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Short Communication

Antibiotics-free stable polyhydroxyalkanoate (PHA) production from carbon dioxide by recombinant cyanobacteria

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ABSTRACT

A practical antibiotics-free plasmid expression system in cyanobacteria was developed by using the complementation of cyanobacterial *recA* null mutation with the *Escherichia coli recA* gene on the plasmid. This system was applied to the production of polyhydroxyalkanoate (PHA), a biodegradable plastic, and the transgenic cyanobacteria stably maintained the *pha* genes for PHA production in the antibiotics-free medium, and accumulated up to 52% cell dry weight of PHA.

1. Introduction

Global warming is the urgent issue of our time, and the carbon dioxide is a greenhouse gas of the major concern. Cyanobacteria are prokaryotic photosynthetic microorganisms which can provide a simple genetic engineering system (Vioque, 2007) for the production of carbon neutral materials from CO_2. There are two foreign gene expression systems for cyanobacteria; one is the plasmid vector mediated expression system, and another one is the integration of the foreign DNA into the cyanobacterial genome through homologous recombination. The advantages of the plasmid system are (i) the higher copy numbers of the foreign genes compared to the genome integration method, (ii) the well established procedure for the modification of the genes on plasmid, such as point mutation, insertion and deletion, and (iii) the wide range of expression host with a shuttle vector system. On the other hand the limitation of plasmid system is the necessity for antibiotics for the maintenance of plasmid. Especially when the genes on plasmid cause a heavy metabolic load to the host cells, the plasmids are easily excluded from the cells in the absence of antibiotics. The use of antibiotics is, however, not realistic for the large scale culture with respect to its cost. In this study, an antibiotics-free stable cyanobacterial expression system was developed by using the complementation of the cyanobacterial lethal *recA* null mutation by the *Escherichia coli recA* gene on the plasmid, and applied to the PHA production. The PHA is a biopolymer accumulated by various microorganisms as reserves of carbon and reducing equivalents, has physical properties similar to those of polyethylene, and can replace the chemical plastics in some applications, such as disposable bulk materials in packing films, containers, and paper coatings.

2. Methods

2.1. Culture conditions and PCR primers

The cyanobacterial strain *Synechococcus* sp. PCC7002 (ATCC 27264) was cultured as described previously (Onizuka et al., 2002). The *Wautersia eutropha* was obtained from the American Type Culture Collection, and cultured at 30 °C in Tryptic soy broth-dextrose free (TSB) medium (Becton Dickinson Microbiology Systems, Cockeysville, MD). A complete list of the PCR primers and their sequences is listed in Table S1 of the Supplementary Information.

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2.2. Cloning of S. PCC7002 recA gene, and preparation of recA gene containing kanamycin resistance (Km^r) cassette

The *recA* gene of *S.* PCC7002 was amplified by PCR from the genomic DNA as a template with the primers recA-S-*Eco*RI and recA-AS-*Eco*RI. The PCR amplified fragment (1.1 kb) was blunted and cloned into the *Sma*I digested pUC18 vector to generate Syn7002-recA-pUC18 plasmid. The kanamycin resistance (Km^r) GenBlok (Pharmacia) was ligated to the *Eco*RV digested Syn7002-recA-pUC18 plasmid, and the *E. coli* JM109 cells were transformed with the ligation mixture. The plasmid was isolated from the kanamycin (Km) resistant transformants, and the DNA fragment of *recA* gene with Km^r cassette was obtained by digesting the plasmid with *Eco*RI.

2.3. Cloning of E. coli recA gene

The *recA* gene of *E. coli* was amplified by PCR by using the genomic DNA as a template and the primers ECOLI recA-S-*Bam*HI and ECOLI recA-AS-*Bam*HI. The PCR amplified fragment (1.7 kb) was blunted and cloned into the *Sma*I digested pUC18 vector to generate E. coli-recA-pUC18 plasmid.

2.4. DNA construct for PHA production and promoter for phaCAB gene expression

The four DNA constructs (Fig. 1) of the *phaCAB* (GenBank Acc. No. AM260479) and *E. coli recA* genes were constructed in the shuttle vector pAQJ4 (Ikeda et al., 2002) (the details of the procedure are shown in the "Supplementary Materials and Methods" and Fig. S1 of the Supplementary Information). The -250 thorough -1 region of ribulose-1,5-bisphosphate carboxylase oxygenase (RuBisCO) gene of *S.* PCC7002 (GenBank Acc. No. D13971) designated as R6 promoter (Onizuka et al., 2002) was used for the expression of *phaCAB* genes.

2.5. RecA mutation

For the homologous recombination, 0.9 mL of the cell suspension (5×10^7 cells mL^{-1}) was mixed with 0.1 mL of the *recA*-Km^r cassette DNA solution (4 μg DNA mL^{-1}), and incubated at 39 °C in light (580 μmol photon cm^{-2} s^{-1}) by bubbling with 1% CO_2 in air for 1.5 h. Nine milliliter of the medium was then added to the culture, and cultured at 32 °C in light (100 μmol photon cm^{-2} s^{-1}). After 48 h, 10 mL of fresh medium was added, and cultured at 32 °C in light by bubbling with 1% CO_2 in air until the cell density became approximately 5×10^8 cells mL^{-1}. An aliquot of the culture was taken, and the culture for homologous recombination was repeated again. The liquid culture was plated on the agar plates containing Km (200 μg mL^{-1}), and the Km resistant colonies were isolated. The presence and absence of *recA* and *recA*-Km^r in the cyanobacterial genome were examined by PCR with the primers recA-S and recA-AS.

3. Results and discussion

3.1. RecA gene complementation as a selection pressure for plasmid maintenance

The bacteria–cyanobacterial shuttle vector pAQJ4 (Ikeda et al., 2002), and the R6 promoter (Onizuka et al., 2002) were used for

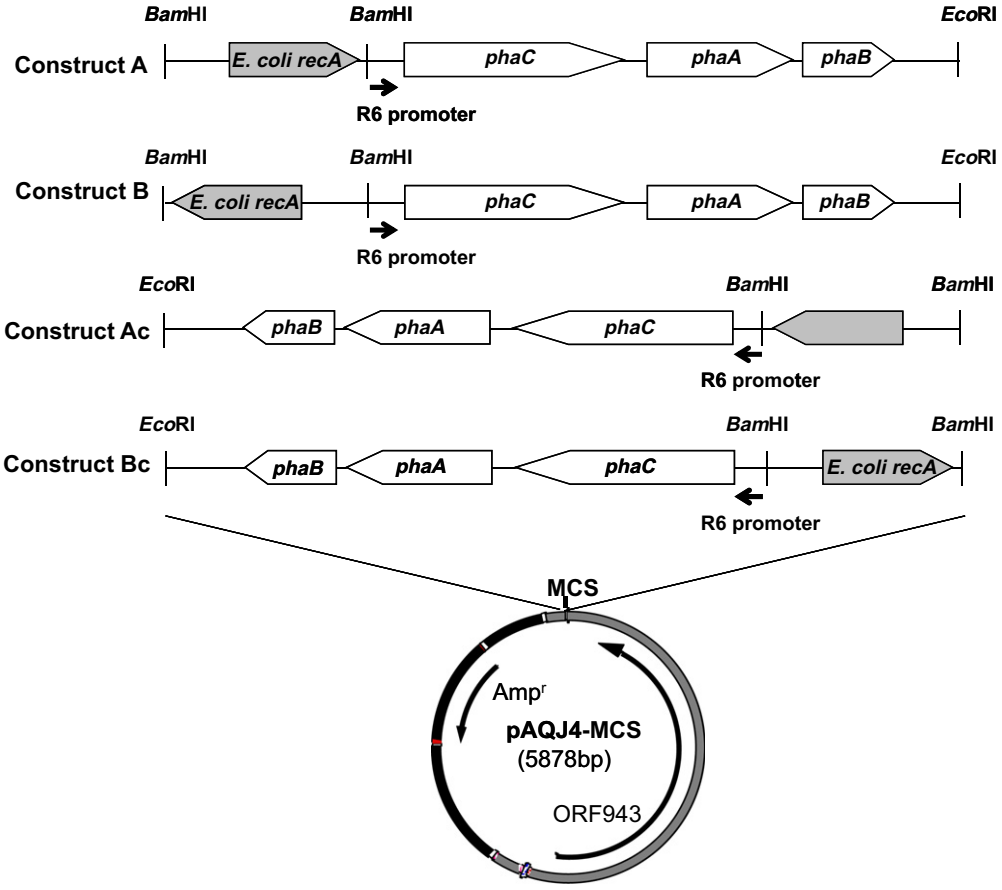

Fig. 1. DNA constructs on pAQJ4 vector for PHA production E. coli_recA-R6_promoter-phaCAB-pAQJ4 (Construct A), E.coli_recA (opposite)-R6_promoter-phaCAB-pAQJ4 (Construct B), E.coli_recA-R6_promoter-phaCAB-pAQJ4 (c) (Construct Ac), and E.coli_recA (opposite)-R6_promoter-phaCAB-pAQJ4 (c) (Construct Bc).