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Isolation of polyhydroxyalkanoate-producing bacteria from a polluted soil and characterization of the isolated strain *Bacillus cereus* YB-4

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ABSTRACT

We describe the characterization of polyhydroxyalkanoate (PHA)-producing bacteria isolated from an ammunition-polluted soil in Kitakyushu City, Japan. Over 270 strains were evaluated for PHA accumulation based on a colony staining method using Nile red. Of these, nine strains were selected based on the intensity of Nile red fluorescence and the cells were quantitatively analyzed for PHA by gas chromatography. PHA accumulation was observed in five strains, all of which are inferred to be close to the *Bacillus cereus* group according to 16S rDNA sequence analysis. Interestingly, these strains produced a PHA copolymer, poly(3-hydroxybutyrae-*co*-3-hydroxyvalerate) [P(3HB-*co*-3HV)], with a 3HV fraction up to 2 mol% with glucose as a carbon source. Further characterization was performed on one isolate, *B. cereus* YB-4. Gel permeation chromatography analysis revealed that the number of average molecular weights of PHA accumulated in *B. cereus* YB-4 drastically changed from 722,000 to 85,000 over a 72-h cultivation period. Furthermore, the PHA synthase genes were cloned and the deduced amino acid sequences were determined. This study provides new insights into PHA biosynthesis by members of the *B. cereus* group.

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1. Introduction

Polyhydroxyalkanoates (PHAs) are biopolyesters synthesized by bacteria as storage compounds for energy and carbon, normally under nutrient-limiting conditions with excess carbon. PHAs are biodegradable thermoplastics that can be obtained from renewable resources such as sugars and vegetable oils [1,2]. They are water insoluble, non-toxic, biocompatible, and have recently received attention because of their applications in the packaging industry, medicine, agriculture, and food industry.

Currently, there are plans to produce some PHAs on an industrial scale employing gram-negative bacteria such as *Ralstonia eutropha* and recombinant *Escherichia coli* [3,4] because these bacteria show better growth and higher accumulation of PHAs than others, including gram-positive bacteria. However, PHAs isolated from gram-negative bacteria contain outer membrane lipopolysaccharide (LPS) endotoxins, which induce a strong immunogenic reaction in humans. Hence, these PHAs are undesirable, particularly for biomedical applications. Gram-positive bacteria have thick outer membranes that do not contain LPS, and therefore, are potentially better sources of PHAs for use in biomedical applications [5]. In addition, some studies have addressed gram-positive bacteria as preferable candidates for host cells of PHA production. For example, members of the genus *Rhodococcus* which frequently resides in arid sites like deserts permits the cells to survive in stressful environments [30], and may therefore adapt to the case of PHA production under stressful conditions. *Corynebacterium glutamicum*, which has long been used for fermentation production of amino acids, has been investigated as a candidate for a safe and established platform for industrial PHA production [31].

Gram-positive bacteria have another potential advantage in terms of raw materials for PHA production. The gram-positive genera *Corynebacterium*, *Nocardia*, and *Rhodococcus* are capable of naturally synthesizing the commercially important copolymer poly(3-hydroxybutyrate-*co*-3-hydroxyvalerate) [P(3HB-*co*-3HV)] from abundant and inexpensive carbon sources such as glucose [4,6]. In contrast, gram-negative bacteria need expensive structurally related substrates such as propionic acid, valeric acid, or other fatty acids with an odd number of carbon atoms to produce 3HV units [4]. The relatively high expenditure involved is a major hindrance in PHA copolymer production. Hence, gram-positive producers could considerably reduce the production cost.

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The best characterized gram-positive bacterial group and the first PHA producer from the genus *Bacillus* was identified as *Bacillus megaterium* in 1926 [7]. Till date, many species of PHA-producing bacilli have been isolated from various environments and characterized. Some of these are able to produce PHA copolymers from inexpensive and structurally unrelated carbon sources. *Bacillus* sp. 88D isolated from a municipal sewage treatment plant is able to produce P(3HB-*co*-3HV) from glucose as a sole carbon source [8]. *Bacillus cereus* SPV is able to use fructose, sucrose, and gluconate to produce P(3HB-*co*-4-hydroxybutyrate) [P(3HB-*co*-4HB)] and P(3HB-*co*-3HV-*co*-4HB) [9]. *Bacillus* sp. INT005 isolated from a gas field soil produces P(3HB-*co*-3-hydroxyhexanoate) [P(3HB-*co*-3HHx)] from glucose [10]. The diversity of PHA products in the genus *Bacillus* are presumed to be due to class IV PHA synthase, which was recently identified as a new class and has relatively broad monomer specificity [4]. This indicates that the genus *Bacillus* could be used for the industrial production of PHA copolymer, which prompted us to isolate new copolymer producers with excellent cell growth and polymer accumulation from a unique environment in Kitakyushu City, Japan. Distinctly evolved bacteria such as toluene-degrading bacteria in an ammunition-polluted soil from this area have been reported [11]. Thus, this is a source of novel bacterial strains.

In this study, hundreds of bacteria isolated from a polluted soil in Kitakyushu City, Japan, were examined for their ability to produce PHAs with glucose as a carbon source. Nine PHA-producing strains were selected, and their products were further characterized. The strain YB-4 that showed the best production of PHA copolymer among the isolates, was further investigated with respect to the molecular weight of the PHA and its time-dependent change during cultivation. Furthermore, genetic analysis of the PHA synthase genes of the strain YB-4 was performed. This study provides a basis for further investigation of PHA biosynthesis by members of the *B. cereus* group.

2. Materials and methods

2.1. Samples

Samples were collected from an ammunition-polluted area located in Fukuoka Prefecture, Japan, in 2008. This area had been used as an ammunition bunker until 1972, and reportedly deformed frogs [12] and 2,4,6,-trinitrotoluene (TNT)-metabolizing bacteria [11], the likely cause for which was residual pollution, were found here. All samples used for this study were transferred to the laboratory and stored at 4 °C.

2.2. Isolation of PHA-producing bacteria

In order to isolate bacteria, a 10 g portion of each sample was homogenized in 30-mL autoclaved distilled water, and serial dilutions were plated onto agar medium (0.5 g yeast extract, 1 g peptone, 10 g glucose, 15 g agar per liter of deionized water) that contained Nile red (Sigma, St. Louis, MO, USA) at 0.5 mg per liter of medium [13]. The plates were incubated aerobically at 30 °C for 2 days, and the colonies were then exposed to UV-light to visualize the strains capable of producing PHA.

2.3. Polymer production

PHA production was carried out in LB medium (5 g yeast extract, 10 g tryptone, 10 g NaCl per liter of deionized water) plus 20 g/L glucose in a 500-mL shaking flask containing 100-mL medium at 30 °C for 72 h or less. After cultivation, the cells collected were washed with distilled water to remove the remaining carbon sources, and then lyophilized.

2.4. Polymer characterization

The PHA content of the lyophilized cells was determined by gas chromatography (GC) after methanolysis of the lyophilized cells in the presence of 15% sulfuric acid [14]. Methanolyzed 3HA was also analyzed by electron impact ionization gas chromatography–mass spectrometry (GC–MS) on a GCMS-QC2010 system (Shimadzu Co., Ltd., Kyoto, Japan) using an Inter Cap 1 column (GL Science Co., Ltd., Tokyo, Japan). The polymers accumulated in the cells were extracted with chloroform for 72 h at room temperature and purified by precipitation with methanol. Molecular weight data were obtained by gel permeation chromatography (GPC) as described previously [15].

2.5. PCR amplification, sequencing, and similarity analysis of 16S rDNA sequences

Eubacterial 16S rDNA was amplified using the following primer sets: 341F (5′-CCT ACG GGA GGC AGC AG-3′) and 907R (5′-CCG TCA ATT CCT TT[A/G] AGT TT-3′), corresponding to positions 341–357 and 907–926 in *E. coli*, respectively, and 27F (5′-AGA GTT TGA TCC TGG CTC AG-3′) and 1525R (5′-AAA GGA GGT GAT CCA GCC-3′), corresponding to positions 8–27 and 1543–1525 in *E. coli*, respectively. Bacterial colonies were picked using a sterile pipette tip and dipped into a PCR reaction mixture consisting of 5 U/μL Ex-Taq DNA polymerase (Takara Bio Inc., Shiga, Japan), 10 μM primers, 10 mM $MgCl_2$, and 10 mM reaction buffer (Takara Bio) in a final reaction volume of 50 μL. PCR was performed in a thermal cycler (MIR-D40, Sanyo Co., Osaka, Japan) using the following reaction conditions: 94 °C for 5 min, followed by 30 cycles at 94 °C for 30 s, 55 °C for 30 s and 74 °C for 1 min. Then, the PCR products were cloned into *E. coli* using a TOPO TA Cloning Kit (Invitrogen, Hilden, Germany). The cloned plasmids were extracted and purified using a Miniprep Plasmid Extraction Kit (Qiagen Co., Hilden, Germany). The DNA sequences of the plasmids were obtained using a DNA sequencing service (Hokkaido System Science Co., Ltd., Sapporo, Japan). All highly accurate sequences were compared to sequences deposited in the Genbank DNA database using the BLAST algorithm [16]. The full sequence of 16S rDNA (1544 bp) from the strain YB-4 has been deposited in DDBJ database (accession number AB535529). The formation of cereulide, the emetic toxin of *B. cereus*, was determined using a PCR-based assay in which the cereulide synthetase (*ces*) gene was targeted [17].

2.6. Biochemical characterization of the strain YB-4

The strain YB-4 was tested using an API 50 CH system (bioMérieux Co., Marcy l'Etoile, France). The API 50 CH strips were inoculated, incubated, and interpreted according to the manufacturer's instructions. Then, the data were analyzed with API labo software (bioMérieux). To analyze growth temperature, the strain YB-4 was cultured at 30–50 °C with constant shaking at 120 rpm for 72 h. Growth was monitored by measuring turbidity using a spectrophotometer (Gene quant *proS*; GE Healthcare UK Ltd., Buckinghamshire, England) at 600 nm.

2.7. Cloning of PHA synthase genes from the strain YB-4

The chromosomal DNA of the YB-4 strain was isolated and used as a template for PCR amplification of the PHA biosynthesis operon involving PHA synthase genes (*phaR* and *phaC*) and the 3-ketoacyl-CoA reductase gene (*phaB*). Oligonucleotide primers for

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