Isolation of a Gram-positive poly(3-hydroxybutyrate) (PHB)-degrading bacterium from compost, and cloning and characterization of a gene encoding PHB depolymerase of Bacillus megaterium N-18-25-9

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Abstract
A Gram-positive poly(3-hydroxybutyrate) (PHB)-degrading bacterial strain was isolated from compost. This organism, identified as Bacillus megaterium N-18-25-9, produced a clearing zone on opaque NB-PHB agar, indicating the presence of extracellular PHB depolymerase. A PHB depolymerase gene, PhaZBm, of B. megaterium N-18-25-9 was cloned and sequenced, and the recombinant gene product was purified from Escherichia coli. The N-terminal half region of PhaZBm shared significant homologies with a catalytic domain of other PHB depolymerases. Although the C-terminal half region of PhaZBm showed no significant similarity with those of other PHB depolymerases, that region was necessary for the PHB depolymerase activity. Therefore, this enzyme's domain structure is unique among extracellular PHB depolymerase domain structures. The addition of PHB to the medium led to a sixfold increase in PhaZBm mRNA, while the presence of glucose repressed phaZBm expression. The maximum activity was observed at pH 9.0 at 65°C.

Introduction
Polyhydroxyalkanoates (PHAs) are polyesters that are accumulated as energy and carbon storage materials by numerous microorganisms, when a nutritional component such as nitrogen, phosphorus, sulfur, oxygen, or magnesium is limited in the presence of an excess carbon source (Anderson & Dawes, 1990; Steinbuchel & Fuchtenbusch, 1998). PHAs have attracted industrial attention for use in the production of biodegradable and biocompatible thermoplastics.

PHAs can be divided into two classes: short-chain-length (SCL) PHA, which consists of three to five carbon atoms, and medium-chain-length (MCL) PHA, which consists of six to 14 carbon atoms (Jendrossek et al., 1996; Jendrossek & Handrick, 2002). Recently, several PHA-producing bacteria containing both SCL and MCL monomer units, have been identified. Aeromonas caviae and Aeromonas hydrophilia each produce a copolymer composed of 3HB and 3HHx [P(3HB-co-3HHx)] (Fukui & Doi, 1997; Fukui et al., 1998; Lee et al., 2000). This copolymer, which consists of SCL and MCL, possesses much better properties than SCL or MCL PHA polymers. The most prominent PHA is poly(3-hydroxybutyrate) (PHB). PHB exists as amorphous granules (native PHB) in the cells, but is transformed into a semicrystalline state (denatured PHB) after being extracted from the cell. Denatured PHB can be degraded only by specialized PHB-degrading bacteria that secrete extracellular PHB depolymerases into the environment (Jendrossek & Handrick, 2002).

Many extracellular PHA depolymerases have been purified from various microorganisms and characterized. However, most purified depolymerases are specific for SCL-PHA, such as PHB. The PHB depolymerases of several Gram-negative PHB-degrading bacteria, such as Alcaligenes faecalis AE122 (Kita et al., 1997), Comamonas sp. (Jendrossek et al., 1995a), Pseudomonas lemoignei (Jendrossek et al., 1993, 1995b), and Pseudomonas putida (Ohura et al., 1999), have been extensively studied, whereas a PHB depolymerase gene of a Gram-positive bacterium has been cloned only from Streptomyces exfoliates K10 so far (Klingbeil et al., 1996).

In this study, we isolated and characterized a Gram-positive PHB-degrading bacterial strain, Bacillus megaterium N-18-25-9. We also cloned a PHB depolymerase gene,
**PhaZ_{Bm}** of *B. megaterium* N-18-25-9, and expressed it in *Escherichia coli* to analyze its function.

**Materials and methods**

**Chemicals**

The PHB granules were purchased from Sigma-Aldrich (Steinheim, Germany).

**Bacterial strains, plasmids, and culture**

*Bacillus megaterium* N-18-25-9 was grown in nutrient broth (NB) medium at 30 °C. *Escherichia coli* XL10-Gold was used for genomic library construction. *Escherichia coli* DH5α was used as host for heterologous expression of PHB depolymerase. *Escherichia coli* strains carrying plasmids were grown at 30 °C in Luria–Bertani medium containing 50 μg mL⁻¹ ampicillin.

**Isolation and identification of bacterial strain**

Eighty-seven strains isolated from compost samples in the previous study (Takaku et al., 2006) were streaked onto NB containing 0.3% (w/v) PHB agar plate and incubated at 30 or 55 °C for 2 days under aerobic conditions. PHB-degrading bacteria were identified by the appearance of translucent halos on opaque PHB agar after 1–2 days.

For analyzing the 16S rRNA gene sequence, a part of 16S rDNA of the isolate N-18-25-9 was amplified using two oligonucleotide primers, 27f (5'-GTT TGA TCM TGG CTC AG-3') and 1389r (5'-ACG GGC GGT GTG TGT ACA AG-3'). The resultant PCR product was purified by agarose gel electrophoresis. The PCR product sequence was determined as described previously (Takaku et al., 2006). The closest relatives of nucleic acid sequences and deduced amino acid sequences were obtained using the sequence similarity search tools BLASTN and BLASTP.

**Northern blot analysis**

Total RNA was purified using RNasy kit (Qiagen, Hilden, Germany). The total-RNA samples (10 μg lane⁻¹) were separated by 1% formaldehyde-agarose gel electrophoresis and blotted onto a nylon membrane (Amersham Biosciences, Piscataway, NJ). Membranes were subsequently hybridized with digoxigenin (DIG)-labeled full-length PhaZ_{Bm} cRNA as a probe for PhaZ_{Bm} mRNA. The specific signals were detected with a digoxigenin system (Roche Diagnostics, Tokyo, Japan), according to the instruction manual from the manufacturer. The quantification results were obtained with LAS3000 mini system (Fuji Photo Film, Tokyo, Japan).

**Cloning of the PHB depolymerase gene of isolate N-18-25-9**

Genomic DNA of *B. megaterium* N-18-25-9 was isolated as described previously (Takaku et al., 2006) and partially digested with the restriction endonuclease Sau3AI. The partially digested DNA fragments (8–10 kb) were ligated into the BamHI-digested and dephosphorylated pUC19-vector. *Escherichia coli* XL10-Gold was transformed into the plasmids. A transformant, which showed a translucent clear zone on the indicator plate, was separated as a positive clone and purified, and its plasmid was isolated.

**Construction of an expression vector and purification of PhaZ_{Bm} from *E. coli***

The genomic DNA corresponding to Ala25-Lys590 of *B. megaterium* PHB depolymerase was subcloned in the vector pQE80L (Qiagen, Hilden, Germany) to generate pQE-FULL, as described below. A forward primer, 5'-ACA TGC ATG CGC AGG AAG TTT TAC CTC T-3' and reverse primer, 5'-CCC AAG CTT TTA CTT ATA TGA AAC AGT TAA AGT TGG-3', (containing a SphI site) and reverse primer, 5'-CCC AAG CTT TTA CTT ATA TGA AAC AGT TAA AGT TGG-3', (containing a HindIII site) were used. A DNA fragment encoding a putative catalytic domain (Ala25 to Gly331) was amplified from pQE-FULL using the following primers: 5'-ACA TGC ATG CGC AGG AAG TTT TAC CTC T-3'

The activity of P(3HB) depolymerase was determined as follows. The standard assay mixture (1 mL) contained 300 mg L⁻¹ of PHB granules and 1 mM CaCl₂ in 100 mM Tris-Cl buffer (pH 9.0). A stable suspension of purified PHB granules was prepared using a sonic oscillator (at 20 kHz, 250 W) for 20 min. The degradable reaction was started by the addition of enzyme (0.2 μg) and decrease in turbidity of PHB granules was monitored by measuring OD₆₀₀ₐ₅ of the sample in 1-cm-light-path cuvettes.

**Assay of the enzyme activity**

The activity of P(3HB) depolymerase was determined as follows. The standard assay mixture (1 mL) contained 300 mg L⁻¹ of PHB granules and 1 mM CaCl₂ in 100 mM Tris-Cl buffer (pH 9.0). A stable suspension of purified PHB granules was prepared using a sonic oscillator (at 20 kHz, 250 W) for 20 min. The degradable reaction was started by the addition of enzyme (0.2 μg) and decrease in turbidity of PHB granules was monitored by measuring OD₆₀₀ₐ₅ of the sample in 1-cm-light-path cuvettes. One unit of activity is...
defined as the decrease of one \( A_{650 \text{nm}} \) unit in 1 min. Sodium acetate buffer (100 mM) at pH 5 and 6, potassium phosphate buffer (100 mM) at pH 6 and 7, and Tris-HCl buffer (100 mM) at pH 7, 8, 8.5, 9, 9.5, and 10 were used in the depolymerase assay. To investigate the effect of reagents, they were added to the reaction mixture containing the enzyme, and then the mixture was incubated for 30 min at 37 °C. The reaction was started by adding a substrate at 37 °C for 20 min. The esterase activity was measured using \( p \)-nitrophenyl (PNP) esters as the substrate. The reaction mixture (1 mL) contained 50 mM Tris-HCl (pH 8.0) and PNP-alkanoate (final concentration, 0.25 mM). The reaction was initiated by adding 2 \( \mu \)g of enzyme, and the reaction mixture was incubated at 37 °C for 30 min. One unit of esterase activity was defined as the amount of protein required to produce 1 \( \mu \)mol of PNP from PNP-alkanoate per minute.

**DNA sequencing and data analysis**

DNA sequencing was carried out with a Beckman CEQ2000 capillary sequencer (Beckman Coulter, Fullerton, CA). The nucleotide and amino acid sequences were analyzed using the GENETYX-MAC program, version 11.2.2 (Software Development Co. Ltd., Tokyo, Japan), and BLAST on the DDBJ/GenBank/EMBL nucleotide sequence databases.

**Protein assay**

The protein concentration was determined as described by Lowry et al. (1951), and sodium dodecyl sulfate-12% polyacrylamide gel electrophoresis (SDS-PAGE) was performed as described by Laemmli (1970).

**Nucleotide sequence accession number**

The Genbank accession numbers of the 16S rRNA gene sequence of the strain N-18-25-9 and the nucleotide sequence of \( \text{PhaZ}_{Bm} \) are AB190040 and AB258388, respectively.

**Results and discussion**

**Isolation and identification of PHB-degrading microorganisms**

As biodegradable plastic products are decomposed by microorganisms into water and carbon dioxide, they can be composted along with organic waste. Therefore, we tried to isolate the PHB-degrading microorganisms from compost samples from the standpoint of co-treatment with organic waste. As described previously in culture-dependent analysis of a composting process, 87 strains were isolated from compost samples (Takaku et al., 2006). We screened one Gram-positive PHB degrading bacterium, N-18-25-9, from 87 strains based on their ability to form degradation halos on NB containing PHB agar plates. The N-18-25-9 was isolated from a composting bin that had been operating at pH 8.0–8.5 at 40–45 °C (Takaku et al., 2006).

Colony growth and light micrographic investigations indicated that N-18-25-9 belongs to the group of *Bacillus* spp. The N-18-25-9 cells were straight and rod-shaped and formed heat-resistant endospores. Cells were 0.5 \( \mu \)m wide and 4–5 \( \mu \)m long. The isolate N-18-25-9 grew at temperatures ranging from 20 to 50 °C, but not at 55 °C. These observations, together with the data obtained by 16S rRNA gene sequence determination, suggested that N18-25-9 belongs to a species of the genus *Bacillus*. Furthermore, phylogenetic analysis of the N-18-25-9 strain implied a close link with *B. megaterium* IAM 13418, with the highest sequence similarity of 99.5%. Based on these results, the isolated strain N-18-25-9 was identified as *B. megaterium*.

**Cloning and sequencing analysis of the PHB depolymerase gene \( \text{PhaZ}_{Bm} \) of isolated *B. megaterium* N-18-25-9**

About 3000 recombinant strains of *E. coli* DH5\( _{x} \) harboring partially Sau3AI-digested genomic DNA (8.0–10.0 kb) of *B. megaterium* N-18-25-9 in pUC19 were screened for PHB depolymerase activity on NB agar supplemented with 0.3% PHB. One clone produced a clearing zone on opaque PHB agar and indicated functional expression of PHB depolymerase activity. An analysis of the recombinant plasmid (pUCBM1) of the clone revealed the presence of a 9.7 kb Sau3AI fragment, of which a 2.7 kb SacI–HincII subfragment was cloned into pUC19 (pUCBMSh). Recombinant *E. coli* clones harboring pUCBMSh produced large clearing zones on opaque NB-PHB agar. The DNA sequence of a 2.7-kb fragment was determined (accession No AB258388). As a result, one ORF of 1773 bp was assigned to \( \text{PhaZ}_{Bm} \). The putative initiation ATG at nucleotide 1 was preceded at a spacing of 9 bp by a potential ribosome-binding protein at nucleotides –18 to –10 (5′-TAAGGAGGA-3′), and the sequences at nucleotides –128 to –123 (5′-TAGATT) and at nucleotides –105 to –100 (5′-CATCTT-3′) with 17 bp spacing showed some homology to the –35 and –10 *E. coli* σ\(^{70}\)-like promoter sequences (Fig. 1).

The encoded polypeptide is of 590 amino acids with a predicted molecular mass of 62.3 kDa. The first 24 amino acids revealed features of well-known signal peptides, and a signal peptidase cleavage site was predicted between Ala24 and Ala25 by the SignalP algorithm (Nielsen et al., 1997). A typical catalytic center, a lipase box containing a putative essential serine residue (Ser142) was located in the upper part of the amino acid sequence of \( \text{PhaZ}_{Bm} \). The positions of the catalytic triad (Asp218 and His291) and putative oxygen ion hole (His59) accorded with those in the type I catalytic
Fig. 1. Nucleotide sequence of the PhaZBm gene and deduced amino acid sequence of the gene product. A putative ribosome-binding [Shine–Dalgarno (S/D)] site and the /C0 35 and /C0 10 regions of a possible promoter sequence are in italics. The deduced amino acid of PHB depolymerase shows the signal peptide (underline), a catalytic domain (boxed) containing the lipase consensus sequence (shaded).
domains of some other extracellular PHB depolymerases (Fig. 1). Mature extracellular PHB depolymerases usually have served common domains such as a catalytic domain, a linker domain, and a substrate-binding domain (Jendrossek et al., 1995b; Romen et al., 2004). The BLAST analysis of the N-terminal half region with the catalytic domain of PHB depolymerase PhaZBm showed significant homologies with extracellular PHB depolymerases of many bacteria. The amino acid sequence of the catalytic domain showed sequence similarity with those of Bacillus sp. NRRL B-14911 PHB depolymerase (ZP_01169502, 61.6% identity), Azotococcus sp. EbN1 PHB depolymerase (YP_158658, 45.6% identity), and Pseudomonas lemoignei PHB depolymerase B (AAB17150, 41.7% identity). However, the C-terminal half region of PHB depolymerase PhaZBm showed no significant similarity with any PHB depolymerase except for Bacillus sp. NRRL B-14911 PHB depolymerase (ZP_011695, 47.1% identity). A part (332–412 amino acids) of C-terminal half region of PHB depolymerase PhaZBm showed significant similarity to the C-terminal domain of α-glucosidase of Clostridium acetobutylicum (AAK80209, 56.1% identity).

**Regulation of the expression of extracellular B. megaterium PHB depolymerase**

We examined the expression of the PhaZBm gene by Northern blot analysis at different time points after the addition of PHB. The results shown in Fig. 2a clearly indicate that the PhaZBm gene is weakly expressed in the absence of PHB, but can be induced by the addition of PHB to the medium. The PhaZBm mRNA level was elevated by eightfold 3 h after the addition of PHB. The addition of PHB induced the expression of the PhaZBm gene in B. megaterium N-18-25-9, while the synthesis of PHB depolymerases is derepressed in many strains after exhaustion of the nutrients. These data suggest that transcriptional control is involved. We also tested the expression of the PhaZBm gene after the addition of PHB and glucose. The addition of glucose and PHB completely repressed the expression of the PhaZBm gene after 3 h in B. megaterium N-18-25-9 (Fig. 2b), suggesting a catabolite repression of the PhaZBm gene. These results indicated that the synthesis and secretion of PHB depolymerase are subject to a double regulatory control: by induction in the presence of PHB and by catabolite repression in the presence of a more readily utilized substrate such as glucose.

**Expression and purification of recombinant phaZBm**

The cell lysate fraction from E. coli Origami B (DE3) harboring pQE-FULL showed strong PHB degradation activity in in vitro PHB depolymerase assay, though that from E. coli Origami B (DE3) harboring pQE80L (control) showed no activity. Two kinds of PhaZBm derivatives were constructed as histidine-tagged proteins to investigate the relation between domain structure and function. One is the wild type PHB depolymerase without signal peptides (PhaZBmFULL), and the other is the protein only with the catalytic domain (PhaZBmCAT). The purity of N-terminal histidine-tagged proteins was very high, as revealed by SDS-PAGE. Molecular masses of the fusion proteins purified from E. coli were consistent with those calculated based on the amino acid sequences (Fig. 3).

**Characterization of PHB depolymerase**

We determined the optimal temperature and pH for the PHB depolymerase activity. The purified N-terminal histidine-tagged PHB depolymerase PhaZBmFULL was most active at pH 9.0 (Fig. 4a) and 65 °C (Fig. 4b). We also tested whether the C-terminal half region of PhaZBmFULL was necessary for the PHB depolymerase activity. The purified PhaZBmCAT with the catalytic domain resulted in even more dramatic decreases in enzymatic activity under various conditions (Fig. 4). This result implied that the C-terminal half region of PhaZBm has an additional important role in...
the enzyme–substrate interaction, while this region has no apparent sequence similarity to the substrate-binding domain of other PHB depolymerases.

A hydrolysis reaction of soluble PNP-alkanoate (PNP-acetate, PNP-butyrate) by the depolymerases was performed to determine the esterase activity. The esterase activities of two enzymes (PhaZBmFULL, PhaZBmCAT) were almost the same (Table 1), suggesting that hydrolysis of soluble substrates such as PNP-acetate and PNP-butyrate is carried out by the catalytic domain of PHB depolymerase PhaZBm, and that esterase activity is not influenced by the presence of the C-terminal half region of PHB depolymerase PhaZBm.

The presence of a serine residue in the active site and the requirement of a disulfide bond for the activity are characteristic properties that may be conserved among many different SCL-PHA depolymerases (Jendrossek & Handrick, 2002). A serine esterase inhibitor, phenylmethylsulfonylfluoride, inhibited the depolymerase significantly (10% and 70% inhibition at 1 and 10 mM, respectively), suggesting that the active center of the enzyme is a serine residue in the lipase box (Ser142). Furthermore, a reducing agent, dithiothreitol, strongly inhibited the depolymerase reaction (70% and 90% inhibition at 1 and 10 mM, respectively), suggesting the presence of essential disulfide bonds in PhaZBm. Detergents such as Tween 20, Triton X-100, and SDS almost completely inhibited the reaction at 0.1%. Other potential inhibitors, such as KCN and NaN₃, did not affect the enzyme activity significantly even at a high concentration (10 mM) (data not shown).

It is well known that metal ions play a role in the maintenance of stable and active structures of enzymes by binding to the specific sites, which are generally formed by negatively charged amino acid side chains. The effect of
some mono- and divalent metal ions on \textit{B. megaterium} PHB depolymerase was tested in the presence of 1–100 mM of chloride salts of K\textsuperscript{+}, Na\textsuperscript{+}, Mg\textsuperscript{2+}, and Ca\textsuperscript{2+} ions. Up to 100 mM, the K\textsuperscript{+}, Na\textsuperscript{+}, and Mg\textsuperscript{2+} ions stimulated depolymerase activity, whereas 100 mM KCl inhibited depolymerase activity. However, depolymerase activity drastically increased in the presence of 1–100 mM Ca\textsuperscript{2+} (Fig. 5). The dependence of PHB depolymerase activity on Ca\textsuperscript{2+} was also reported for \textit{A. faecalis AE122}, \textit{Pseudomonas lemoignei}, and \textit{Pseudomonas stutzeri} PHB depolymerases (Jendrossek et al., 1996; Kasuya et al., 2000). The stimulation of the activity by Ca\textsuperscript{2+} is evidence that Ca\textsuperscript{2+} is necessary as a cofactor for PHB depolymerase activity.

Composting is a microbial process that is thought to be one of the most promising technologies for the biological management of solid wastes, and PHB is a good material for disposable plastic when used in combination with a solid-waste composting system. Because \textit{B. megaterium} N-18-25-9 was isolated at the maturation stage, when the supply of utilizable substrate such as glucose is scarce, and considering that it can grow at high temperature and that the optimal temperature and pH of its enzyme \textit{PhaZ}_{\text{rim}} are 60\,\degree C and 9, respectively, N-18-25-9 may be a utilizable bacterium for PHB degradation during the maturation stage of composting.

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**References**


