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Full Length Research Paper

Combinations of spectrofluorometry and polymerase chain reaction for rapid detection of medium-chain-length polyhydroxyalkanoate-accumulating (Mcl-PHA) bacteria

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Medium-chain-length polyhydroxyalkanoates (Mcl-PHAs) share physical and material properties that are considered as an alternative to nonbiodegradable plastics. In this report, a new method was developed for detection of Mcl-PHA-producing bacteria. The procedure was composed of, first staining with Nile red and screen under fluorescence microscope at a wavelength of 565 nm, then further confirmation by amplifying the partial nucleic acid sequence of the PHA synthase gene *pha* with polymerase chain reaction (PCR). The stained positive Mcl-PHA-producing bacteria were also detected while subjected to transmission electron microscopy (TEM). This new method should therefore be useful for screening Mcl-PHA-producing bacteria from large numbers of microbial cultures time-efficiently.

Key words: Fluorescence spectroscopy, polyhydroxyalkanoates, *Pseudomonas* sp., polymerase chain reaction.

INTRODUCTION

Polyhydroxyalkanoates (PHAs) is a family of microbial intracellular biopolymers, which are synthesized by a variety of microorganisms as a temporary storage material of carbon and energy compounds under growth limited conditions (Rehm and Steinbüchel, 1999; Sun et al., 2007; Castilho et al., 2009). Mcl-PHAs share physical and material properties that recommend them for application in various areas, and are considered as an alternative to nonbiodegradable plastics produced from fossil oils (Spiekermann et al., 1999; Solaiman et al., 2000). PHA synthase is the critical enzyme in PHA biosynthesis. It was divided into four classes on the basis of the structure-function properties of the PHA synthase (polymerase) enzymes. In which, the second class (type II) *pha* operon was the main gene for Mcl-PHA synthesis (Kim et al., 2007). The type II system was commonly found in Mcl-PHA-producing *Pseudomonas* (Madison and Huisman, 1999; Solaiman and Ashby, 2005; Hazer and Steinbüchel, 2007).

Several screening protocols have been developed in the past to detect PHAs, but they were suffered from certain drawbacks and limitations. They were rather time-consuming and unavailable when cells were not producing or accumulating the polymers (Rehm and Steinbüchel, 1999; Chen et al., 2009). These applications depend on several prerequisites and constellations, only few strategies will allow the identification of PHA synthase genes encoding enzymes with unusual and or novel features. Hence, the main purpose of the present study was to investigate a potential method in the detection of type II genes and application to further

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Table 1. Bacteria positively cloned and characterized with PHA synthase genes.

Microorganisms	Origin	Synonyms or identities	Classes	References
Pseudomonas glathei	DSM50014	*Burkholderia glathei*	*pha* Ⅱ	Kung et al. (2007)
Pseudomonas oleovorans	DSM1045	-	*pha* Ⅱ	Potter and Steinbüchel (2005) Kim et al. (2007)
Pseudomonas putida	DSM291	*Arthrobacter siderocapsulatus*	*pha* Ⅱ	Kim et al. (2007), Sun et al. (2007)
Pseudomonas aeruginosa	MCCC1A00099	-/G-	*pha* Ⅱ	Rehm and Steinbüchel (1999)
Pseudomonas sp. KOR	From fossil oil	*Pseudomonas koreensis*	*pha* Ⅱ	This paper

investigations of Mcl-PHA synthases.

MATERIALS AND METHODS

The strains used in this study for PHAs analysis were all listed in Table 1. Three strains were purchased from DSMZ. *Pseudomonas aeruginosa* was provided by Marine Culture Collection Center of China (MCCC). *Pseudomonas* sp. KOR was isolated and identified from fossil oil samples (Shengli Oil Field, China). The medium used for PHAs accumulation was identical with inorganic salts medium (IS medium) utilizing D-glucose as sole carbon source (D-glucose 20.0 g/L, yeast powder 1.0 g/L, KH_2PO_4 0.5 g/L, NH_4Cl 0.5 g/L, $MgSO_4$ 0.5 g/L, pH7.0). *Escherichia coli* Top 10 was used as negative control. The enrichment medium was Luria-Bertani culture when required.

Solution of Nile red (Sigma, St. Louis, Mo. USA) (0.25 mg/mL in DMSO) was added to the sterilized IS medium to give a final concentration of 0.5 mg/L dye (Wu et al., 2003). PHA-accumulated bacteria incubated on solid medium Nile red plates were suspended in water and subjected to fluorescence microscope (Nikon TE2000-S/U, Japan). It was observed at a wavelength of 565 nm after appropriate cultivation periods to detect accumulation of PHAs and other lipid storage compounds.

Template DNA samples used for PCR were prepared as follows: a single colony was picked and resuspended in 15.0 µL sterile water. The cell suspension was heated to 99°C for 10 min. Five microliters of the reaction solution (*Taq* DNA polymerase 1U (Takara), 10 × PCR buffer (Mg^{2+} plus) 2 µL, dNTP Mixture (2.5 mM) 2 µL and primers 0.8 µL) was added. Then the PCR were performed as follows: initial denaturation at 94°C for 4 min, 30 cycles of 94°C for 30 s, 55°C for 30 s and 72°C for 1 min, and with a final extension at 72°C for 7 min. Forward (II-F; 5'-ACAGATCAACAAGTTCTACATCTTCGAC-3') and reverse (II-R; 5'-GGTGTTGTCGTTGTTCCAGTAGAGGATGTC-3') primers were used, which were based on two highly conserved sequences that were deduced from a multiple alignment analysis of the *Pseudomonad phaC* genes (Solaiman et al., 2000; Solaiman and Ashby, 2005). The PCR products were analyzed by agarose gel electrophoresis and patterns were visualized and photographed using a digital camera (GIS-2010; Tanon) under UV light.

The cells used for thin-section electron microscopic images were cultured in IS medium supplemented with D-glucose. Incubation was at 30°C with 180 rpm rotary shaking. The cells were shaken for about 72 h and then centrifuged at 3,000 rpm for 5 min. The sediment was washed three times with sterile sea water. The pellet was resuspended and fixed for 2 h in glutaraldehyde (2.5% in 0.1 M phosphate buffer, pH7.4), then washed three times with 0.1 M phosphate buffer (pH7.4) for 15 min. The cells were post-fixed with 1% osmium tetraoxide for 2 h and then dehydrated using 50% ethanol, 70% ethanol, and 90% acetone sequentially for 15 to 20 min each at 4°C. After that, the cells were dehydrated three times for 20 min with 100% acetone at room temperature (RT). After dehydration, cells were embedded in Durcupan's ACM epoxy resin. Polymerization of the resin to form specimen blocks was performed in an oven at 60°C for 24 h, and then the blocks were thin-sectioned, contrasted with 1% (w/v) uranyl-acetate and 1% (w/v) lead citrate. In the end, the sections (50 to 60 nm) were observed and photographed using a transmission electron microscope (JEM-1230; JEOL Company, Japan). In this study, the detection experiments were repeated three times with the same results, confirming reproducibility.

RESULTS AND DISCUSSION

Nile red is a promising fluorescence dye in rapid qualitative analysis of cellular lipids. Morphology of Nile red stained *Pseudomonad* excited by green light (565 nm) was observed under fluorescence microscope. *Pseudomonas* cells showed red fluorescence characteristics and were distinguished from negative control by the specific emission (Figure 1). Nile red fluorescence measurement could eliminate the negative effect of environment on fluorescence measurement. The direct PHA assays (lipophilic-dye staining) were not applicable when cells are not producing or accumulating the polymers. Under this specific excitation wave length, the Mcl-PHA producing cells stained Nile red were quickly and easily distinguished. The traditional direct assays needs much more time in incubations (Kung et al., 2007). So this method was fast.

Four different PHA-positive bacteria reported to accumulate Mcl-PHA were used in this study, while one PHA-negative strain (*E. coli*) was used to evaluate the detection efficiency of the PCR. The four detected strains belonged to the genus *Pseudomonas*. Based on the preceding report by (Potter and Steinbüchel 2005 Sheu et al., 2000), the *phaC* genes were detected. Among these bacterial strains, PCR products of about 500 bp found in five isolates showed that the genes contained *phaC1* and *phaC2* genes (Figure 2).

Although more and more genomic sequences data are available for the study of *pha* genetics, many other bacteria with interesting metabolic backgrounds still have

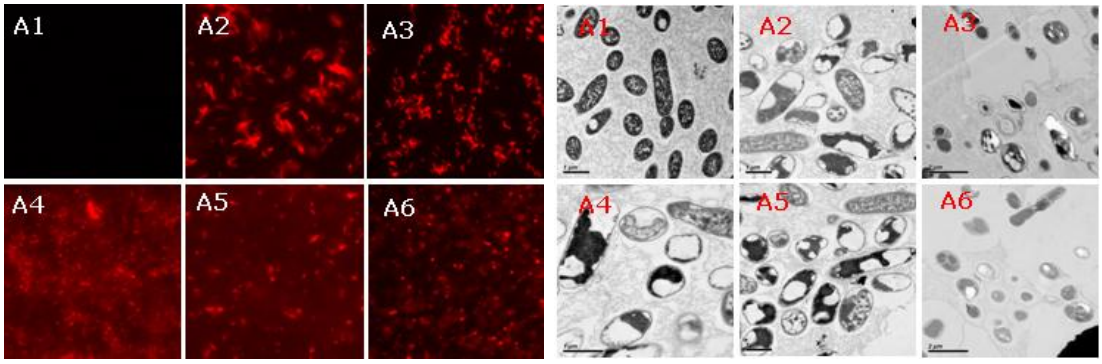

Figure 1. Fluorescence microscope images of Nile-red-strained *Pseudomonas* cells in ARE medium for 3 days and thin-section electron microscopic images for PHA granules in *Pseudomonas* cells. A1: *E. coli*; A2-A6: *Pseudomonas glathei, Pseudomonas oleovorans*, *Pseudomonas putida*, *P. aeruginosa* and *Pseudomonas* sp. KOR, respectively.

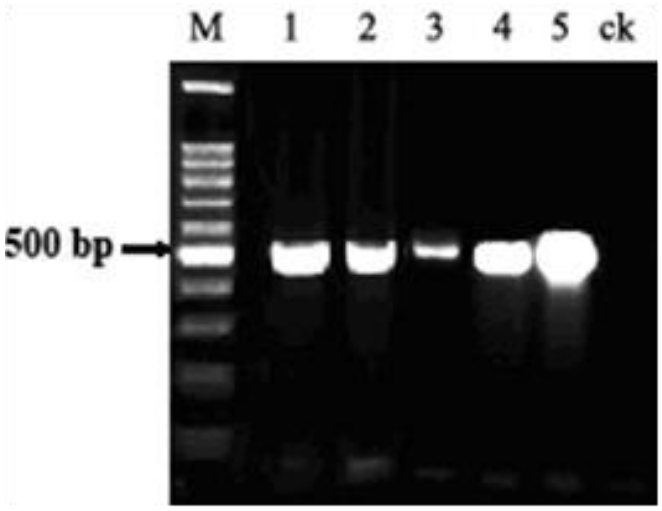

Figure 2. Results of type Ⅱ *phaC* gene amplification from DSMZ, MCCC and fossil oil strains by agarose gel electrophoresis. Lane M: DNA Maker 100bp; lane ck: *E. coli*; lanes 1 to 5: *Pseudomonas glathei, Pseudomonas oleovorans*, *Pseudomonas putida*, *Pseudomonas aeruginosa* and *Pseudomonas* sp. KOR, respectively.

not been sequenced (Huang et al., 2009). On the other hand, morphology observation of ultra-thin section under transmission electron microscopy (TEM) will provide more solid evidence of PHA production. When visualized by TEM imaging, the cells were grown for 72 h under PHA-inducing conditions exhibited the typical polymer-containing inclusion bodies (Figure 1). From the determination of Mcl-PHA producers by transmission electron microscope, we further confirmed the reliability and validity of the method. The method was especially important for the identification and verification of organisms that harbored Mcl-PHA biosynthesis gene of *Pseudomonas*. Moreover, this study indicated that the screening method was rapid and specific for identifying the Mcl-PHA polymer produced by *Pseudomonas koreensis* that has less been reported to date. This bacterium would be used for the production of high molecular weight Mcl-PHAs.

This method is of high sensitivity that enabled it a useful tool in detecting the accumulation of Mcl-PHA production. The method for the detection of *pha* II genes, combined with the genotypic detection and phenotypic detection overcame the major drawbacks set as a result of the increase in screening efficiency. The screening protocol described in this paper was a rapid, simple, and specific method for identifying Mcl-PHA-producing

microbes. Combinations of spectrofluorometry and polymerase chain reaction method for type II *pha* genetics detection were high efficiency to identify potential Mcl-PHA-producing microorganisms.

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