *B. subtilis* DB104 [24,25]. PHA accumulation in fermentation medium by recombinant *B. subtilis* was 4.6% [24]. In addition, *B. subtilis* 1A304(Φ105MU331) was used to express *B. megaterium phaPQRBC* genes, yielding 2.53% PHB in malt waste medium [25]. We speculated that expressing different

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Table 1
Bacterial strains and plasmids used in this study.

	Characteristic	Source
Strains		
E. coli		
DH5α	*deoR endA1 gyrA96 hsdR17 supE44 thi1 recA1 lacZM15*	Lab. collection
B. subtilis		
PT5	DB428, Δ *wpr::T7gene 1*	[25]
PT5(ReA)	PT5, Δ*mpr*:: P_{T7} /*RephaA*	This study
PT5(ReAB)	PT5, Δ*mpr*:: P_{T7} /*RephaA* and *RephaB*	This study
PT5(ReA-BmB)	PT5, Δ*mpr*:: P_{T7} /*RephaA* and *BmphaB*	This study
Plasmid		
pMT1	Integration vector contains P_{T7} and *aprN* SP	[25]
pMT-ReA	Integration vector contains P_{T7}/RePhaA	This study
pMT-ReAB	Integration vector contains P_{T7}/RePhaA-RePhaB	This study
pMT-ReA-BmB	Integration vector contains P_{T7}/RePhaA-BmPhaB	This study
pHT01	*B. subtilis/E. coli* shuttle vector contain P_{grac} promoter and LacI	MoBiTec GmbH
pReC	pHT01 with ReC operon driven by P_{grac}	This study
pBmRBC	pHT01 with BmPhaCAB operon driven by P_{grac}	This study
pBmRC	pHT01 shuttle vector with BmPhaR and BmPhaC driven by P_{grac}	This study

Abbreviations: BmPhaR, *phaR* from *B. megaterium*; BmPhaB, *phaB* from *B. megaterium*; BmPhaC, *phaC* from *B. megaterium*; RePhaA, *phaA* from *R. eutropha*; RePhaB, *phaB* from *R. eutropha*; RePhaC, *phaC* from *R. eutropha*.

combinations of PHB synthesis genes in *B. subtilis* could influence PHB yield. To test this hypothesis, PHB genes from *R. eutropha* and *B. megaterium* genomes were amplified by PCR [11,12,14,26] and expressed in *B. subtilis* in different combinations in this study. We found that coexpression of *phaA* from *R. eutropha* and *phaB*, *phaC*, and *phaR* from *B. megaterium* resulted in a higher PHB yield.

2. Materials and methods

2.1. Bacterial strains and culture condition

Bacterial strains and plasmids used in this study are listed in Table 1. *R. eutropha* (BCRC 13,036) and *B. megaterium* (BCRC 11,595) cells were purchased from Bioresource Collection and Research Center, Taiwan. All recombinant PHB-producing strains were derived from *B. subtilis* PT5 [27]. After overnight cultivation on an orbital shaker at 200 rpm and 37 °C, cultures were inoculated to obtain an optical density at 600 nm (OD_{600}) of 0.1. Ampicillin (100 μg/mL for *E. coli*), chloramphenicol (10 μg/mL for *B. subtilis*), and erythromycin (10 μg/mL for *B. subtilis*) were used to screen *E. coli* or *B. subtilis* strains harboring plasmids or *B. subtilis* integrants. Unless otherwise noted, recombinant *B. subtilis* was cultured in Luria-Bertani (LB) broth with 1% glucose and induced by isopropyl β-D-1-thiogalactopyranoside (IPTG) when OD_{600} reached 0.3.

2.2. Construction of shuttle and integrant vectors

Primers used in this study are listed in Table 2. Using the genomic DNA of *R. eutropha* as template, *phaA* (RePhaA) and *phaA-phaB* operon (RePhaAB) genes were amplified using primer pairs ReA01-ReA02 and ReA01-ReB01, respectively. The integrant vector MT0102 was generated using plasmid pMT1 and primers MT01-MT02. Amplicons were digested with *Hin*dIII and *Xho*I and ligated into MT0102 to obtain pMT-ReA (Fig. 1A) and pMT-ReAB (Fig. 1B). The *phaB* gene sequence from the *B. megaterium* genome (BmPhaB) and the ReA02MT03 sequence from the vector pMT-ReA were amplified with primer pairs BmB01-BmB02 and ReA02-MT03, respectively. Amplicons were digested with *Xho*I and *Bam*HI, and the BmPhaB fragment was ligated into ReA02MT03 to obtain the integrant vector pMT-ReABmB (Fig. 1C). The shuttle vectors pReC (Fig. 1D) and pBmRBC (Fig. 1E) were constructed using pHT01 as a backbone. The *phaC* gene from *R. eutropha* and *pha*RBC operon fragment from *B. megaterium* were amplified from chromosomal DNA with primer pairs ReC01-ReC02 and BmR01-RmC01, respectively, and cloned into pHT01 via *Bam*HI and *Xho*I restriction sites. Using the shuttle vector pBmRBC as template, the PCR amplicon generated using primers BmC02 and BmR02 was self-ligated into the *Spe*I site to obtain vector pBmRC (Fig. 1F).

Table 2
Primers used in this study.

Primer name	Primer sequence
MT01	TTATAACTCGAGCACCACCACCACCACCACTG (*Xho*I)
MT02	TGCAGTAAGCTTGGGAAACCGTTGTGGTCT (*Hin*dIII)
MT03	TAACAAGGATCCAAAGGAAGCTGAGTTGGCTG (*Bam*HI)
ReA01	CCGTACAAGCTTGGAGGACTACACAATGACTG (*Hin*dIII)
ReA02	GCTGAACTCGAGTTATTTGCGCTCGACTGCCA (*Xho*I)
ReA03	CCGTACGGATCCGGAGGACTACACAATGACTG (*Bam*HI)
ReB01	GCAATCTCGAGGAGGTCAGCCCATATGCAGG (*Xho*I)
ReB02	GCAATTCTAGAGAGGTCAGCCCATATGCAGG (*Xba*I)
ReC01	GGAGATGGATCCATGGCGACCGGCAAAGG (*Bam*HI)
ReC02	GCTGCCTCGAGTCATGCCTTGGCTTTGACG (*Xho*I)
BmB01	ATCATACTCGAGGAAGCAAGGAGGAAATTTTTCATG (*Xho*I)
BmB02	GGCCAGGGATCCTTACATGTATAAGCCGCCGTT (*Bam*HI)
BmC01	ATGCACTCTAGATTATTTAGAGCGTTTTTCTAGCC (*Xba*I)
BmC02	ACTAACACTAGTGGAGGTGAAAGGGTGTGTATATTCGT (*Spe*I)
BmR01	GAGGAAGGATCCGTGTCCATATTGAAAGCCATC (*Bam*HI)
BmR02	ATCCCCACTAGTTTACTTGCGAGCTGGCTGCT (*Spe*I)

2.3. PHB extraction

Cells were collected by centrifugation at 8000 rpm and 4 °C for 30 min and dried at 60 °C for 48 h Dried cells were subjected to a modified version of acid propanolysis to extract PHB [28]. Dried cell pellets (40 mg) were immersed in a solution of 2 mL 1,2-dichloroethane and 2 mL propanol (propanol: 12 N HCl = 4: 1) and 200 μL benzoic acid (4%) as an internal standard. The solution was incubated for 4 h at 100 °C in an oil bath and was cooled to room temperature, then mixed with 4 mL double-distilled water by vortexing and allowed to stand overnight at room temperature. PHB was recovered from the organic layer. PHB and benzoic acid standards were purchased from Sigma-Aldrich (St. Louis, MO, USA).

2.4. Analysis of PHB content

To determine the PHB content of cells, the extracted PHB was analyzed by gas chromatography (Hewlett-Packard model 5890A chromatograph; Agilent Technologies, Santa Clara, CA, USA) with a Zebron ZB-5 column (Phenomenex, Torrance, CA, USA) and a

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