PAPER

Preparation of genetic engineered *Enterobacter* strain TF to produce green plastic from xylose
バイオマスからのグリーンプラスチック生産を目指した遺伝子組換え微生物の作製

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Abstract：Polyhydroxyancanoate (PHA) is a bacterial polyester which is thermoplastic, flexible and biodegradable. PHA is expected as "green plastic" material therefore many researchers have been studying the development of new type PHAs, their physical property, biodegradability and the production method. In commercial production of PHA, use of substances obtained from biomass as economic and ecological carbon sources is necessary. Hemicellulose, which is mainly composed of glucose, xylose and other monosaccharaides is an important biomass, however xylose is not very feasible sugar to be used as substrate in fermentation. Our isolated bacterium, *Enterobacter* sp.TF grow vigorously on xylose, arabinose and other many kind of sugars but it does not accumulate PHA. Hence, we made genetic engineering to the strain TF by inserting the genes for PHA biosynthesis from *Ralstonia eutropha* which is well known as PHB producer, and from *Pseudomonas* sp.61-3 which is one of the few bacteria to produce PHA copolymer from glucose. As, result, our recombinant of *Enterobacter* sp.TF did not produce PHA copolymer but produced about 20wt% PHB homopolymer per dried cells.

キシロースは、農産廃棄物や廃材など植物性バイオマスに含まれるヘミセルロースの主要構成単糖であり、発酵原料等への有効利用法の開発が急がれている。しかしながら、キシロースは微生物にとって利用しやすい糖であるとは言い難く、また工業的に利用価値のある発酵生産物も少ないのが現状である。ある種の微生物が産生するポリヒドロキシ酪酸（PHB）やポリヒドロキシアルカン酸（PHA）などのポリエステルは、熱可塑性と生分解性を併せ持つため"グリーンプラスチック"として期待されており、植物由来の有機物を原料基質として使えばCO2の排出量を増やさない"カーボンニュートラル"なプラスチックにもなりえる。本研究では、独自に分離したキシロース高資化性の*Enterobacter*属細菌にPHAやPHBの生合成遺伝子を導入した組換え株を作製し、実際にキシロースからこれらグリーンプラスチックを合成するか検証した。

Key words : *Enterobacter*, green plastic, PHB, xylose
キーワード：*Enterobacter*、　グリーンプラスチック、　PHB、　キシロース

1. Introduction

Polyhydroxyalkanoates (PHAs) are the polyesters accumulated in bacterial cells under imbalanced nutritional condition like nitrogen limitation. PHAs are expected as raw material for manufacturing of green plastic (biodegradable plastic) because the polyesters are thermoplastic, flexible and biodegradable. Poly-D-3-hydroxybutyrate (PHB) was the first reported and well known bacterial polyester. However, PHB is inferior in flexibility, then it is difficult to mold and fragile to stress of physical force. Therefore, PHA copolymer which is composed of both D-3-hydroxybutyrate and other types of hydroxyalkanoate have been studied. They are synthesized by using various kinds of bacteria and carbon sources to improve the physical characteristics. Especially, mcl-PHA which contains medium chain length D-3-hydroxyalkanoic acid ($C_6 \sim C_{14}$) as the monomer unit is expected due to its feasibility in molding and durability of the products[1]. For practical application of PHAs as biodegradable plastics, cheap and "carbon neutral" organic substances like biomass existing abundantly in nature should be used as the carbon source for the production of PHAs. Glucose, fructose and some oligosaccharides like sucrose and lactose are converted

to PHB by many bacteria while the other saccharides like xylose or arabinose which are often contained in the hydrolysate of hemicellulose are easily converted to PHB. On the other hand, only a few bacterial strains can synthesize the PHA copolymers from sugars such as *Pseudomonas* sp. 61-3[2]. Therefore, the most of PHA copolymers are produced using organic acids or fatty acids in commercial scale. We isolated a bacterium growing vigorously on xylose, arabinose or other saccharides contained in hemicellulose. However, the bacterium does not accumulate any types of polyesters in the cells.

In this study, we prepared the gene recombinant to synthesize PHA from xylose by introducing the genes for biosynthesis of PHA from *Ralstonia eutropha* and *Pseudomonas* sp.61-3 into our xylose-utilizing bacterium. The accumulation of PHA from xylose in the recombinants and the composition of monomer unit were investigated.

Materials and methods

Xylose-utilizing bacterium and culture condition

Entertobacter sp. TF was used as the host for genetic engineering for biosynthesis of PHA from xyose. This gram-negative bacterium was isolated from Onga River on Kyushu island using the xylose-mineral salts medium which was composed of $(NH_4)_2SO_4$ 2.0g, $MgSO_4 \cdot 7H_2O$ 0.2g, KH_2PO_4 1.0g, xylose 20g, agar 15g and trace elements solution 0.1ml per 1 L of distilled water. The composition of the trace elements solution was $FeSO_4 \cdot 7H_2O$ 16.0g, $CrCl_3 \cdot 6H_2O$ 0.13g, $NiCl_3 \cdot 6H_2O$ 0.18g, sodium citrate dihydrate 15.6g, $CuSO_4 \cdot 5H_2O$ 0.1g, $CoCl_2 \cdot 6H_2O$ 15.6g in 100ml of 1M HCl. pH of the culture medium was adjusted to 7.0.

The nucleotide sequence of the 16S rRNA gene of the isolated bacterium was determined and the homology analysis was carried out by a BLAST program using the GenBank database [3]. The nucleotide sequence of the 16S rRNA gene of the isolated bacterium showed 98% identities to *Enterobacter* sp.CCBAU15488, *E.cloacae*, *E.agglomerans* and other strains of *Enterobacter* species. Then, we name our strain *Enterobacter* sp. strain TF. The growth of the isolated bacterium on xylose was fast and the specific growth rate in liquid culture was about $0.70h^{-1}$ (the data is not shown). Then we thought that the strain

TF was attractive as the host in genetic engineering for production of PHA.

DNA manipulation and plasmid construction

The plasmid pRKmKSc-C1GAB was constructed to introduce the following genes for biosynthesis of PHA into *Enterobacter* sp.TF :- β-ketothiorase gene (*phb*A) and acetoacetyl-CoA reductase gene (*phb*B) from *R.eutropha* to provide D-3-hydroxybutyrate, D-3-hydroxyacyl-acyl carrier protein (ACP)-CoA transferase gene (*pha*G_{Ps}) from *Pseudomonas* sp. 61-3 to provide various D-3-hydroxyalkanoates, PHA synthase gene (*pha*$C1_{Ps}$) having broad substrate specificities from *Pseudomonas* sp. 61-3 to polymerize various D-3-hydroxyalkanoates, and *lac* promotor.

The plasmid pRKmKSc-C1GAB was constructed as follows. pBSKB-C1AB was first constructed by PCR using pJBB49-phb (pJRD215[4] derivative including *phb*$_{Re}$ promoter, *pha*$C1_{Ps,}$ *phb*AB_{Re}) as a template with primers phaC1-KpnI(f) (5'-CGG<u>GGTACC</u>CAATGGAG CGTCGTAGATG-3') and phbCAB-r3(BamHI) (5'-C G<u>GGATCC</u>CTGCAGCCTCGCCCCCGCGAGGGC CGCGCTGCA-3') (underlined sequence indicate *Kpn*I and *Bam*HI recognition sites, respectively), followed by the 0.9-kb KpnI-BamHI fragment excluding P_{Re} promoter (native promoter for biosynthesis of PHA in *R. eutropha*) was introduced into pBluescript II KS+ (including Ap^r *lac*POZ T7 and T3 promoter) at the KpnI-BamHI sites. pRKmKSc-C1AB was constructed by cloning the 4.1-kb fragment of pBSKB-C1AB digested with *Kpn*I and *Sac*I to pBBR1MCS-2[4]. Next, the *pha*G_{Ps} gene was amplified by PCR using pRKmHS32 as a template with primers phaG-SD(HindIII)-f (5'-CCC<u>AAGCTT</u>GCATAC CCGCTTGCCAGGAGT-3') and phaG-EcoRI(TGA)-r (5'-CG<u>GAATTC</u>CTCAAATTG CCAATGCATGGTG-3') (underlined sequence indicate *Hind*III and *Eco*RI recognition sites, respectively). The PCR product was digested with *Hind*III and *Eco*RI, and the 0.9-kb *Hind*III-*Eco*RI fragment was introduced into pBluescript II KS$^+$ at the HindIII-EcoRI sites. We referred the plasmid as pBSHE9. The *pha*G_{Ps} gene was amplified by PCR using pBSHE9 as a template with primers phaG-SD(HindIII)-f and phaG-SmaI(TGA)-f (5'-TCC<u>CCCGGG</u>TCAAATTGCCA ATGCATGGTG-3') (underlined sequence indicate *Hind*III and *Sma*I recognition sites, respectively). Finally, plasmid

pRKmKSc-C1GAB was constructed by digesting the PCR product with *Sma*I and *Hind*III, then introducing the 0.9-kb *Sma*I-*Hind*III fragment into the pRKmKSc-C1AB with the HindIII-SmaI sites prepared by inverse PCR. The pRKmKSc-C1GAB was introduced to *Enterobacter* sp.TF by conjugal transfer via *E.coli* S17-1. To isolate recombinant colony harboring the plasmid, kanamycin (50 mg·L^{-1}) was added to xylose-mineral salts agar plate medium.

All DNA manipulations including the isolation of total genomic DNA and plasmids, digestion of DNA with restriction endonuleases, agarose gel electrophoresis and transformation of *E. coli* S17-1 were performed by standard procedures [5].

Analysis of polyester

Recombinant of *Enterobacter* sp.TF was cultivated in liquid medium composed of xylose and mineral salts in a 300-mL Erlenmeyer flask. The concentration of $(NH_4)_2SO_4$ was reduced to 0.5 g·L^{-1} to promote PHA accumulation under nitrogen limitation. The cultivation was carried out by reciprocal shaking at 170rpm and 30℃. Cell concentration of the recombinants of the strain TF grown in xylose-mineral salts medium was monitored by measuring the absorbance of the culture broth at 600nm and the dry cell weight was also determined after centrifugation of the broth and drying the harvested cells at 105℃. Concentration of PHA accumulated in the bacterial cells and the monomer composition were determined according to the method reported by Hokamura et al. [4].

Results and discussion
Effect of pRKmKSc-C1GAB on PHA accumulation

Many colonies of recombinant strain TF harboring pRKmKSc-C1GAB were obtained on the xylose agar plate. Among them, the three strains (*Enterobacter*/ pRKmKSc-C1GAB strain No.5, 6, 8) were picked up and grown in xylose-mineral salts liquid medium added with kanamycin (50 mg·L^{-1}) and the accumulation of PHA in the cells was investigated. The result is shown in Table 1. In the two strains No.5 and No.6, the copolymer PHA

Table 1 Intracellular content of PHA produced from xylose and the composition of monomer unit in the recombinant *Enterobacter* sp.TF harboring *lac* promoter, *phb*AB from *R.eutropha*, *phaG*$_{Ps}$ and *phaC1*$_{Ps}$ from *Pseudomonas* sp.61-3

Strains/plasmid	PHA content (wt%)	PHA composition (mol%)				
		C_4 (3HB)	C_6 (3HHx)	C_8 (3HO)	C_{10} (3HD)	C_{12} (3HDD)
Enterobacter/pRKmKSc-C1GAB⑤	1.67	87.2	0	0	12.8	0
Enterobacter/pRKmKSc-C1GAB⑥	2.02	87.9	0	0	12.1	0
Enterobacter/pRKmKSc-C1GAB⑧	1.53	100	0	0	0	0

3HB, D-3-hydroxybutyrate; 3HHx, D-3-hydroxyhexanoate; 3HO, D-3-hydroxyoctanorate; 3HD, D-3-hydroxydecanoate; 3HDD, D-3-hydroxydodecanoate;
The data column which are described with "0" mean that GC analysis was carried out but the substance was not detected.

Table 2 Intracellular content of PHA produced from xylose and the composition of monomer unit in the recombinant *Enterobacter* sp.TF harboring *phb*CAB and the native promoter P$_{Re}$ from *R.eutropha*

Strains/plasmid	PHA content (wt%)	PHA composition (mol%)				
		C_4 (3HB)	C_6 (3HHx)	C_8 (3HO)	C_{10} (3HD)	C_{12} (3HDD)
Enterobacter/pRKmBB-phbCAB①	19.5	100	0	0	0	0
Enterobacter/pRKmBB-phbCAB②	27.3	100	0	0	0	0

3HB, D-3-hydroxybutyrate; 3HHx, D-3-hydroxyhexanoate; 3HO, D-3-hydroxyoctanorate; 3HD, D-3-hydroxydecanoate; 3HDD, D-3-hydroxydodecanoate;
The data column which are described with "0" mean that GC analysis was carried out but the substance was not detected.

containing about 12mol% D-3-hydoxydakanoate was accumulated in the cells while the intracellular content of the PHA was just only1.67 and 2.02wt%, respectively. In the other strain No.8, the homopolymer PHB accumulated in the cells but the content was 1.53wt%. Thus, the recombinant harboring the plasmid pRKmKSc-C1GAB showed very low intracellular content in both PHB and PHA.

Effect of pRKmBB-phbCAB on PHA accumulation

Hence, we changed the target of our study on PHB production from xylose. Then, the new plasmid pRKmBB-phbCAB was introduced to *Enterobacter* sp.TF and the accumulation of PHB was tested. pRKmBB-phbCAB was incorporated with PHB biosynthesis operon in *R. eutropha* that contains *phb*AB, PHB synthase gene from ($phbC_{Re}$) and native promoter (phb_{Re} promoter). The fermentation result is shown in Table 2. The obtained recombinant *Enterobacter*/ pRKmBB-phbCAB accumulated PHB in the cells and the intracellular content of polyester was much higher than *Enterobacter*/pRKmKSc-C1GAB.

In *Enterobacter*/pRKmKSc-C1GAB, we introduced $phaG_{Ps}$. The enzyme PhaG is known as a D-3-hydroxyacyl-acyl (3HA) carrier protein (ACP)-CoA transferase then it was expected to provide efficiently various D-3-hydroxyacyl-CoA from fatty acid biosynthetic pathway as the substrate for polymerization into copolymer PHA in the recombinant strain TF. However, the accumulation of PHA/PHB was very poor. On the other hand, any polyesters did not accumulate from xylose in the recombinant cells harboring plasmid pRKmKSc-C1G without *phb*AB (the data is not shown). The *Enterobacter*/ pRKmBB-phbCAB accumulated PHB over 20wt % in the cells. Form these results, it is suspected that $PhaC1_{Ps}$, which was incorporated in *Enterobacter*/ pRKmKSc-C1GAB, somewhat functions the polymerization activity but hydroxyacyl-CoA as the substrate is not almost provided. Recently, Wang et al. have reported that PhaG actually has another function as a 3-hydroxyacyl-ACP thioesterase[6]. This suggests that PhaG produces free D-3-hydroxyalkanoic acids rather than (R)-3HA-CoA. Actually, the decrease in culture pH was bigger in case of *Enterobacter*/ pRKmKSc-C1GAB than that of *Enterobacter*/ pRKmBB-phbCAB and the wild strain.

Several researchers reported the microbial production of biodegradable plastics from xylose for PHB production by recombinant[7] and engineered yeast[8], mcl-PHA production from xylose and octanoic acid by engineered *Pseudomonas putida* KT2440[9], screening of bacteria to produce PHB from xylose[10], PHA production from sugar maple hemicellulosic hydrolysate by *Burkholderia cepacia*[11], and PHA production from lignocellulosic materials[12]. In their reports, yield, content and productivity of PHB/PHA from xylose were still in low levels compared to those from glucose or organic acids. Therefore, it seems that further progress is necessary to manufacture biodegradable plastic from xylose.

It is expected that the introduction of D-3-hydroxyacyl (3HA)-CoA ligase gene to our strain TF may be available to incorporate mcl-3HA units into the polymer chain through fatty acid biosynthesis pathway and increase the polymer content in the cells.

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