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Construction of Recombinant *Bacillus subtilis* for Production of Polyhydroxyalkanoates

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Abstract

Polyhydroxyalkanoates (PHAs) are polyesters of hydroxyalkanoates synthesized by numerous bacteria as intracellular carbon and energy storage compounds and accumulated as granules in the cytoplasm of cells. In this work, we constructed two recombinant plasmids, pBE2C1 and pBE2C1AB, containing one or two PHA synthse genes, respectively. The two plasmids were inserted into *Bacillus subtilis* DB104 to generate modified strains, *B. subtilis*/pBE2C1 and *B. subtilis*/pBE2C1AB. The two recombinants strains were subjected to fermentation and showed PHA accumulation, the first reported example of mcl-PHA production in *B. subtilis*. Gas Chromatography analysis identified the compound produced by *B. subtilis*/pBE2C1 to be a hydroxydecanoate-co-hydroxydodecanoate (HD-co-HDD) polymer whereas that produced by *B. subtilis*/pBE2C1AB was a hydroxybutyrate-co-hydroxydecanoate-co-hydroxydodecanoate (HB-HD-HDD) polymer.

Index Entries: *Bacillus subtilis;* cloning and expression; P (HB-co-mclHA); PHA synthase gene; polyhydroxyalkanoates (PHAs).

Introduction

Polyhydroxyalkanoates (PHAs) function as carbon and energy reserves in prokaryotic cells *(1)*. They are accumulated by a wide range of bacteria when a carbon resource is provided in excess and at least one essential growth nutrient is limited *(2,3)*. Because their physical characteristics are similar to those of petrochemical polymers such as polypropylene, PHAs have been studied intensively by academic and industry and are considered good candidates for biodegradable plastics and elastomers *(4)*. The synthesis of PHA requires the enzyme PHA synthase *(phaC)*, which uses β-hydroxyacyl-coenzyme as a substrate for polymerization. The production of such substrates can occur by a variety of pathways *(5)*, including the simplest using the enzymes β-ketothiolase (encoded by *phaA*), acetoacetyl-CoA

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reductase (encoded by *phaB*), β-oxidation *(6)*, and a fatty acid *de novo* synthesis pathway *(7)*. Several genes have been cloned and expressed in *Escherichia coli*. However, the expression levels were much lower than that in the parent organisms and the expressed enzymes were accumulated as inclusion bodies inside the cells, which was a limiting factor in continuous-culture fermentation *(8–10)*. Because the method of transforming *Bacillus subtilis* with plasmid DNA *(11)* was discovered, *B. subtilis* has become an attractive alternative to *E. coli* as a host for the expression of cloned genes because it has several advantages over *E. coli*. Secretion of proteins may circumvent the formation of inactive inclusion bodies, which occurs during the overexpression of foreign genes in *E. coli*. Furthermore, *B. subtilis* is not a human pathogen and can be considered biologically safe. In this study, we report the expression of the PHA synthase gene(s) in *B. subtilis* DB104.

Methods

Strains and Plasmids

B. subtilis DB104 and *E. coli* HB101 were used as hosts. pBHR71 contains the *phaC1* gene from *Pseudomonas aeruginosa (12)*, pJM9131 contains the *phaAB* gene from *Ralstonia eutropha (13)*, pBE2 is an *E. coli–B. subtilis* shuttle vector.

Media and Culture

PHA fermentation medium was described by Ramsay et al. *(2)*. Nutrient broth, nutrient agar, and R2A agar were purchased from Sigma (Germany). The strain was first inoculated into 5-mL nutrient broth, and then 1% inoculum was used in fermentation. Fermentation was carried out in a 500-mL or 1-L shaking flask.

Construction of Recombinants

Plasmids were isolated using the Wizard Plus SV Minipreps DNA Purification System (Promega, Madison, WI). The isolated plasmids and digested DNA fragments were analyzed by electrophoresis in horizontal slab gels containing 0.7% (w/v) agarose, and a 1 kb DNA ladder (Promega, Madison, WI) was used as standard marker. DNA restriction fragments were isolated from the agarose gel using the QIAEX II Gel Extraction Kit (Qiagen, Valencia, CA). Restriction enzymes and T4 DNA ligase (Promega) were used according to the instructions provided by the supplier. A 1.8 kb *Bam* HI/*Xba* I restriction fragment containing *phaC1* gene obtained from plasmid pBHR71 was used as an insert, and plasmid pBE2 was employed as vector. A 2.5 kb *pst* I restriction fragment containing *phaAB* gene obtained from plasmid pJM9131 was used as an insert, and plasmid pBE2C1 was employed as vector. Ligation products were mixed with competent *B. subtilis* DB104 cells, and the resulting transformants were selected on nutrient agar plates containing 100 μg/mL of ampicillin. The positive transformants were proved by restriction digestion followed by agarose gel electrophoresis.

Gas Chromatography Analysis

Almost 1 mL esterification solution (3 mL 95–98% H_2SO4, 0.29 g benzoate, and 97 mL methanol), 15 mg freeze-dried cells, and 1 mL chloroform were mixed and heated at 100°C for 4 h, 1 mL of double-distilled H_2O (ddH_2O) was added to the cooled mixture, which was then vortexed for phase separation. A 1 µL portion of the lower organic phase was subjected to gas chromatography (GC) analysis, which was performed on a Hewlett Packard 5890 Series II Gas Chromatograph, using a 6-ft Supelco (10% Carbowax 20 *M* with 80/100 in mesh size Chromosorb WAW) Packed Column. Nitrogen was used as the carrier gas at the flow rate of 20 mL/min. The analysis was started at 135°C and the temperature was kept stable for 10 min to determine both the content and composition of the polymer.

Fourier Transform Infrared Spectroscopy

Almost 2–5 mL of the cell culture was centrifuged at 2610*g* for 15 min. The cells were transferred onto an IR window (ZnSe Disc, Spectratech) and dried on it. A mirror was used to give the reflected infrared signal to the horizontally laid window. With a scan number of 32, resolution of 16 and autogain, spectra were recorded at wavenumbers (/cm) from 400 to 4000 using a Mangna-IR spectrometer 750 (Nicolet) *(14)*. The PHA peak was observed at wavelengths of 1726–1740/cm *(15)*.

Results

Construction of the Recombinant Strain

The recombinant plasmid pBE2C1 was obtained by using a 1.8 kb *Bam* HI/*Xba* I restriction fragment *phaC1* gene from *P. aeruginosa* obtained from plasmid pBHR71 as an insert, and plasmid pBE2 as vector. The recombinant plasmid pBE2C1AB was obtained by using a 2.5 kb *pst* I restriction fragment *phaAB* gene obtained from plasmid pJM9131 as an insert, and plasmid pBE2C1 as vector (Fig. 1). The constructed plasmids were confirmed by restriction digestions and agarose gel electrophoresis. The newly cloned plasmids were amplified in *E. coli*. The size of the vector pBE2 is around 6.2 kb and the inserts around 1.8 kb (*phaC1* gene) and 4.3 kb *(phaC1AB* gene). These two newly constructed plasmids were transformed into *B. subtilis*. The positive transformants were checked by restriction digestions followed by agarose gel electrophoresis (Fig. 2). These result showed that the foreign fragment had been successfully inserted into the plasmids.

Gene Expression in Recombinant Strain

The culture was incubated at 28°C and shaken at 280 rpm. The cells were subjected to Fourier transform infrared spectroscopy (FTIR) and appeared to have a PHA peak with wavelengths about 1726–1740/cm. The result suggested that the recombinant strains could produce PHA. After

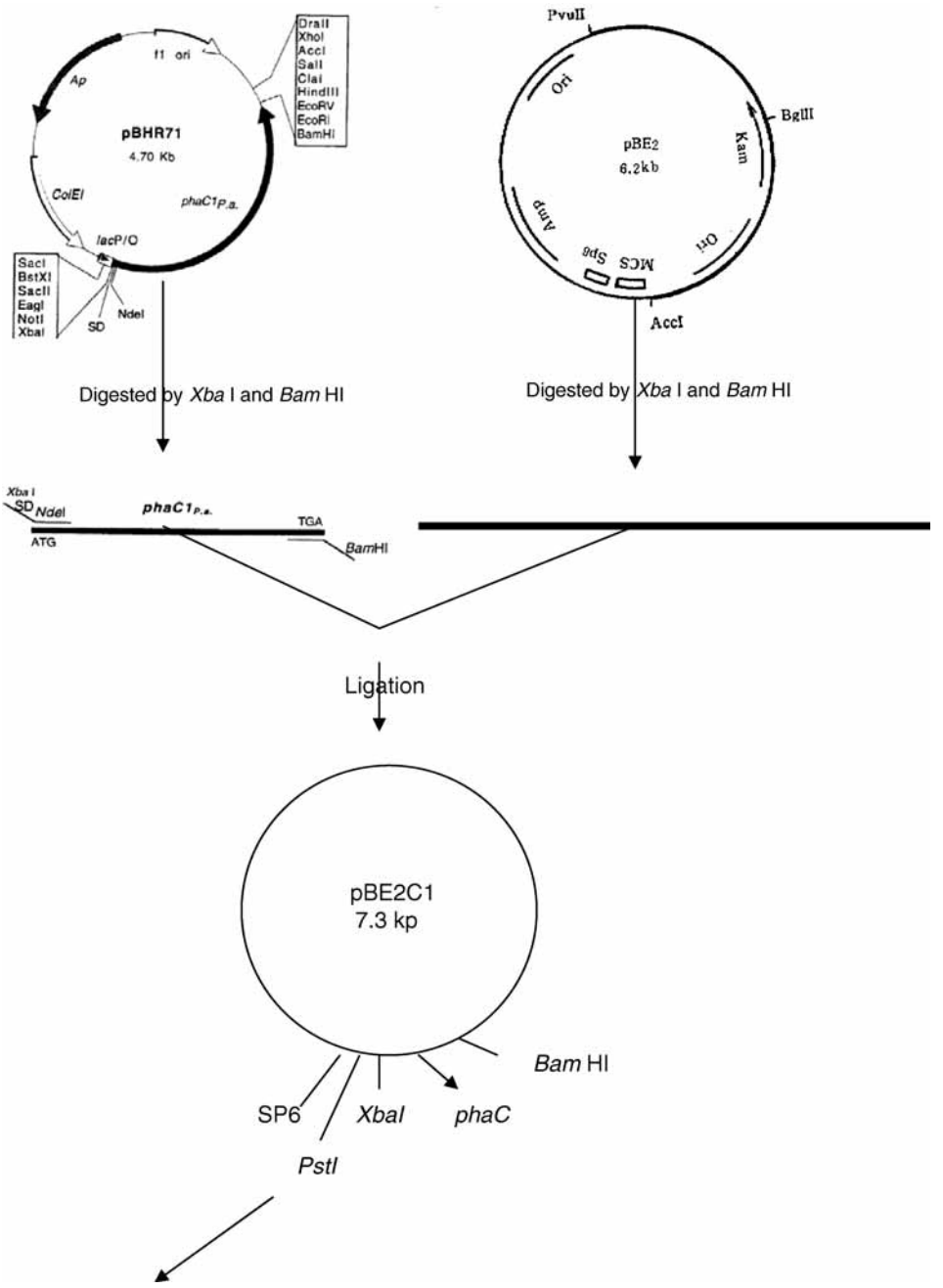

Fig. 1. *(Continued)*

fermentation, the cells were freeze-dried and the composition of the biopolymer was analyzed by GC; the result is showed in Fig. 3. The expression in the recombinant strain is controlled by the promoter SP6 of pBE2.

There was no PHA accumulation in *B. subtilis* DB104. However, *B. subtilis* DB104/pBE2C1 and *B. subtilis* DB104/pBE2C1AB, recombinants

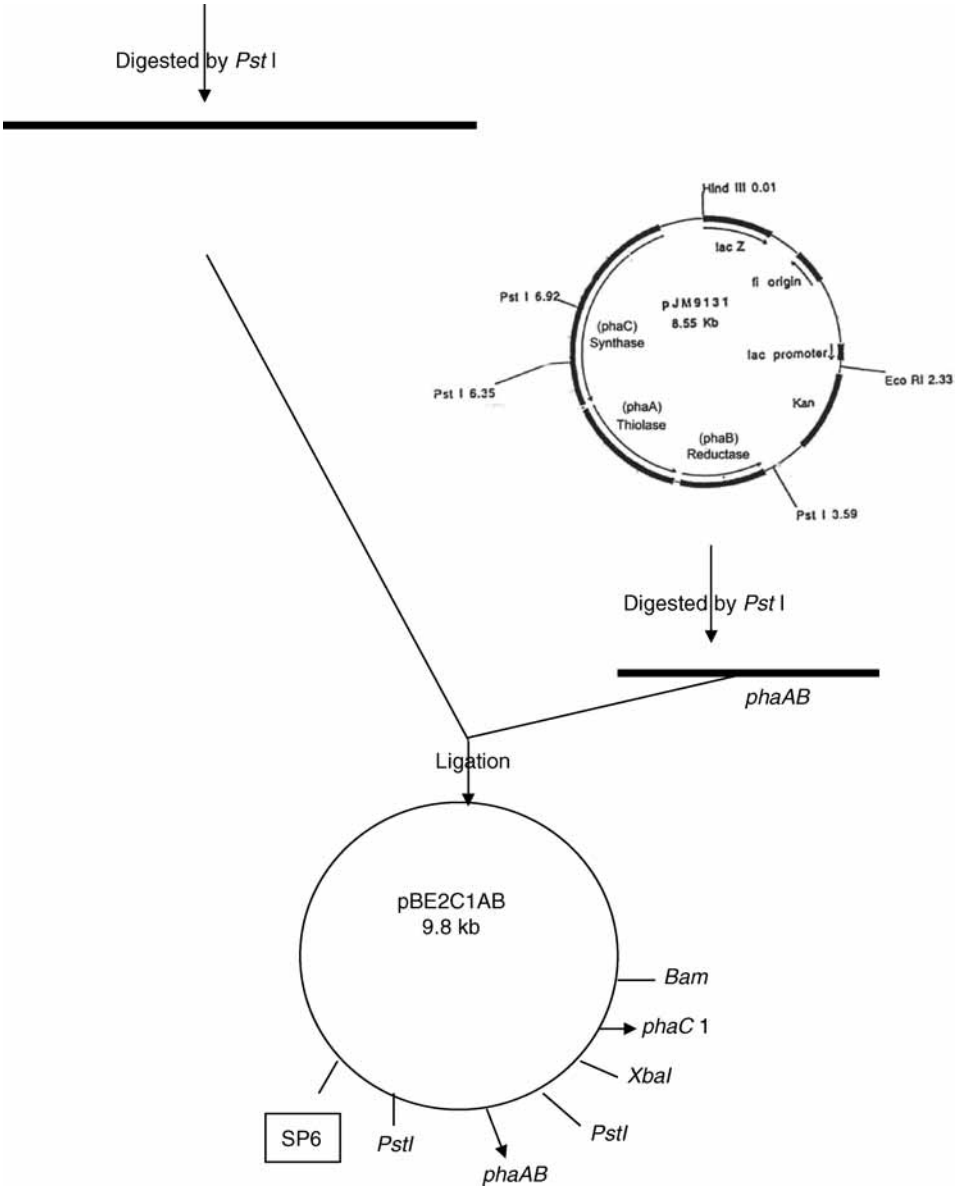

Fig. 1. Construction of plasmids pBE2C1 and pBE2C1AB.

containing *pha* genes, showed PHA accumulation—the first reported expression of *pha* genes from *P. aeruginosa* and *R. eutropha* into *B. subtilis*. As shown in Fig. 3, PHA production by the recombinant *B. subtilis* DB104/pBE2C1AB is higher than that by *B. subtilis* DB104/pBE2C1. GC analysis further identified the product synthesized by *B. subtilis* DB104/pBE2C1 to be a HD-HDD polymer whereas the product synthesised by *B. subtilis* DB104/pBE2C1AB was identified to be a HB-HD-HDD polymer (Table 1).

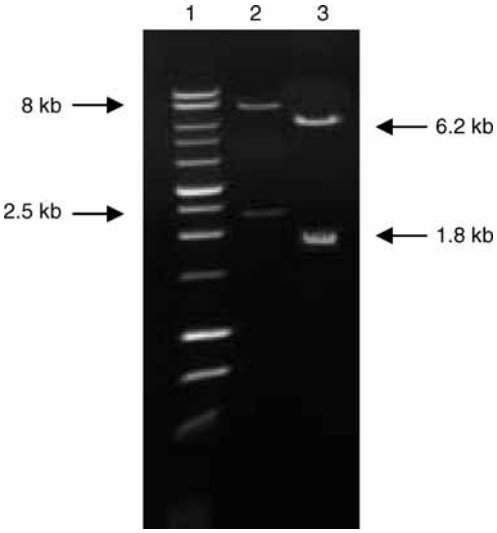

Fig. 2. Agarose gel electrophoresis. 1. DNA ladder 2. pBE2C1AB/*Pst* I 3. pBE2C1/*Xba* I and *Bam* HI.

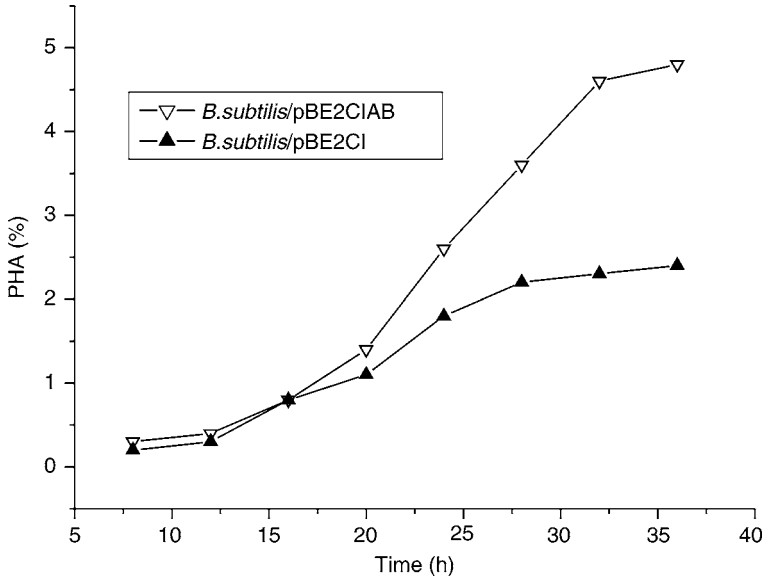

Fig. 3. Percentage the CDW of PHA-time curve of recombinants.

The *B. subtilis* DB104/pBE2C1 and *B. subtilis* DB104/pBE2C1AB carrying *pha* gene were subjected to fermentation and showed PHA accumulation, providing the first evidence that the *pha* gene from *P. aeruginosa* and *R. eutropha* can be expressed in *B. subtilis*.

Table 1
Monomer Composition of the PHA Accumulated by Recombinant Strains

Strains	HB (%)	HD (%)	HDD (%)
B. subtilis DB104/pBE2C1	0	58	42
B. subtilis DB104/pBE2C1AB	16	62	22

Discussion

In this research, we used *B. subtilis* as a host because it offered advantages such as short generation time, absence of endotoxin, and secretion of amylases and proteinases that utilize food wastes for nutrients, reducing the cost of production of PHAs. Our work is the first to report the expression of the mcl-PHA synthase gene obtained from *P. aeruginosa* into *B. subtilis*. The recombinant strains successfully produced mcl-PHA. *P. aeruginosa* can synthesize mcl-PHA by β-fatty acid oxidation using fatty acid as substrate, and by the *de novo* fatty acid synthesis pathway, which uses simple molecules such as glucose as substrate, but production of the latter is lower. In this work, glucose was used as a substrate, so the production of the PHA is low. Some mcl fatty acids can be added in future experiments to improve the production of the PHA.

As the results indicate, the recombinant *B. subtilis* DB104/pBE2C1 and *B. subtilis* DB104/pBE2C1AB can synthesize monomer mcl-PHA, which agrees with the character of PHA synthase gene *phaC1*. The synthase (encoded by *phaC1*) is referred to as type II PHA synthase and prefers 3-hydroxyacyl CoA with chain lengths of 6–14 carbon atoms (mcl-HA) as substrates *(16)*. Because the melting point temperature was low and the crystallization rate of mcl-PHA was slow, the recombinant can produce P (HB-co-mclHA). We are interested in the monomer composition of PHAs and monomer percentage. To follow up, we subcloned β-ketothiolase gene *(phbA)* and acetoacetyl-CoA reductase gene *(phbB)* from *R. eutropha* into plasmid pBE2C1 to obtain plasmid pBE2C1AB. The recombinant *B. subtilis* DB104/pBE2C1AB was found to produce PHB.

Recently, there had been growing interest in P (HB-co-mcl HA) because of its enhanced physical and chemical character. Our research results suggest that recombinant strain *B. subtilis* DB104/pBE2C1AB could produce P (HB-co-mcl HA) polymer.

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