MANIPULATION OF THE GENES FOR POLY-β-HYDROXYBUTYRIC ACID SYNTHESIS IN ALCALIGENES EUTROPHUS.

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SUMMARY

In order to establish the molecular breeding system in Alcaligenes eutrophus producing poly-β-hydroxybutyric acid (PHB), phbCAB genes from A. eutrophus were recombined into the E. coli-A. eutrophus shuttle vector and directly transferred into A. eutrophus by the electroporation. In A. eutrophus transformants, recombinant plasmids were stably maintained and enzyme activities for PHB biosyntheses were elevated 1.4-2.7 fold by the cloned genes.

INTRODUCTION

Alcaligenes eutrophus synthesizes poly-β-hydroxybutyric acid (PHB), a biopolymer, as a carbohydrate storage material. The biosynthesis and degradation of PHB in A. eutrophus occurs via a cyclic metabolism and the synthesized PHB is accumulated as granules (Shively, 1974) in the bacterial cells.

The biosynthetic pathway started from acetyl-CoA is accomplished by the three sequential enzymatic reactions (Steinbuchel and Schlegel, 1991). In the initial step, 3-ketothiolase as a product of the phbA gene catalyzes the reversible condensation of two acetyl-CoA molecules to acetoacetyl-CoA and the following reduction step generates D(-)-β-hydroxybutyl-CoA by the NADPH-linked acetoacetyl-CoA reductase encoded by the phbB gene. Then PHB polymerase, product of the phbC gene, catalyzes the polymerization of D(-)-β-hydroxybutyl-CoA to poly-β-hydroxybutyric acid (Peoples and Sinskey, 1989a, b).

The accumulation rate of PHB in Alcaligenes eutrophus is stimulated when the carbon/nitrogen ratio in the medium is high, therefore a two step cultivation process is being employed for the achievement of high cell mass and elevations of the intracellular PHB content (King, 1982). In order to improve the bacterial strain of A. eutrophus, the genes for PHB
biosynthesis were isolated and recombined into the E. coli A. eutrophus shuttle vector. The recombinant plasmids constructed were directly transferred into A. eutrophus. In this report, we first expressed the cloned phbCAB genes in A. eutrophus and demonstrate the effect of cloned genes on the enzyme activities for PHB biosynthesis.

**MATERIALS AND METHODS**

**Bacterial strains and plasmids**

Alcaligenes eutrophus ATCC 17699 was used as a bacterial host strain and plasmid pHR52 (Kim et al., 1993) was used as a source for the phbCAB gene of A. eutrophus. Broad host range plasmid pKT230 (Franklin et al., 1981) in Escherichia coli DH5α was isolated and used as an E. coli-A. eutrophus shuttle vector (Park, et al., 1995) for transferring the genes into A. eutrophus.

**Recombinant DNA method**

Plasmid DNAs were isolated by the alkaline lysis method (Bimboim and Doly, 1979) and further purified by the ethidium bromide-CsCl density gradient centrifugation. Restriction enzymes, Taq-DNA ligase and klenow enzyme were purchased from Boehringer Mannheim (Indianapolis, IN, U.S.A.). Their reaction conditions followed the protocols suggested by the manufacturer. Other chemicals were of the highest grade commercially available.

**Culture conditions and media**

A. eutrophus was cultured in NR medium (10 g/l, polypeptone; 10 g/l, yeast extract; 5 g/l, beef extract and 5 g/l, ammonium sulfate, pH 7.0) (Sambrook et al., 1989) or a synthetic medium (3.8 g/l, Na₂HPO₄, 2.65 g/l, KH₂PO₄, 2 g/l, NH₄Cl; 20 g/l, fructose; 0.2 g/l, MgSO₄ and 1 ml/l, trace element solution). The medium was inoculated with a 5 % (v/v) inoculum of an overnight culture in NR medium. E. coli strain was grown in LB medium (10 g/l, bactotryptone; 5 g/l, yeast extract and 5 g/l, NaCl, pH 7.0). Liquid cultivations were carried out with shaking at 30 °C for A. eutrophus and 37 °C for E. coli.

**Transformation of A. eutrophus**

Bacterial transformation for A. eutrophus was accomplished by the electroporation method of Park et al. (1995). For preparing the competent cells, A. eutrophus cells grown in NR media to OD₆₀₀= 0.8 were washed three times with 50 ml of ice-cold deionized water and resuspended in 400 µl sterile distilled water containing 10% (v/v) glycerol. The cells were immediately frozen at -70 °C, thawed on ice and an aliquot of cell suspension (50 µl) in 0.2 cm width ice cold cuvette was mixed with 1.0 µg plasmid DNA dissolved in 5 µl of TE buffer (10 mM Tris-Cl, 1 mM EDTA, pH 7.0). For the electroporation, single pulses at 11.5 kV/cm of the electric field strength and 25 µF of capacitance with 5 msec of pulse time were performed by the electroporator (EASYJECT, Equibio S.A., Belgium). The pulsed cells were transferred to 1ml of fresh NR media and cultured for 2 h at 30 °C. Transformants were selected after cultivation for 48 h at 30 °C on a NR-agar plate containing kanamycin (200 µg/ml).

**Enzyme assay**

For assay of the enzyme activities involved in PHB biosynthesis, A. eutrophus strains including transformants were cultured in a 1.5 liter fermentor with synthetic media, pH 7.0 at 30 °C with air flow rate of 1vvm. The activities of β-ketothiolase (EC 2.3.1.9) and NADPH-depndant acetoacetyl-CoA reductase (EC 1.1.1.36) in crude cellular extract were determined.