

**Relation of 3-Hydroxybutyrate Oligomer Hydrolase and
3-Hydroxybutyrate Dehydrogenase to Polyhydroxyalkanoate
Degradation in *Paracoccus denitrificans***

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Abstract

Microbial polyesters are high-molecular-weight compounds with degradability in natural habitat. They are attractive in industrial or commercial fields. Polyhydroxyalkanoates (PHAs) are examples of such polymers. It has well been known that PHAs are intracellularly synthesized and degraded by many bacteria because they are carbon and energy reserve materials. *Paracoccus denitrificans* used in this study is a methylotrophic bacterium which synthesizes different type of PHAs from different alcohols. Also, the strain was previously used for analyzing PHA synthesis in genetic and biotechnological studies. Intracellular PHA depolymerase gene (*phaZ*) of *P. denitrificans* was previously isolated and analyzed only for poly(3-hydroxybutyrate) (PHB) degradation. Although there are many studies on PHA synthesis, many things on the intracellular PHA degradation system has still remained to be cleared.

In this study, genes encoding 3-hydroxybutyrate oligomer hydrolase (3HBOH or PhaZc) and 3-hydroxybutyrate dehydrogenase (Hbd) were isolated from *P. denitrificans*. *P. denitrificans* NBRC13301 (PD01) was used. *P. denitrificans* Pd1222 genome sequence is available in the NCBI data base. By referring to its nucleotide sequence, PCR was performed with the *P. denitrificans* PD01 DNA. PCR products obtained were cloned in *E.coli* cells and sequenced, and the ORFs were named *phaZc* and *hbd*. When the *phaZc* and *hbd* cloned in pUC19 or pTAC-1 were expressed, two proteins with predicted molecular sizes were produced. 3HBOH activity was detected with 3-hydroxybutyrate (3HB) oligomers as a substrate. Also, Hbd activity was detected with 3HB as a substrate. It was demonstrated that the gene chosen as one of “hydrolases” was for 3HBOH. pQE30-*phaZc* and pQE30-*hbd* were then constructed in *E.coli* M15 to purify the enzymes as His-tagged proteins, His-PhaZc and His-Hbd.

For purifying His-PhaZc and His-Hbd, IPTG concentrations were optimized to avoid inclusion body formation. IPTG was used at 10 μ M and 0.1 mM for expressing His-PhaZc and His-Hbd, respectively. His-PhaZc and His-Hbd were purified via two-step

chromatography using affinity and gel filtration columns to a single band on SDS-PAGE. Purified His-tagged proteins had molecular masses of 31 kDa and 120 kDa (a tetramer of 29-kDa subunits). Enzymatic characteristics (substrates specificity, pH, temperature, and inhibitors) of His-PhaZc were determined and were almost the same as the enzyme previously purified as 3HBOH from *P. denitrificans*. Especially, the purified His-PhaZc catalyzed the hydrolysis of 3-hydroxyvalerate (3HV) oligomers in addition to 3HB oligomers. His-Hbd catalyzed the dehydrogenation of 3-hydroxyvalerate as well as 3-hydroxybutyrate at similar rate. When both enzymes were included in the same enzymatic reaction system with 3HV dimer, sequential reaction occurred, suggesting PhaZc and Hbd play an important role in the intracellular degradation of poly(3-hydroxyvalerate) (PHV) homopolyester and PHB-PHV copolyester.

The *phaZc* gene of *P. denitrificans* PD01 was disrupted in order to know the function of PhaZc in PHA degradation. Kanamycin resistant (Km^r) gene was introduced inside the *phaZc* region of *P. denitrificans* PD01 genome by a traditionally and commonly used procedure of homologous recombination. Colonies displaying Km^r were obtained on selective agar plates. One of them was chosen and named *P. denitrificans* PD01Km. Introduction of the Km^r gene into the *phaZc* region was confirmed by Southern blotting and PCR. A 1.2-kb *SalI* fragment (Km^r gene) from pUC4K was used as a probe for hybridization. The probe hybridized with the mutant DNA, but did not hybridize with the wide type DNA. When PCR was performed, the size of PCR product amplified with the mutant was approximately 1.3 kb larger than that with the wild type. PhaZc activity was not detected in *P. denitrificans* PD01Km. When the *phaZc*-disrupted *P. denitrificans* mutant was complemented with the *phaZc* gene, PhaZc activity was restored. These results suggest that *P. denitrificans* carries a single *phaZc* gene. Effect of the *phaZc* disruption on intracellular PHA degradation in *P. denitrificans* PD01 was examined. PHA contents in *P. denitrificans* PD01 and PD01Km were measured in both PHA synthesis and degradation phases. *P. denitrificans* PD01 and PD01Km were cultivated for analyzing cellular PHA contents. The strains were first grown to late exponential growth phase. The second cultivation stage was under carbon-sufficient and nitrogen-deficient. The third cultivation stage was under carbon-depleted conditions. In the second stage, the wild and

mutant strains produced PHA in a similar way. In the third stage, especially, in the first 5 h of this stage, PHA content in the wild strain decreased by 40.5% (w/w), whereas that in the mutant strain decreased by 26.3% (w/w). PHA degradation in the mutant strain preceded more slowly than in the wild type strain. It was demonstrated that PhaZc activity affects PHA degradation rate in *P. denitrificans* PD01.

Chapter 1

Introduction

1.1. PHA properties

In recent years, biodegradable plastics have been developed (Fig. 1). Polyhydroxyalkanoates (PHAs) are biodegradable and a family of polyhydroxyesters which are composed of 3-, 4-, 5- and 6-hydroxyalkanoic acids. In 1923, PHAs in bacteria first was recognized by Lemoigne who reported the formation of poly(3-hydroxybutyrate) (PHB) as a cytoplasmic inclusion inside bacterial cells (1). There are more than 150 different PHA constituents have been described during the last 20 years (2). PHAs have been divided into two groups: short-chain-length (SCL) PHAs and medium-chain-length (MCL) PHAs, based on the number of constituent carbon atoms in their monomer units. SCL PHAs contains 3-hydroxyfatty acids with 3 to 5 carbon atoms in the side chain. In contrast, MCL PHAs have side chains composed of 3-hydroxy fatty acids with 6 to 14 carbon atoms (3). PHAs are produced by a variety of bacterial species as intracellular storage compounds under nutrient-limiting conditions with excess carbon source. PHAs are biodegradable and exhibit thermoplastic and elastomeric properties. PHAs can renewable and recyclable, which can be degraded to carbon dioxide and water. Hence, they attracted much attention for using as new environmentally compatible materials in place of conventional petroleum-derived plastics.

1.2. Application of PHAs

PHAs have received more attention on account of their biodegradability and

biocompatibility, and they are applicable in chemical, medical, and pharmaceutical industries. Although the production cost of PHAs is still higher, they are considered to be replacement of conventional petrochemical products such as paper coatings, hot-melt adhesives, non-woven fabrics, performance additives, cosmetic materials, films, and diaphragms. PHAs are possible to use as scaffolding material in tissue engineering (4, 5) and surgical material (6). PHAs can be used for targeted drug delivery (7). The use of nanoparticles in many biomedical fields is rapidly increasing, and PHA granules have great potential candidate for the same applications. PHAs can also be used as sources to produce chiral hydroxyalkanoates (8). Blending PHAs with other high molecular weight compounds can improve their material properties and reduce production costs. PHA blends with natural fibres (9), rubber (10), and cellulose acetate phthalate (11) are reported. The microbial polymers are water-insoluble and highly polymerized, but the blends might exhibit improved material properties.

1.3. PHA synthesis and degradation in *Paracoccus denitrificans*

PHAs are produced and degraded in many bacteria strains (12). Poly(3-hydroxybutyrate) (PHB) and poly(3-hydroxyvalerate) (PHV) homopolyesters and poly(3-hydroxybutyrate-co-3-hydroxyvalerate) (PHB-PHV) copolyesters are well-known examples. Many studies of microbial PHA production have been carried out. PHAs are synthesized by a wide variety of bacteria including the species of *Aeromonas*, *Alcaligenes*, *Bacillus*, *Burkholderia*, *Pseudomonas*, *Ralstonia*, and *Rhodobacter*. Because of the useful application of PHAs as biodegradable polymeric materials, microbial and enzymatic degradation of PHAs have been studied (13-16).

P. denitrificans is classified as a facultatively methylotrophic bacterium (17, 18),

which can intracellularly synthesize and degrade PHAs. Intracellular pathway for the synthesis and degradation of PHB in PHB-producing bacteria is shown in Fig. 2. *P. denitrificans* synthesizes PHAs from several alcohols, such as methanol, ethanol, *n*-butanol, and *n*-pentanol (19, 20) (Fig. 3). It synthesized PHB homopolymer from ethanol or *n*-butanol (Fig. 3). PHV homopolymer was produced when *n*-pentanol was used as a carbon source. Copolymer of PHB-PHV was synthesized in the presence of methanol and *n*-propanol. Fig. 4 shows chemical structures of PHAs produced by *P. denitrificans*. When carbon sources were automatically fed to the culture with a fed-batch cultivation technique, cellular polyester content became much higher than that in batch cultivation. Cellular PHA content was relatively low under the nutrition-rich conditions. However, under carbon-sufficient and nitrogen-deficient conditions, PHA content was increased (21). Intracellular PHA degradation in *P. denitrificans* was also studied. PHB were degraded under carbon source starvation conditions. PHA depolymerase gene (*phaZ*) of *P. denitrificans* was identified and characterized. D-(-)-3-hydroxybutyrate (3HB) oligomer hydrolase (17) and 3HB dehydrogenase (22) were purified from *P. denitrificans*, but the genes of which were still unidentified.

1.4. Proteins and genes involved in PHB synthesis pathway

Proteins and genes related to PHB synthesis are investigated: PHB synthase (*phaC*), acetoacetyl-Coenzyme A (CoA) reductase (*phaB*), and β -ketothiolase (*phaA*).

The first step of PHB synthesis is catalyzed by β -ketothiolase. Two molecules of acetyl-CoA are condensed to produce acetoacetyl-CoA by this enzyme. Acetoacetyl-CoA is reduced to D-(-)-3-hydroxybutyryl-CoA by acetoacetyl-CoA reductase. As a final step, PHB synthase catalyzes the polymerization of

3-hydroxybutyryl-CoA to PHB. Genes encoding these enzymes have been isolated from PHA-producing microorganisms.

1.5. Proteins and genes involved in intracellular PHB degradation pathway

Proteins and genes related to intracellular PHB degradation have been investigated: PHB depolymerase (*phaZ*), 3HB-oligomer hydrolase (*phaZb* or *phaZc*), 3-hydroxybutyrate dehydrogenase (*bdh* or *hbd*), acetoacetyl-CoA synthase (*acsA*), and β -ketothiolase (*phaA*). PHB accumulated in cells serves as carbon and energy reserve materials for PHB-producing bacteria under some physiological conditions. PHB is first depolymerized to 3-hydroxybutyrate (3HB) monomers or 3HB oligomers (e.g., 3HB dimers, 3HB trimers, and 3HB tetramers) by PHB depolymerase. 3HB oligomers are then hydrolyzed to 3HB by 3HB oligomer hydrolase. 3HB is converted to acetoacetate by 3HB dehydrogenase. Acetoacetate is activated to acetoacetyl-CoA by acetoacetyl-CoA synthase. Acetoacetyl-CoA is then cleaved to produce two molecules of acetyl-CoA by β -ketothiolase. Finally, acetyl-CoA is metabolized via tricarboxylic acid or glyoxylic acid cycle.

1.6. PHA depolymerase

PHA depolymerase consists of two groups, intracellular depolymerases (iPhaZ) and extracellular depolymerases (ePhaZ). The former degrades amorphous PHA within granules accumulated in microbial cells, and the latter degrades denatured or crystalline PHA. Investigation of iPhaZ has been much less than that of ePhaZ.

ePhaZs are secreted from microbial cells through their cell membranes by a wide variety of bacteria in the environment. ePhaZ can degrade PHB into 3HB oligomers

and/or 3HB monomers. ePhaZ has been purified and characterized from various microorganisms such as *Alcaligenes* sp. (23), *Comamonas* sp. (24), *Acidovorax* sp. (25), *Paucimonas* sp. (26), *Rhodospirillum* sp. (27), and *Bacillus* sp. (14). Most ePhaZs catalyze denatured PHB (dPHB) with some exceptions. ePhaZ7 of *Paucimonas lemoignei* and ePhaZ of *R. rubrum* have been reported to degrade amorphous PHB (26, 27). Analysis of the primary structures of ePhaZ revealed that they were composed of catalytic, linker, and substrate-binding domains. The catalytic domain contains a catalytic triad (Ser-His-Asp). A serine residue exists at the center of “lipase box” pentapeptide (Gly-X-Ser-X-Gly) which has an important function for hydrolases such as lipases, esterases, and serine proteases. The substrate-binding domain is an important part in binding to the surface of crystalline PHB.

iPhaZs have been reported from several bacterial strains. In *P. denitrificans* (28), *R. eutropha* H16 (29), *B. thuringiensis* (30), *P. putida* (31), *B. megaterium* (32), and *Rhizobium (Sinorhizobium) meliloti* (33), iPhaZs were identified and characterized. iPhaZ from *R. eutropha* H16 degrades artificial amorphous PHB granules mainly to 3HB oligomers. iPhaZ from *P. denitrificans* degraded protease-treated PHB granules mainly to 3HB dimer. Purified His-tagged iPhaZ from *B. thuringiensis* efficiently degraded both native PHB granules and artificial amorphous PHB granules to 3HB monomer, but it could not hydrolyze denatured semicrystalline PHB. iPhaZ from *P. putida* KT2442 could catalyze MCL-PHAs to chiral 3-hydroxyalkanoic acids when the bacteria are cultured under carbon limitation. iPhaZ of *B. megaterium* can hydrolyze both native PHB (nPHB) granules and semicrystalline dPHB to mostly 3HB monomers. The *phaZ* gene of *S. meliloti* was disrupted in the cells. The mutant lacking iPhaZ activity revealed significant increase of PHB accumulation relative to that of wild type.

1.7. 3HB oligomer hydrolase

3HB oligomer hydrolase (3HBOH) is considered to be important for intracellular PHB metabolism. Although PhaZs were widely investigated for many years and from many bacteria, reports on 3HBOH are not so many. As in the case of PhaZ, 3HBOH divided into two groups: extracellular 3HBOH (e3HBOH) and intracellular 3HBOH (i3HBOH).

e3HBOH of *A. faecalis* (34) was purified to homogeneity and had a molecular mass of 74 kDa. The purified e3HBOH highly hydrolyzed 3HB oligomers, but did not act on PHB. The enzyme hydrolyzed the substrates from the carboxyl terminus, releasing D-(-)-3HB unit one by one. e3HBOH from *Pseudomonas* sp. strain A1 hydrolyzed D-(-)-3HB dimer and trimer at similar rates (15). Molecular mass of the hydrolase was estimated to be approximately 70 kDa.

i3HBOH have been reported from *Zoogloea ramigera* I-16-M (35), *P. lemoignei* (formerly *Pseudomonas lemoignei*) (36), *R. eutropha* (other names: *Wautersia eutropha* and *Cupriavidus necator*) H16 (37-39), *Acidovorax* sp. strain SA1 (16), and *P. denitrificans* (17). Genes encoding i3HBOH activity were investigated with a few strains (16, 36, 37, 39). These enzymes have been classified to PhaZb and PhaZc. Molecular mass of PhaZb from *R. eutropha* H16 was relatively large (78 kDa) and it hydrolyzed 3HB oligomers. The enzyme also degraded PHB granules to 3HB monomers. In case of PhaZc, the molecular mass of PhaZc from different bacteria ranged from 28 to 32 kDa. It was reported that PhaZc from *R. eutropha* H16 did not degraded native PHB granules and semicrystalline PHB. However, the PhaZc efficiently hydrolyzed various 3HB oligomers. PhaZc exhibited stronger 3HBOH activity than PhaZb (39).

1.8. 3HB dehydrogenase

3HB dehydrogenase (BDH) has widely been investigated with a variety of organisms. It is also important in PHB metabolism, in which it catalyzes the dehydrogenation of 3HB to acetoacetate. The enzyme has been investigated since 1960s. Biochemical works have been carried out with *P. lemoignei* (40), *Rhodopseudomonas spheroides* (41), *P. denitrificans* (42), *R. meliloti* (43), and *R. pickkettii* T1 (44).

The BDH genes were cloned from *R. meliloti* and *R. pickkettii* T1. This is the first example of genetic and molecular characterization of BDH (43), except for mammals. *R. pickkettii* T1 has two BDHs, BDH1 and BDH2 (44). The BDH1 and BDH2 were expressed in *Escherichia coli* and characterized. BDH1 activity increased when the cells were grown with organic acids, but not 3HB. In contrast, BDH2 activity significantly increased when grown with 3HB or PHB as a carbon source. It was suggested that the two BDHs had different biochemical properties and physiological roles.

1.9. Purposes of this research

In this study, 3HBOH (or PhaZc) and Hbd of *P. denitrificans* were investigated. Genetic and biotechnological studies on PHA synthesis in *P. denitrificans* have been carried out. PHA depolymerase has also been investigated. Although 3HBOH was previously purified from *P. denitrificans* and characterized in part, the investigation was not sufficient. 3HBOH and Hbd are considered to be important to understand the intracellular PHA degradation in *P. denitrificans*.

The purposes of this study are: (1) To isolate and analyze the 3HBOH (PhaZc) gene from *P. denitrificans*, because the gene has not reported from *P. denitrificans*. (2) To know some enzymatic properties of 3HBOH and Hbd. It was mainly focused on the

catalytic activities of 3HBOH toward 3HV oligomers and of Hbd toward 3HB. *P. denitrificans* can synthesize 3HV-containing PHAs such as PHV homopolyester and PHB-PHV copolyester as carbon-energy reserve materials. They are intracellularly degraded. Therefore, it was interested in whether 3HBOH and Hbd are related to the degradation of 3HV-containing PHAs. (3) To know the effect of disruption of 3HBOH gene (*phaZc*) on the PHA synthesis and degradation in *P. denitrificans*. In addition to the *in vivo* examination, the effect of the *phaZc* disruption on the PHA synthesis and degradation was examined *in vivo*. To do it, the *phaZc*-disrupted *P. denitrificans* mutant was constructed. Wild type and mutant strains were compared for their growth and PHA content.

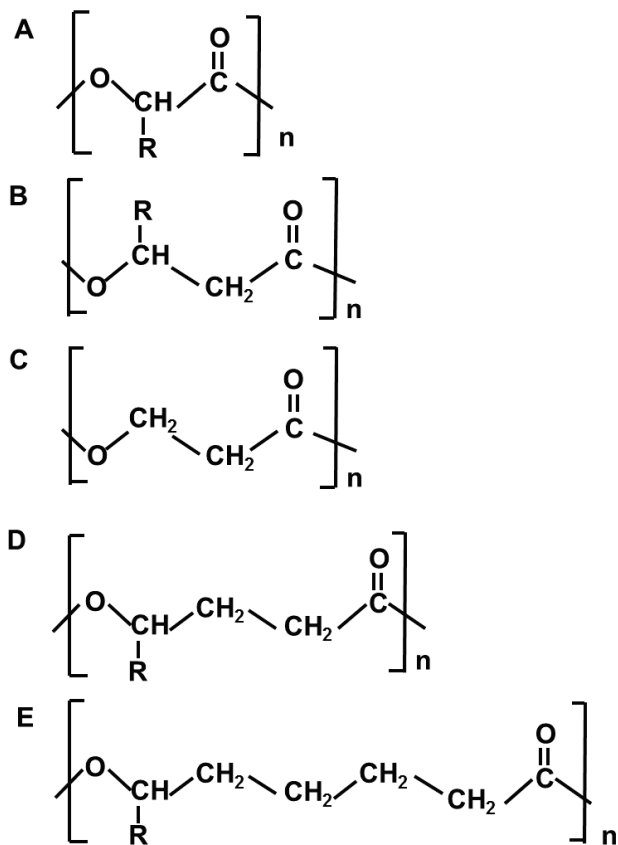


Fig. 1 Chemical structures of representative biodegradable plastics

A : (R=CH₃)PLA, poly(lactide)

B : (R=CH₃) PHB, poly(3-hydroxybutyrate)

C : PPL, poly(propiolactone)

D : (R=H) P(4HB), poly(4-hydroxybutyrate)

E : (R=H) PCL, poly(ε-caprolactone)

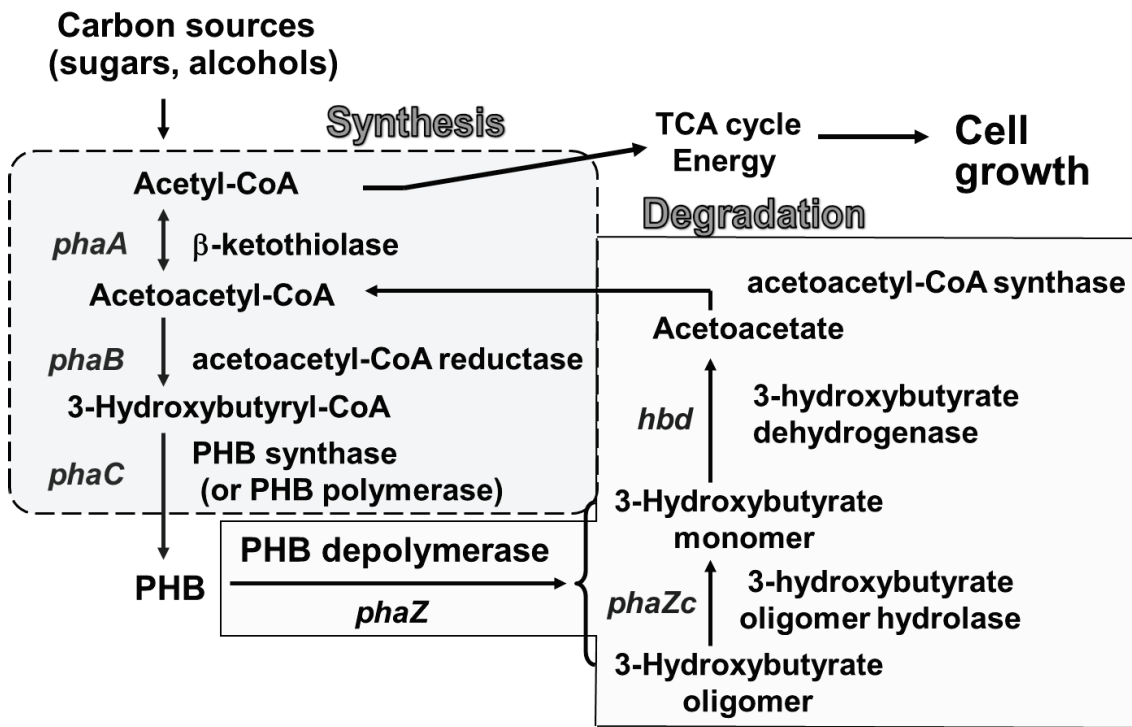


Fig. 2 Proposed PHB cycle in PHB-producing bacteria

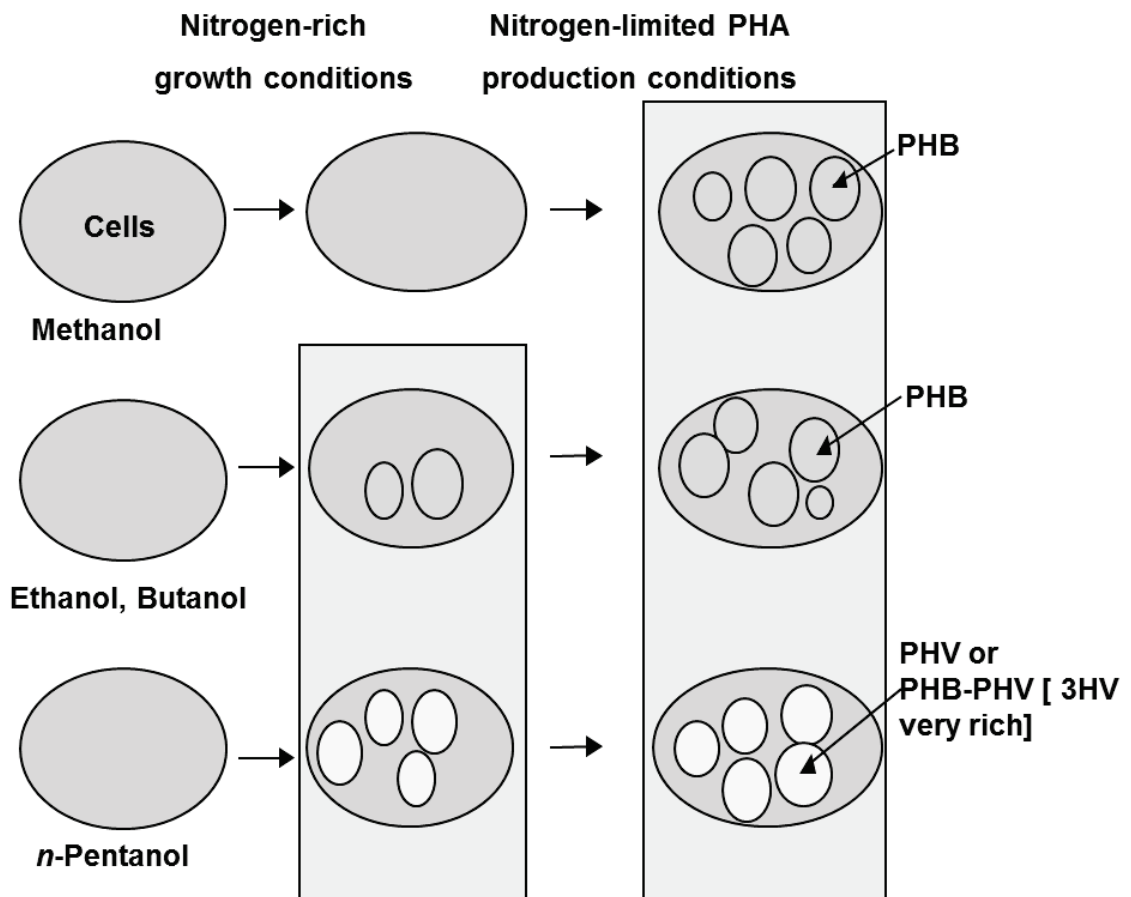


Fig. 3 PHA production profiles from alcoholic compounds by *P. denitrificans*

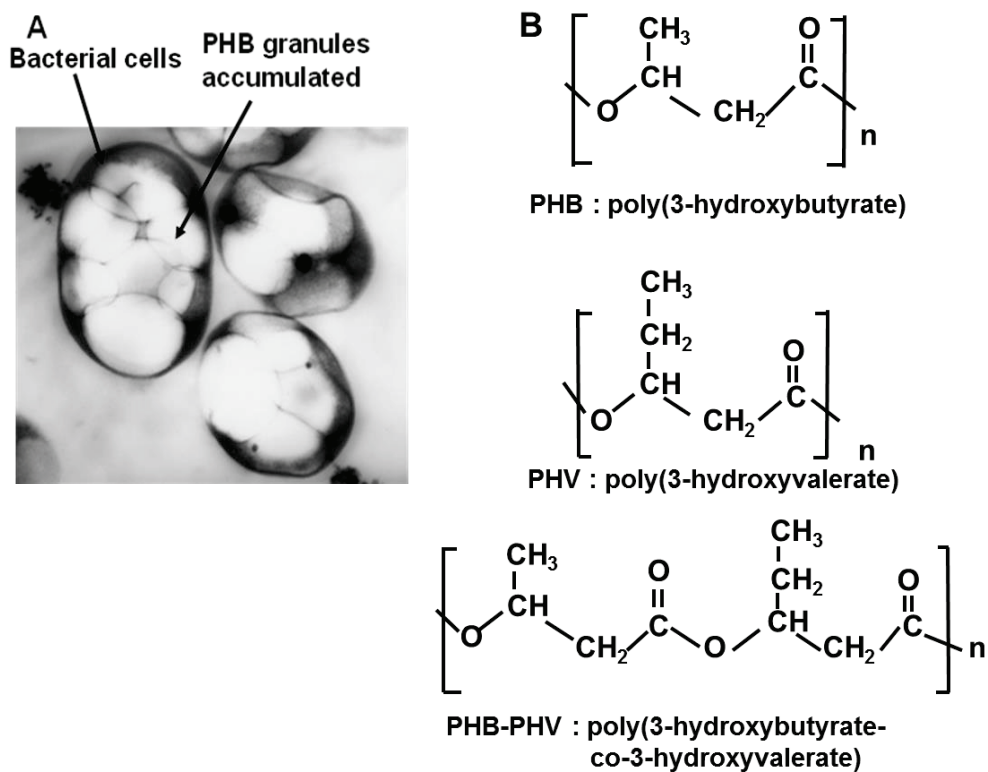


Fig. 4 PHB granules accumulated in the cells of *Paracoccus denitrificans* (A) and chemical structures of typical polyhydroxyalkanoates (B)

Chapter 2

Analysis of the *phaZc* gene of *P. denitrificans* PD01

2.1. Introduction

P. denitrificans is classified in a group of facultatively methylotrophic bacteria which are able to grow on one-carbon compounds such as methanol as a sole carbon and energy source. The bacterial strain can produce PHB homopolymer, PHV homopolymer, and PHB-PHV copolymers (PHB-PHV) from methanol, *n*-pentanol, and a mixture of methanol and *n*-pentanol, respectively. Ethanol can also serve as a carbon source for PHB production.

Up to date, genes related to intracellular PHA synthesis and degradation in *P. denitrificans* have previously been investigated with *P. denitrificans* ATCC17741. The *phaC* and *phaZ* of this bacterium has been cloned and analyzed. PHA synthase (PhaC) and PHA depolymerase (PhaZ) have also been characterized in detail.

It has been considered that 3HB oligomer hydrolase should play an important role on the intracellular degradation of PHA in some PHA-producing bacteria such as *R. eutropha* H16. The enzyme was previously purified from *P. denitrificans* ATCC17741 and analyzed for some enzymatic properties. Nevertheless, its gene has remained to be unknown. In order to complete the metabolic pathway for the intracellular PHA degradation in *P. denitrificans*, the 3HB oligomer hydrolase gene (*phaZc*) was investigated in this chapter.

2.2. Materials and methods

2.2.1. Bacterial strains, growth conditions, and plasmids

In Tables 1 and 2, all bacterial strains and plasmids used not only in this chapter but also in the other chapters are listed.

P. denitrificans NBRC13301 (named *P. denitrificans* PD01) was aerobically grown at 30°C in an inorganic salt medium. The medium (PD medium) was composed of (per liter) 3 g of (NH₄)₂SO₄, 1.4 g of KH₂PO₄, 3 g of Na₂HPO₄, 0.2 g of MgSO₄·7H₂O, 0.2 g of yeast extract, 0.3 g of NaHCO₃, 5.25 mg of MnCl₂·4H₂O, 5.25 mg of ZnSO₄·7H₂O, 0.53 mg of CuSO₄·5H₂O, 31.5 mg of Fe³⁺ citrate, 31.5 mg of CaCl₂·2H₂O, and 1% (v/v) ethanol. When specified, *n*-pentanol was used instead of ethanol at 0.05% (v/v). pH of the medium was adjusted to 7.0 with 3 M KOH. The PD medium without MgSO₄·7H₂O, Fe³⁺ citrate, CaCl₂·2H₂O, and ethanol (or *n*-pentanol) was sterilized by autoclaving at 121°C for 20 min. MgSO₄·7H₂O, Fe³⁺ citrate, and CaCl₂·2H₂O were separately autoclaved and aseptically added to the medium. Ethanol and *n*-pentanol were sterilized with a 0.2-µm filter membrane. The strain was grown aerobically at 30°C, and its growth was monitored by measuring the optical density (OD) at 660 nm with a spectrophotometer.

E. coli DH5α and JM109 were grown at 37°C in Luria-Bertani (LB) medium (10 g of bactotrypton, 5 g of yeast extract, and 5 g of NaCl per liter). When needed, the medium was supplemented with ampicillin (Amp, 50 µg/ml). pUC19 and pTAC-1 were used as plasmids for *E. coli*.

2.2.2. DNA techniques

The following DNA techniques were commonly used throughout this study. Glass and plastic wares and reagent solutions used were sterilized by autoclaving or with sterilized membrane filters.

2.2.2.1. Plasmid preparation

Plasmid DNAs were prepared by using a NucleoSpin Plasmid QuickPure (Macherey Nagel). A microcentrifuge tube containing 1.2 ml of overnight culture of *E. coli* cells was centrifuged at $11,000 \times g$ for 1 min. Cell pellet was suspended in 250 μ l of Buffer A1 containing 0.4 mg/ml of RNase A. To the cell suspension were added 250 μ l of Buffer A2. The tube was gently inverted several times, followed by the addition of 300 μ l of Buffer A3. After mixing well, the tube was centrifuged at $11,000 \times g$ for 5 min. The supernatant was transferred to a NucleoSpin Plasmid column placed in a collection tube. The column was centrifuged at $11,000 \times g$ for 1 min and washed with 450 μ l of Buffer AQ by centrifuging at $11,000 \times g$ for 3 min. DNA bound to the column was eluted with 50 μ l of Buffer AE by centrifuging at $11,000 \times g$ for 1 min. DNA solution was stored at -20°C .

2.2.2.2. Agarose gel electrophoresis

DNA fragments were separated on 0.8% (w/v) Agarose LE with a Mupid-2 minigel electrophoresis apparatus (Advance). TAE buffer (40 mM Tris, 20 mM acetic acid, and 1 mM EDTA) was used as a running buffer. Molecular size markers used were λ DNA-*Hind*III digest and 2-Log DNA ladder (New England Biolabs). DNA fragments in the gels were stained with ethidium bromide and visualized under ultraviolet light at 254 nm or 365 nm.

2.2.2.3. Recovery of DNA fragments from agarose gels

A NucleoSpin Extract II kit (Macherey Nagel) was used. After electrophoresis, a small piece of gel containing a target DNA was excised from the gel and put into a

microcentrifuge tube. NT buffer (200 μ l per 100 mg of the gel piece) was added to the tube. The tube was incubated at 50°C for 10 min to melt the gel piece. The melted sample solution was transferred to the silica column to bind DNA and centrifuged at 11,000 $\times g$ for 1 min. The column was washed with 600 μ l of NT3 buffer by centrifuging at 11,000 $\times g$ for 1 min and for additional 2 min to dry silica membrane. DNA bound to the membrane was eluted with 20 μ l of NE buffer by centrifuging at 11,000 $\times g$ for 1 min.

2.2.2.4. Determination of DNA concentrations

DNA concentrations were fluorometrically determined by using a Qubit dsDNA BR Assay kit (Invitrogen).

2.2.3. Preparation of the genomic DNA from *P. denitrificans* PD01

The *P. denitrificans* PD01 genomic DNA was prepared by using a Genomic-tip 100/G and buffer set (Qiagen).

P. denitrificans PD01 was cultivated in 4 ml of the PD medium at 30°C overnight. The cells were collected by centrifugation at 10,000 $\times g$ for 10 min and suspended in 3.5 ml of Buffer B1. To the suspension were added 7 μ l of RNase A solution (100 mg/ml), 80 μ l of lysozyme solution (100 mg/ml), and 100 μ l of proteinase K solution (600 AU/ml). After incubating at 37°C for 30 min to lyse cells, 1.2 ml of Buffer B2 were added to the lysate. The lysate was inverted gently several times and incubated at 50°C for 30 min. To remove insoluble debris, the lysate was centrifuged at 6,000 $\times g$ for 10 min. The supernatant solution was carefully transferred into a clean tube and then loaded onto a Genomic-tip 100/G column pre-equilibrated with 4 ml of Buffer QBT.

The column was washed twice with 7.5 ml of Buffer QC. The genomic DNA bound on the resin of Genomic-tip 100/G was elute with 5 ml of Buffer QF and precipitated at room temperature by adding 3.5 ml of isopropanol. The sample was gently inverted several times and stored at 4°C overnight. Precipitated DNA was collected by centrifugation at 11,000 ×g and 4°C for 15 min, and washed with 5 ml of 70% (v/v) ethanol. The precipitated DNA was air-dried, dissolved in 200 µl of TE buffer (10 mM Tris-HCl (pH8.0)-1 mM EDTA), and stored at -20°C.

2.2.4. Cloning of the *phaZc* gene of *P.denitrificans* PD01

2.2.4.1. PCR primers

In order to obtain the *phaZc* gene of *P.denitrificans* PD01 by referring the nucleotide sequence of *P.denitrificans* Pd1222 genomic DNA as described in the Result section. The following PCR primes were designed.

PDOHF2 (forward): 5'-CGAAGCTTGATGGCCGTCCAGTTCTTCC-3'

*Hind*III Start

PDOHR2 (reverse): 5'-CGGGATCCGTCTCTTCAGGTGCTTCCGC-3'

*Bam*HI

2.2.4.2. PCR conditions

PCR was performed with a KAPA Taq EXtra PCR with dNTPs kit (KAPA Biodynamics) as follows. A 0.8-kb fragment (named OHF2R2) was amplified.

PCR mixture (in 50 μ l)	
Sterilized Milli-Q water	33.25 μ l
5 \times KAPATaq EXtra Buffer	10 μ l
dNTPs (10 mM each)	1.5 μ l
MgCl ₂ (25 mM)	3.5 μ l
PDOHF2 (20 mM)	1.25 μ l
PDOHR2 (20 mM)	1.25 μ l
PD01 genomic DNA (46 μ g/ml)	1.0 μ l
KAPATaq EXtra DNA Polymerase	0.25 μ l

PCR conditions	
Initial denaturing	95°C, 4 min
Denaturing	95°C, 1 min
Annealing	51°C, 1 min
Extension	72°C, 1 min
Final extension	72°C, 5 min

} 30 cycles

2.2.4.3. Construction of pTAC1-OHF2R2

TA cloning was performed by using a DynaExpress TA PCR Cloning kit (Biodynamics Laboratory). Ligation mixture was incubated at 16°C for 30 min.

Ligation reaction mixture (in 10 μ l)	
pTAC-1 vector (50 μ g/ml)	1 μ l
OHF2R2 (50.2 μ g/ml)	3 μ l
2 \times Ligation Buffer	5 μ l
Ligase Mixture	1 μ l

After the ligation reaction, the reaction mixture was used for transforming Jet competent cells (*E. coli* DH5 α , Biodynamics Laboratory). The mixture (2 μ l) was mixed with 50 μ l of the competent cells, incubated on ice for 5 min, and transferred to a 1.5-ml microcentrifuge tube containing 0.45 ml of Recovery Medium. After vortexed briefly, the tube was incubated at room temperature for 5 min. Aliquots (50 μ l) of the cell suspension in the tube were spread onto LB agar plates containing Amp and 5-bromo-4-chloro-3-indolyl β -D-galactopyranoside (X-Gal) (40 μ g/ml). The plates were incubated at 37°C overnight. Colonies grown on the agar plates were screened for plasmid isolation. A plasmid named pTAC1-OHF2R2 was obtained and used for nucleotide sequencing with primers of T7 (5'-AATACGACTCACTATAG-3') and M13R (-20) (5'-GCGGATAACAATTTTCACACAGG-3'). Nucleotide sequence of the *phaZc* was deposited in the GenBank/EMBL/DDBJ databases under accession no. AB839771.

2.2.4.4. Construction of pUC19-*phaZc*

A 0.8-kb *Hind*III-*Bam*HI fragment (OHF2R2) was excised from pTAC1-OHF2R2 and inserted in the same restriction enzyme sites of pUC19. Restriction enzyme digested pUC19 was treated with bacterial alkaline phosphatase (BAP) (Takara).

Restriction enzyme reaction mixture (in 20 μ l)

Milli-Q water	6 μ l
<i>Bam</i> HI	1 μ l
<i>Hind</i> III	1 μ l
10 \times Buffer	2 μ l
pUC19 (22.4 μ g/ml)	10 μ l
or pTAC1-OHF2R2 (50.2 μ g/ml)	

The reaction mixture was incubated at 37°C for 3 h, and *Hind*III/*Bam*HI-digested pUC19 and OHF2R2 fragments were recovered from agarose gel after electrophoresis.

Ligation reaction was performed at 16°C for 30 min by using a DynaExpress DNA Ligation kit (Biodynamics Laboratory).

Ligation reaction mixture (in 20 μ l):

<i>Hind</i> III/ <i>Bam</i> HI-digested pUC19 (2.4 μ g/ml)	7 μ l
<i>Hind</i> III/ <i>Bam</i> HI-digested OHF2R2 (34.9 μ g/ml)	2 μ l
2 \times Ligation Buffer	10 μ l
Ligase Mixture	1 μ l

After the ligation reaction, 2 μ l of the ligation reaction mixture was mixed with 50 μ l of *E. coli* JM109 competent cells (Takara) in a microcentrifuge tube. The tube was incubated on ice for 30 min and heated for 45 sec at 42°C. After 0.45 ml of SOC medium (2% tryptone, 0.5% yeast extract, 10 mM NaCl, 2.5 mM KCl, 10 mM MgCl₂, 10 mM MgSO₄, and 20 mM glucose) was added, the tube was incubated at 37°C for 1 h.

Aliquots (50 μ l) of the cell suspension were spread onto LB agar plates containing Amp, isopropyl- β -thiogalactopyranoside (IPTG), and X-Gal. The plates were incubated at 37°C overnight, and colonies grown on the plates were screened for plasmid isolation. A plasmid named pUC19-phaZc was obtained.

2.2.5. Preparation of protease-treated PHA granules

Native PHA (PHB and PHV) granules were isolated from PHB-rich *Sinorhizobium fredii* NBRC14780 grown on mannitol and from PHV-rich *P. denitrificans* ATCC17741 grown on *n*-pentanol, respectively. *S. fredii* NBRC14780 can synthesize PHB in a growth-associated fashion (unpublished results) and was used to prepare native PHB granules. The bacterium was cultivated aerobically at 30°C in a medium (1L, pH 7.0) containing 0.1 g of KH₂PO₄, 0.7 g of K₂HPO₄, 0.5 g of MgSO₄·7H₂O, 1.0 g of yeast extract, and 10 g of mannitol. *P. denitrificans* ATCC17741 was cultivated as described for *P. denitrificans* PD01 (Chapter 2, Section 2.2.1.). The medium was the PD medium containing 0.05% (v/v) *n*-pentanol as a sole carbon source. PHA contents in the cells were determined by gas chromatography as described in Chapter 4 (4.2.4.). After cultivated, the cells were washed with 50 mM Tris-HCl buffer (pH 7.5), suspended in the same buffer, and disrupted by sonication at 4°C for 10 min three times. The sonicated suspension was centrifuged to remove cell debris at 2,000 \times g and 4°C for 10 min. Crude PHA granules contained in the turbid supernatant fractions were recovered as white pellets by centrifugation at 8,000 \times g and 4°C for 15 min. The precipitated PHA granules were treated with protease (0.1% (v/v) Alcalase 2.4L, Novo Nordisk A.S.) in a solution composed of 50 mM Tris-HCl (pH 7.5), 10 mM EDTA, 30 mM NaCl, and 0.2% (w/v) sodium oleate (as a protective agent) at 50°C for 60 min. After this

treatment, protease-treated PHA granules were washed with water twice and stored as a white suspension in water at 4°C.

2.2.6. Preparation of 3HA oligomers

3HA (3HB and 3HV) oligomers were prepared by degrading the protease-treated PHA granules with extracellular PHB and PHV depolymerases prepared from *P. lemoignei* ATCC17989. The bacterium was aerobically cultivated at 30°C in a mineral salt medium (1L, pH 6.8) containing 2.3 g of KH_2PO_4 , 5.8 g of K_2HPO_4 , 1.0 g of NH_4Cl , 0.5 g of $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$, 0.1 g of $\text{FeCl}_3 \cdot 6\text{H}_2\text{O}$, and 4.0 g of sodium succinate (for PHB depolymerase) or 2.0 g of sodium valerate (for PHV depolymerase)). Sodium valerate was prepared from valeric acid as described by Ch'ng et al. (45). After cultivating for 2 to 3 days, the culture supernatants were recovered by centrifugation at $8,000 \times g$ for 20 min. The supernatants were dialyzed against 20 mM Tris-HCl (pH7.5) overnight and used as crude enzyme solutions. PHB and PHV granules were degraded in the reaction mixture composed of 50 mM Tris-HCl (pH8.0), 1 mM CaCl_2 , and PHB or PHV depolymerase at 30°C.

2.2.7. Analysis of 3HA oligomers

2.2.7.1. Chromatographic analysis (HPLC)

After the degradation reactions of PHA granules, pH of the reaction mixture was adjusted to 2.0 with 1M H_2SO_4 . Degradation products in the mixture were recovered by liquid-liquid extraction with a Soxhlet extractor and diethyl ether. Ether layer was recovered, and ether was evaporated. The viscous residue was diluted with water and used for HPLC analysis with an Aminex Ion Exclusion HPX-87H column (0.75×30

cm, Bio-Rad). Five millimolar of H_2SO_4 was used as a mobile phase. Flow rate and temperature were 0.6 ml/min and 60°C , respectively. Detection was performed at 210 nm. Under these conditions, typical retention times for 3HB monomer, 3HB dimer, 3HB trimer, 3HV monomer, and 3HV dimer were 14.1 min, 15.7 min, 17.5 min, 16.7 min, and 23.5 min, respectively. Monomer, dimer, and trimer (a trace amount) of 3HB were identified as the products from PHB granules. Monomer and dimer of 3HV were products from PHV granules. 3HB dimer and 3HV dimer fractions were recovered and used as substrates for 3HB oligomer hydrolase reactions.

2.2.7.2. Colorimetric analysis (Hestrin's method)

Concentrations of the oligomers were determined as water-soluble ester compounds by the alkaline hydroxylamine procedure (Hestrin's method) (46) as follows. To 0.2 ml of solutions containing the oligomers was added 0.4 ml of alkaline hydroxylamine solution (an equal volume mixture of 2 M hydroxylamine-HCl and 3.5 M NaOH). After 1 min, 0.2 ml of 4 M HCl and 0.2 ml of 0.37 M ferric chloride (in 0.1 M HCl) were added. Optical density of the mixture was measured at 540 nm.

2.2.7.3. Correlation between HPLC analysis and colorimetric analysis

In order to examine the correlation between peak areas of the oligomers in HPLC analysis and the values of OD_{540} in the alkaline hydroxylamine method, the same volume of 3HB dimer (or 3HV dimer) solution was used for the alkaline hydroxylamine assay and HPLC analysis. 3HB trimer was a previous gift from Prof. T. Saito of Kanagawa University. It was also used for this purpose. The significant correlation was obtained (Fig. 5). Since spectrophotometric assay was much convenient, the amount of

3HB or 3HV dimers in enzymatic reactions was routinely measured at OD₅₄₀.

2.2.8. Preparation of cell-free extracts

E.coli JM109 harboring pUC19-phaZc was cultivated in LB medium in the presence of Amp. To prepare cell-free extracts, cells were suspended in 50 mM Tris-HCl (pH 7.0) and disrupted by sonication at 4°C for 10 min three times. After the sonication, supernatant was recovered by centrifuging at 11,000 × *g* and 4°C for 10 min. The supernatant was dialyzed against 20 mM Tris-HCl (pH 7.0) at 4°C overnight and stored at -20°C.

2.2.9. 3HB oligomer hydrolase (PhaZc) assay

PhaZc activity was assayed based on the amount of 3HB or 3HV oligomers consumed during enzymatic reactions. The reaction mixture (1 mL) contained 50 mM Tris-HCl (pH 8.0), substrate solution, and cell-free extracts (74 μg protein). Enzymatic reactions were performed at 30°C. The amount of oligomers remaining in 0.2 ml of the reaction mixture was measured by the method of Hestrin (46) as described above.

2.2.10. Other methods

2.2.10.1. Protein concentrations

Protein concentrations were determined by the method of Lowry et al. (47) with bovine serum albumin as a standard.

2.2.10.2. Sodium dodecyl sulfate (SDS)-polyacrylamide gel electrophoresis (PAGE)

SDS-12.5% (w/v) PAGE was performed as described by Laemmli (48). Compositions of running and stacking gels were as follows.

Running gel (12.5%, 15 ml):

Acrylamide-Bis mixture* ¹	4.2 ml
1.5 M Tris-HCl (pH 8.8)	3.75 ml
Milli-Q water	6.75 ml
APS (0.1 g/ml)* ²	0.15 ml
SDS	0.15 ml
TEMED* ³	15 µl

*¹ 30% (w/v) acrylamide-0.8% (w/v) *N,N'*-methylene-bis(acrylamide)

*² Ammonium persulfate

*³ Tetramethylenediamine

Stacking gel (5%, 5 ml):

Acrylamide-Bis mixture	0.55 ml
1.5 M Tris-HCl (pH 6.8)	0.625 ml
Milli-Q water	3.725 ml
APS (0.1 g/ml)	0.05 ml
SDS	0.05 ml
TEMED	5 µl

Electrophoresis was performed at 20 mA with a running buffer (3.03 g of Tris, 14.4 g of glycine, and 1 g of SDS, pH 8.3, 1 L). Sample buffer (0.8 ml) composed of 0.05 ml of 1 M Tris-HCl (pH 6.8), 0.2 ml of glycerol, 0.02 ml of 0.5% (w/v) bromophenol blue,

0.2 ml of 10% (w/v) SDS, and 0.33 ml of Milli-Q water. An equal volume mixture of enzyme solution and sample buffer was mixed and subjected to electrophoresis. M.W. Marker of “Daiichi” III (for Laemmli’s method) (Daiichi Pure Chemicals) was used as a molecular mass standard. Protein bands in the gel were visualized with Imperial Protein Stain (Thermo Scientific).

2.3. Results

2.3.1. Correlation between colorimetric and HPLC assays for measuring 3HB and 3HV oligomers

It was examined whether colorimetric method (alkaline hydroxylamine method) described by Hestrin is applicable for assaying 3HB and 3HV oligomers. The colorimetric method was first developed to assay acetylcholine and other carboxylic acid derivatives (e.g., ethyl acetate, methyl butyrate, tributyrin, and triacetin). 3HB oligomers have usually been assayed as monomeric 3HB after 3HB oligomers were converted to 3HB monomer in 3HB oligomer hydrolase reaction. HPLC assay has also been used.

As described in the methods section of this chapter (2.2.7.3.), significant correlation was obtained between colorimetric and HPLC assays (Fig.5). Therefore, the assay of 3HB and 3HV oligomers was performed by the colorimetric methods. HPLC assay was also used along with the colorimetric assay.

2.3.2. Cloning of *phaZc* gene

In our preliminary experiments, 3HBOH activity was detected in crude cell-free extracts of *P. denitrificans* PD01. However, any genes encoding 3HBOH activity have

not been identified. *P. denitrificans* DNA did not hybridize with the *phaZc* regions from *R. eutropha* H16 and *Acidovorax* sp. strain SA1 (data not shown). The complete nucleotide sequence of the *P. denitrificans* Pd1222 genomic DNA is available in the NCBI database (accession number NC_008686.1). Based on BLAST searching (blastp) with PhaZc from *R. eutropha* H16 (39), an open reading frame (ORF) annotated as “hydrolase or acyltransferase” (gene id: 4580834, protein id: YP_915445.1) in the nucleotide sequence of *P. denitrificans* Pd1222 DNA might encode PhaZc activity (Fig. 6). According to this result, PCR primers for cloning the *phaZc* of *P. denitrificans* PD01 were designed. PCR was performed.

A 0.8 kb DNA fragment was amplified with the primers of PDOHF2 and PDOHR2 by PCR. The amplified DNA fragment (OHF2R2) was introduced into a pTAC-1 plasmid and its restriction map was constructed with the restriction enzymes of *Sph*I and *Sal*I. The map coincided with that for the nucleotide sequence region of “hydrolase or acyltransferase” of *P. denitrificans* Pd1222. The constructed plasmid was named pTAC1-OHF2R2 and the nucleotide sequence of the OHF2R2 fragment was determined.

2.3.3. Nucleotide sequence analysis of the OHF2R2 fragment

Size of the nucleotide sequence of OHF2R2 was 879 bp. There was an 828-bp open reading frame (ORF) which starts with ATG start codon and terminates with TGA stop codon. Nucleotide sequence of the ORF was identical to that of “hydrolase or acyltransferase” of *P. denitrificans* Pd1222. Estimated molecular mass of the translated product composed of 275 amino acids was 30.1 kDa (Fig. 7). Lipase box-like motif (Gly-Thr-Ser-Arg-Gly) existed in the deduced amino acid sequence. A PHA-producing

bacteria, *R. eutropha* H16 contains two 3HBOH named PhaZb and PhaZc. The deduced amino acid sequence of the ORF was similar to PhaZc of *R. eutropha* H16 as described in Discussion section of this chapter. From these results, the ORF should code for 3HBOH activity of *P. denitrificans* PD01 and the ORF was named *phaZc*.

2.3.4. PhaZc expression

In order to confirm that the 828-bp ORF encodes 3HBOH activity, pUC19-*phaZcF2R2* was constructed. In this plasmid, the ORF was in-frame with the *lacZc* gene of pUC19. 3HBOH activity was assayed with cell-free extracts of *E. coli* JM109 harboring pUC19-*phaZcF2R2*.

As shown in Fig. 8A, molecular mass of expressed protein was about 30 kDa. PhaZc reactions were performed. When 3HB dimer was used as a substrate, a decrease in the substrate concentration was clearly observed, indicating that the cloned ORF encodes PhaZc activity (Fig. 8B).

2.4. Discussion

Intracellular 3HB oligomer hydrolases (3HBOHs) have been isolated and characterized from several bacterial. In preliminary experiments, 3HBOH activity was detected in cell-free extracts of *P. denitrificans* PD01. However, the 3HBOH gene has not been isolated. The complete nucleotide sequence of the *P. denitrificans* Pd1222 genome is available in the NCBI database (accession number NC_008686.1). Based on BLAST searching (blastp) with PhaZc from *R. eutropha* H16 (39), we hypothesized that an open reading frame (ORF) annotated as “hydrolase or acyltransferase” (gene id: 4580834, protein id: YP_915445.1) in the nucleotide sequence of *P. denitrificans*

Pd1222 DNA might encode 3HBOH activity. PCR primers (PDOHF2 and PDOHR2) were designed so as to amplify the *P. denitrificans* PD01 DNA region corresponding to the “hydrolase or acyltransferase” of *P. denitrificans* Pd1222. A 0.8 kb DNA fragment was obtained by PCR. From the nucleotide sequence analysis of the amplified DNA fragment, it was expected that the 3HBOH gene of *P. denitrificans* PD01 was cloned in *E. coli*. It contained 879 nucleotides and an ORF of 828 nucleotides was found. The estimated molecular mass of the translated product (275 amino acids) was 30.1 kDa. The nucleotide sequence of the ORF was identical to that of *P. denitrificans* Pd1222. The pentapeptide motif (Gly-Thr-Ser-Arg-Gly) was found in the deduced amino acid sequence of the ORF. The motif (Gly-X-Ser-X-Gly) has been known as a lipase box, which plays an essential role in lipase, esterase, and protease activities (49). The lipase box-motif has also been found in some 3HBOHs.

P. denitrificans DNA did not hybridize with the *phaZc* regions from *R. eutropha* H16 and *Acidovorax* sp. strain SA1 (data not shown). Nucleotide sequence of the *phaZc* from *P. denitrificans* PD01 was compared with those of the *phaZc* genes from *R. eutropha* H16 and *Acidovorax* sp. strain SA1 using the blastn program. Similarity was very low. The codon usage may be significantly different among these strains. It is the reason why *phaZc* regions of these strains did not hybridize with the *P. denitrificans* PD01 DNA. However, with the blastp program, it was found that the deduced amino acid sequences of *phaZc* from *P. denitrificans* PD01 and other strains were relatively similar. The deduced amino acid sequence of *P. denitrificans* PD01 PhaZc was compared with those reported for *R. eutropha* (PhaZc), *Acidovorax* sp. strain SA1 (i3HBOH), and *P. lemoignei* (PhaZc). Identities and similarities were 36%/48%, 34%/47%, and 32%/47%, respectively (Fig. 9).

Since the expressed protein had 3HBOH activity, it was concluded that an ORF found in the PCR-amplified fragment encoded PhaZc of *P. denitrificans* PD01.

The *phaZc* and *hbd* (described in Chapter 5) genes identified here were found by using the genome sequence of *P. denitrificans* Pd1222. Their nucleotide sequences were identical to those from *P. denitrificans* Pd1222, which is a derivative of *P. denitrificans* DSM413 (50). *P. denitrificans* PD01 resides in the same group and is closely related to *P. denitrificans* DSM413 on the basis of 16S rRNA gene sequence comparisons, DNA-DNA hybridization, and cytochrome *c* profiles (18). Therefore, it was not unexpected that the nucleotide sequences of the *phaZc* and *hbd* genes from the two strains are identical.

Table 1 Bacterial strains used in this study

Strains	Descriptions	Sources or references
<i>P. denitrificans</i>		
PD01	Wild type	NBRC 13301
PD01Km	PD01 derivative, $\Delta phaZc$, Km ^r	This study
PD01KmOH	PD01Km derivative harboring pBBR1MCS4-phaZc, Km ^r , Amp ^r	This study
PD41	PHV producer	ATCC17741
<i>E. coli</i>		
JM109	PhaZc expression host strain	Takara
DH5 α	TA-cloning host strain	BioDynamics
M15/pREP4	His-PhaZc and His-Hbd expression host strain	Qiagen
<i>S. fredii</i>	PHB producer	NBRC14780
<i>P. lemoignei</i>	Source of extracellular depolymerases	ATCC17989

Table 2 Plasmids used in this study

Plasmids	Descriptions	Sources or references
pUC19	Cloning vector, Amp ^r	Takara
pTAC-1	TA-cloning vector, Amp ^r	BioDynamics
pQE30	Expression vector, Amp ^r	Qiagen
pSUP5011	Source of <i>mob</i> ; Amp ^r , Km ^r , Cm ^r , <i>Tn5-mob</i>	51
pRK2013	Helper plasmid, <i>traRK2</i> , Km ^r	52
pUC4K	Source of Km ^r (<i>Tn903</i>) cassette; Amp ^r , Km ^r	53
pBBR1MCS-4	Broad-host-range plasmid vector, <i>mobRP4</i> , Amp ^r	54
pTAC1-OHF2R2	pTAC-1 carrying a 0.8-kb fragment (OHF2R2) amplified by PCR	This study
pUC19-phaZc	pUC19 carrying a 0.8-kb <i>Bam</i> HI- <i>Hind</i> III fragment containing <i>phaZc</i>	This study
pTAC1-OHF2BR2H	pTAC-1 carrying a 0.8-kb fragment (OHF2BR2H) amplified by PCR	This study
pQE30-phaZc	pQE30 carrying a 0.8-kb <i>Bam</i> HI- <i>Hind</i> III fragment containing <i>phaZc</i>	This study
pUC19-phaZc-Km	pUC19 carrying <i>phaZc::Km^r</i> (a 1.2-kb <i>Sal</i> I fragment of <i>Km^r</i> gene)	This study
pUC19-phaZc-Km-mob	Suicide vector carrying fragments of the <i>phaZc::Km^r</i> and <i>mob</i> genes	This study
pTAC1-phaZc-Km	pTAC-1 carrying an amplified PCR fragment containing <i>phaZc::Km^r</i>	This study
pBBR1MCS4-phaZc	pBBR1MCS4 carrying a 0.8-kb <i>Bam</i> HI- <i>Hind</i> III fragment containing <i>phaZc</i>	This study
pTAC1-hbd	pTAC-1 carrying a 0.8-kb fragment (HbdF1R) amplified by PCR	This study
pQE30-hbd	pQE30 carrying a 0.8-kb <i>Bam</i> HI- <i>Hind</i> III fragment containing <i>hbd</i>	This study

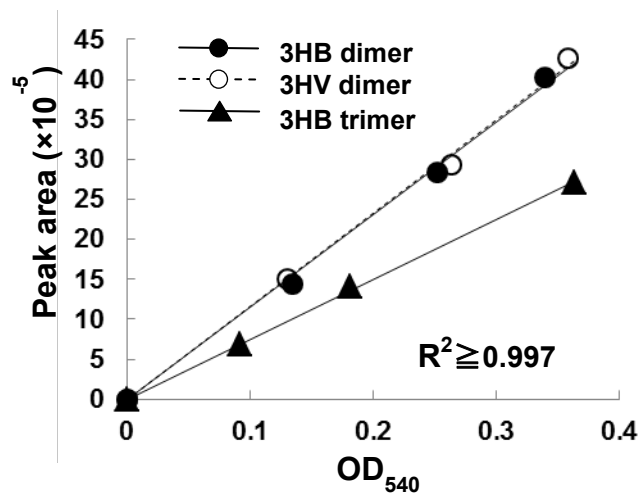


Fig. 5 Correlation between OD₅₄₀ and peak areas in the HPLC analysis with the Aminex HPX-75H column. The data are means for three independent measurements. R^2 : coefficients of determination.

MSASPRLGFVQCISPAGLHRMAYHEWGD PANPRVLVCAHGLTRTGRDFDTVASALCGDYR	ReH16
-----MAVQFFQAEDGARLAYRDEGEGLPVLAL <u>PGLTRTGRDFDY</u> LAPHLP-GVR	Pd1222
VVCPDVAGRGRSEWLADANGYVVPQYVSDMVTLIARLNVEKVDWFGTSMGGLIGMGLAGL	ReH16
LIRPDYRGRGSDWTGSDS-YTVPQEARDVLVLLDHLGVAQAAVL <u>GTSRGGIIGMLLAAT</u>	Pd1222
PKSPVRKLLLNDVGPKLAPSAVERIGAYLGLPVRFKTFEEGLAYLQT-ISASFGRHTPEQ	ReH16
ARERLLGL <u>CLNDVGPVLQR</u> SLERIFDYVGRNPAAKNLEDLAQRLPAAMPG-FANVPASR	Pd1222
WRELNAAILKPVQGTGLEWGLHYDQLAVPFRKSTPEAIAAGEAALWRSFEAIEGPVLV	ReH16
WLEEAARHYVETPSGL----SVNYDPALREAF-LAAFDGPEVDLWPLFDATQG--LPLAL	Pd1222
VRGAQSDLLLRETVAEMVARGKHVSSVEVPDVGHAPTFVDPAQIAIAPQFFTGA-	ReH16
<u>IRGANSDLLS</u> HETATEMQRRRPDMILAEARDRAHVPFLEPESLAAIHAWLAAMR	Pd1222

Fig. 6 Alignment of amino acid sequences between PhaZc from *R. eutropha* H16 and from *P. denitrificans* Pd1222. Conserved sequences are underlined.

HindIII 10 20 30 40 50 60 70 80 90
AAGCTTGATGGCCGTCAGTTCCTTACAGGCCGAGGACGGCGCCGGCTGGCCTATCGCGACGAGGGCGAGGGCCTACCCGTCCTGGCCCT
 M A V Q F L Q A E D G A R L A Y R D E G E G L P V L A L
 100 110 120 130 140 150 160 170 180
 GCCGGGGCTGACCCGGACGGGACGGGATTTGACTATCTGGCGCCGCATCTGCCGGGTGTGCGCCTGATCCGGCCGGACTATCGCGGTGCG
 P G L T R T G R D F D Y L A P H L P G V R L I R P D Y R G R
 190 200 210 220 230 240 250 260 270
 CGGGGATTCGGACTGGACCCGGCTCGGACAGCTATACGGTCCCGCAAGAGGCCCGCGACGTTCTGGTGTGCTGGATCACCTGGGCGTGGC
 G D S D W T G S D S Y T V P Q E A R D V L V L L D H L G V A
 280 290 300 310 320 330 340 350 360
 TCAGGCAGCCGTGCTGGGCACCTCGCGCGGGGGATCATCGGCATGCTTCTGGCAGCGACGGCGCGGAAACGGCTTCTGGGCCTGTGCCT
 Q A A V L G T S R G G I I G M L L A A T A R E R L L G L C L
 370 380 390 400 410 420 430 440 450
 GAACGATGTCGGTCCCCTGCTGCAACGCAGCGGGCTGGAACGGATCTTCGACTATGTGGGCCGGAACCCGGCCGAAAGACCTTGAGGA
 N D V G P V L Q R S G L E R I F D Y V G R N P A A K D L E D
 460 470 480 490 500 510 520 530 540
 TCTGGCGCAGCCGCTGCCCGCCGCATGCCTGGTTTCGCCAATGTCCCAGCCGATGGCTGGAGGAGGCCCGCGCTCACTATGTGCA
 L A Q R L P A A M P G F A N V P A S R W L E E A A R H Y V E
 550 560 570 580 590 600 610 620 630
 GACGCCCTCGGGCCTGAGCGTCAACTATGATCCCGCGCTGCGCGAGGCGTTCCTGGCGGGCCTTCGACGGTCCCAGGTTCGACCTGTGGCC
 T P S G L S V N Y D P A L R E A F L A A F D G P E V D L W P
 640 650 660 670 680 690 700 710 720
 GTTGTTCGACGCCACGGGATTCGCGCTGCGCGTATCCGGGGCGCAAACCTCGGACCTGCTGTGCGATGAGACCGCGACGGAAATGCA
 L F D A T Q G L P L A L I R G A N S D L L S H E T A T E M Q
 730 740 750 760 770 780 790 800 810
 GCGCCGTCGCCCAGACATGATCTTGGCCGAGGCTCGGGACCGCGCGCATGTCCCCTTCCTCGATGAACCGGAATCCCTGGCCGCGATCCA
 R R R P D M I L A E A R D R A H V P F L D E P E S L A A I H
 820 830 840 850 860 870 880 890
 TGCCTGGCTTGACGCGATGAGGTGAAACCACACCGGCCCGGTCGCATATCTGACGCGGAAGCACCTGAAGAGACGGATCC
 A W L A A M R * BamHI

Fig. 7 Nucleotide sequence of the 879-bp PCR product (OHF2R2) and the deduced amino acid sequence of the 828-bp ORF. Deduced amino acid sequence of OHF2R2 (30.1 kDa) is shown under the nucleotide sequence. The *phaZc* gene of *P. denitrificans* PD01 was amplified with the primers of PDOHF2 and PDOHR2 as described in text. *HindIII* and *BamHI* restriction sites of the primers are shown. Lipase box-like sequence is boxed.

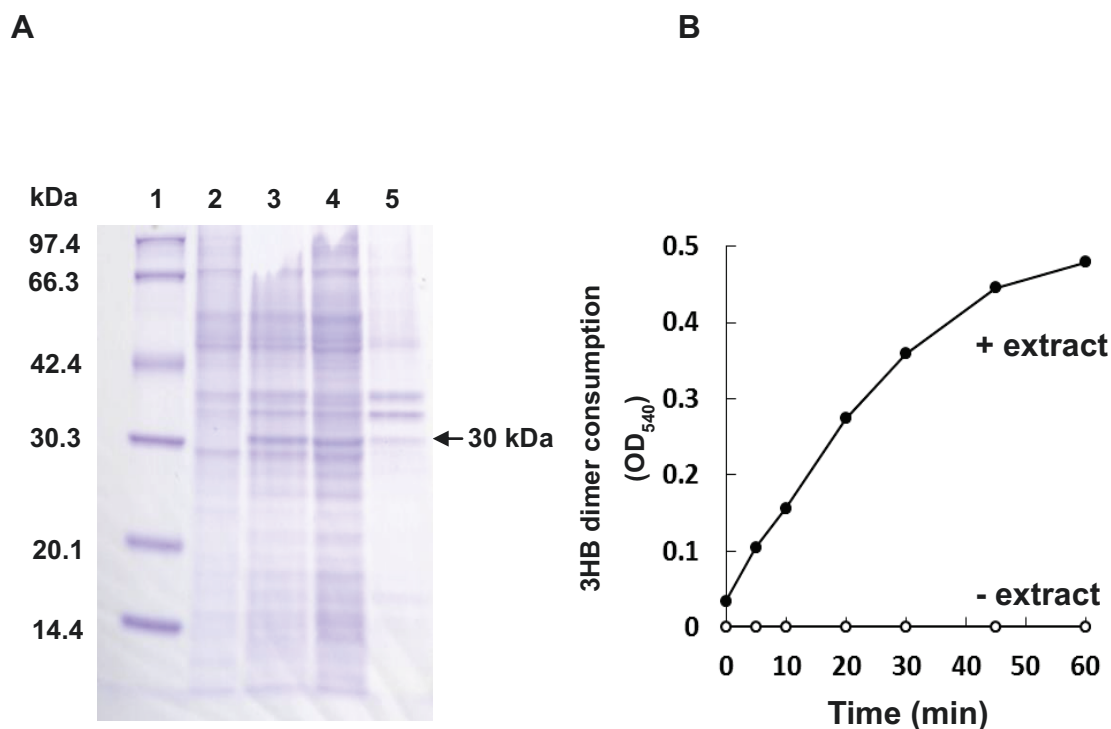


Fig. 8 SDS-PAGE of pUC19-phaZc expression (A) and PhaZc activity (B). In A, lane 1: molecular mass standards, lane 2: *E. coli* JM109/pUC19 extracts (supernatant), lane 3: *E. coli* JM109/pUC19-phaZc (whole cells), lane 4: *E. coli* JM109/pUC19-phaZc extracts (supernatant), and lane 5: *E. coli* JM109/pUC19-phaZc extracts (precipitate). In B, supernatant fraction of *E. coli* JM109/pUC19-phaZc extracts (lane 4 in A) was used as an enzyme source.

```

Ralstonia      MSASRGVCSAGHRMAYHWGDANRVVCAHGTRTGRDDTVASACGDYRVVCDVAGRGRSWAD
Paucimonas    -MTTKSVCSAGHKMVKWGDINNVCVHGVTRVSDDDNAAACDKYR-VCDVVGRGRSDWR
Paracoccus    -----MAVADGARAYRDGGVAGTRTGRDDYAHGVRRDYR-----GRGDSWT
Acidovorax    -MTSRYTTCAG--YHYMWG-ADAVVAWHGARTGRMDAAHAS-RYR----VCDTGRGSSWA
                . :      * . * . ** . * .

Ralstonia      ANGYVVYVSDMVTARNVKVDWGTSMGGMGAGKSVRNVDVG-KASAVRGAYGVRKTG--
Paucimonas    NAYYVYMSDMMTVARDVKVDWGTSMGGAMASGN--RKVNDVGADMTSVSADYGDRWSYD
Paracoccus    GS-DSYTVARDVVDHGVAAAVGTSRGGGMAATAR-RGCNDVGVRSGRDYVGRNAAKNDAR
Acidovorax    RADYRSYARAADDGKAHWVGTSMGGAGTVCASGKGRVSNDRADARADAAARKAYAGHADTVKA
                : . . . * : : * ** . . .

Ralstonia      AYTSASGRHT--WRNAAK-VGTDGWG-HYDAVRKSTAAAGAAWRTAGVVVRGASDRTVAM
Paucimonas    ATYRASVGHTDAWNKMARSVRDDGWRHYDRASKDAYNNVASSWAYDAATRGASGMTTAA
Paracoccus    AAMGANVASR----WAARHYVTSGSVNYDAR----AAADGVDWATGARGANSDSHTATM
Acidovorax    RVYKYGWSDWR-----TSSTRRDGRVTHYDAMVTHHTNDYWDHYDADVCRGASDVDTAM
                . :      . * : . . . : . . .

Ralstonia      VA-RGKHVSSVVDVGHATVDAATGA-----
Paucimonas    MT-RGR---AKVGVGHATMHDDSVKDD----
Paracoccus    RR-RDMAARDRAHVDSAAHAWAAMR-----
Acidovorax    TRGGSRGAVVVAGCGHAANVHYAVD GASAGG
                . . . * : :

```

Fig. 9 Alignment of deduced amino acid sequences among PhaZc from *P. denitrificans* PD01, *R. eutropha* (PhaZc), *Acidovorax* sp. strain SA1 (i3HBOH), and *P. lemoignei* (PhaZc)

Chapter 3

Purification and characterization of PhaZc

3.1. Introduction

3-Hydroxybutyrate oligomer hydrolase (3HBOH) and PHA depolymerase (PhaZ) are important enzymes in both extracellular and intracellular PHA degradation. Investigations of the extracellular enzymes are considerably large. However, reports on the intracellular enzymes have been very limited when compared with the extracellular ones. Up to date, intracellular 3HBOHs from several bacterial strains have been studied (16, 17, 35-37, 39). The enzymatic properties of the enzyme from *R. eutropha* H16 have especially been elucidated. In these previous investigations, 3HB oligomers have exclusively been used as enzymatic reaction substrates of 3HBOHs. Since not only PHB but also PHAs containing 3HV unit (i.e., PHV and PHB-PHV) are synthesized by some PHA-producers, the involvement of 3HBOH in the metabolism of these PHAs was predicted.

3HB oligomers have been assayed by enzymatic or chromatographic procedures. In the enzymatic procedure, 3HB monomers have been assayed with 3HB dehydrogenase and nicotinamide adenine dinucleotide (NAD) after 3HB oligomer hydrolase reaction. These procedures are somewhat inconvenient. Especially, chromatographic procedure is time-consuming when many samples should be assayed.

In this chapter, enzymatic properties of the 3HBOH from *P. denitrificans* were investigated. Especially, it was intensely focused on 3HV oligomers as a reaction substrate for the enzyme. Application of convenient colorimetric procedure to assay 3HB and 3HV oligomers was also examined in this chapter.

3.2. Materials and methods

3.2.1. Bacterial strains, growth conditions, and plasmids

E. coli strains DH5 α and M15/pREP4 (Table 1 in Chapter 2) were grown at 37°C in LB medium. When needed, the medium was supplemented with Amp (50 μ g/ml), Km (15 μ g/ml), and IPTG (0.1 mM, if not specified). pTAC-1 and pQE30 (Table 2 in Chapter 2) were used as plasmid vectors for *E. coli*.

3.2.2. Construction of pQE30-phaZc

3.2.2.1. PCR primers

To overproduce PhaZc in *E. coli*, pQE30 expression vector was used. The following PCR primers were designed.

PDOHF2B (forward): 5'-CGGGATCCGATGGCCCGTCCAGTTCTTCC-3'

*Bam*HI Start

PDOHR2H (reverse): 5'-CGAAGCTTGTCTCTTCAGGTGCTTCCGC-3'

*Hind*III

3.2.2.2. PCR conditions

PCR was performed with a KAPA Taq EXtra PCR with dNTPs kit (KAPA Biodynamics). Composition of PCR mixture and PCR conditions were as follows.

PCR mixture (in 50 μ l)	
Sterilized Milli-Q water	33.25 μ l
5 \times KAPATaq EXtra Buffer	10 μ l
dNTPs (10 mM each)	1.5 μ l
MgCl ₂ (25 mM)	3.5 μ l
PDOHF2B (20 mM)	1.25 μ l
PDOHR2H (20 mM)	1.25 μ l
PD01 genomic DNA	1 μ l
KAPATaq EXtra DNA Polymerase	0.25 μ l

PCR conditions	
Initial denaturing	95°C, 4 min
Denaturing	95°C, 1 min
Annealing	51°C, 1 min
Extension	72°C, 1 min
Final extension	72°C, 5 min

} 30 cycles

3.2.2.3. TA cloning

A 0.8-kb DNA fragment (named OHF2BR2H) was amplified by PCR.

TA cloning was performed by using a DynaExpress TA PCR Cloning kit (BioDynamics Laboratory). Ligation conditions were 16°C and 30 min.

Ligation reaction mixture (in 10 μ l)	
pTAC-1 vector (50 μ g/ml)	1 μ l
OHF2BR2H (11.1 μ g/ml)	3 μ l
2 \times Ligation buffer	5 μ l
Ligase mixture	1 μ l

After the ligation reaction, 2 μ l of ligation mixture were used for transforming Jet competent cells (*E. coli* DH5 α , Biodynamics Laboratory). The transformation was performed as described in Chapter 2 (2.2.4.3.). Colonies appeared on selective LB agar plates were screened for plasmid isolation. A plasmid named pTAC1-OHF2BR2H was obtained. It was used for nucleotide sequencing with primers of T7 (5'-AATACGACTCACTATAG-3') and M13R (-20) (5'-GCGGATAACAATTTACACAGG-3').

3.2.2.4. Construction of pQE30-phaZc

pQE30-phaZc was constructed with pQE30 and a 0.8-kb *Bam*HI-*Hind*III fragment excised from pTAC1-OHF2BR2H.

Restriction enzyme reaction mixture (in 20 μ l)	
Milli-Q water	4 μ l
<i>Bam</i> HI	2 μ l
<i>Hind</i> III	2 μ l
Buffer 2	2 μ l
pQE30	10 μ l
or pTAC1-OHF2BR2H	

The restriction enzyme reaction was performed at 37°C for 3 h. *Bam*HI/*Hind*III-digested pQE30 and the 0.8-kb OHF2BR2H fragment were recovered and used for ligation reaction. Ligation was performed with a DynaExpress DNA Ligation kit at 16°C for 30 min.

Ligation reaction mixture (in 20 µl)	
<i>Bam</i> HI/ <i>Hind</i> III-digested pQE30 (11.2 µg/ml)	1.5 µl
<i>Bam</i> HI/ <i>Hind</i> III-digested OHF2BR2H (2.0 µg/ml)	8.5 µl
2 × Ligation Buffer	10 µl
Ligase Mixture	1 µl

After the ligation reaction, the ligation mixture was used for transforming *E. coli* M15/pREP4.

3.2.2.5. Preparation of *E. coli* M15/pREP4 competent cells

Overnight culture (0.25 ml) of *E. coli* M15/pREP4 was added to pre-warmed LB medium (25 ml) containing 25 µg/ml Km in a 100-ml flask. The flask was shaken at 37°C until OD₆₀₀ reached about 0.5. The culture was cooled on ice for 5 min and transferred to a round-bottom centrifuge tube. The cells were collected by centrifugation at 4,000 × g and 4°C for 5 min. The supernatant was discarded carefully. The cells were resuspended gently in 7.5 ml of cold TFB1 buffer (100 mM RbCl, 50 mM MnCl₂, 30 mM potassium acetate, 10 mM CaCl₂, and 15% (v/v) glycerol, pH 5.8), and the suspension was kept on ice for an additional 90 min. The cells were collected by centrifugation at 4,000 × g and 4°C for 5 min and resuspended gently in 1 ml of cold

TFB2 buffer (10 mM MOPS, 10 mM RbCl, 75 mM CaCl₂, and 15% (v/v) glycerol, pH 6.8). Aliquots (50 µl) of the competent cells were dispensed to microcentrifuge tubes and stored at -60°C.

3.2.2.6. Transformation

The competent cells of *E. coli* M15/pREP4 (50 µl) were thawed on ice and mixed with 2 µl of ligation reaction mixture in a microcentrifuge tube. The tube was incubated on ice for 20 min and heated for 90 sec at 42°C. To the tube was then added 0.5 ml of Psi medium (LB medium containing 4 mM MgSO₄ and 10 mM KCl). After incubating with shaking for 90 min at 37°C, aliquots (50 µl) of the cell suspension were spread onto LB agar plates containing 50 µg/ml Amp, 25 µg/ml Km, and 1 mM IPTG. The plates were incubated at 37°C overnight and colonies were screened for plasmid isolation. *E. coli* M15/pQE30-phaZc was obtained.

3.2.3. His-PhaZc overexpression in *E. coli*

Overnight preculture (0.5 ml) of *E. coli* M15/pQE30-phaZc was added to LB medium (50 ml) containing 50 µg /ml Amp and 25 µg/ml Km in a 200-ml flask. The cells were cultivated at 37°C until OD₆₀₀ reached about 0.5. IPTG (10 µM) was added to the culture. Cultivation was continued for 4.5 h. The cells were collected by centrifugation at 11,000 × *g* and 4°C for 10 min and washed with 50 mM Tris-HCl buffer (pH7.0).

3.2.4. Purification of His-PhaZc from *E. coli*

All purification steps were carried out at 4°C. To prepare cell-free extracts, the cells were suspended in lysis buffer (50 mM NaH₂PO₄, 300 mM NaCl, and 10 mM imidazole,

pH8.0) and disrupted by sonication at 4°C for 10 min three times. Cell-free extracts were obtained by centrifugation at 11,000 × *g* and 4°C for 10 min.

The resulting supernatant was used to purify the His-tagged proteins by affinity chromatography on a Ni-NTA Superflow column (1.5 × 6 cm, Qiagen). Flow rate was 0.15 ml/min. The supernatant was applied to the column which was pre-equilibrated with lysis buffer. The column was washed with 30 ml of lysis buffer and washed with 50 ml of wash buffer (50 mM NaH₂PO₄, 300 mM NaCl, and 20 mM imidazole, pH8.0). Proteins bound to the column were then eluted with a linear gradient (30 ml) from 20 mM to 250 mM of imidazole. Fractions (2 ml each) were collected. Elution of proteins was monitored at 280 nm.

Fractions displaying His-PhaZc activity were combined, dialyzed against 20 mM Tris-HCl (pH 7.0), and concentrated by ultrafiltration with Vivaspin 20 (Sartorius).

The concentrated sample was applied to a TSK-gel G3000SW column (0.75 × 30 cm, Tosoh) pre-equilibrated with 50 mM phosphate buffer (pH 7.0). Elution of proteins were performed with the same buffer at 1 ml/min and monitored at 280 nm. Active fractions were collected, concentrated, and stored at -20°C.

3.2.5. His-PhaZc assay

The enzyme activity was assayed based on the amount of 3HB or 3HV oligomers consumed during enzymatic reactions. The reaction mixture (1 mL) contained 50 mM Tris-HCl (pH 8.0), substrate solution, and enzyme solution (74 µg protein). Enzymatic reactions were performed at 30°C. The amount of oligomers remaining in 0.2 ml of the reaction mixture was measured by the method of Hestrin (46) as described above in Chapter 2. Effect of temperature on the enzymatic activity was examined with 3HB

oligomers as a substrate at a temperature range from 10°C to 80°C. Effect of pH on the enzymatic activity was examined with 50 mM acetate buffer (pH 4.0 to 6.0), 50 mM phosphate buffer (pH 6.0 to 8.0), 50 mM Tris-HCl buffer (pH 8.0 to 9.0), and 50 mM glycine-NaOH buffer (pH 9.0 to 11.0). Reaction temperature was 30°C. The amount of 3HB oligomers remaining in the mixture was measured by Hestrin's method.

His-PhaZc activity was measured with *p*-nitrophenyl fatty acid esters of acetic (C2), propionic (C3), butyric (C4), pentanoic (C5), hexanoic (C6), and decanoic (C10) acids (Sigma-Aldrich). The reaction mixture (1 ml) contained 50 mM Tris-HCl buffer (pH 8.0), His-PhaZc (8 µg protein), and 0.4 µM *p*-nitrophenyl fatty acid. Reaction temperature was 30°C. The liberation of *p*-nitrophenol during the enzyme reactions was monitored at 400 nm for 60 sec.

3.2.6. Inhibitors

To test the effect of chemical reagents on the His-PhaZc activity, 0.5 ml of reaction mixture containing 50 mM Tris-HCl buffer (pH 8.0), His-PhaZc (8 µg protein), and a chemical reagent was kept on ice. After 10 min, 3HB oligomer solution (20 µl) was added to the mixture. The mixture was then incubated at 30°C for 10 min. To stop the enzymatic reaction, the mixture was heated at 90°C for 3 min. The amount of 3HB oligomers remaining in the mixture was measured by Hestrin's method. Chemical reagents used were: *n*-ethylmaleimide (1 mM), iodoacetamide (1 mM), diisopropylfluorophosphate (DFP) (1 mM), phenylmethanesulfonyl fluoride (PMSF) (1 mM), SDS (0.05%, w/v), and Triton X-100 (0.05%, v/v).

3.3. Results

3.3.1. pQE30-phaZc construction

pUC19-phaZc could be used for purifying PhaZc. However, the expression level was not high. Therefore, pQE30 was used for purifying the enzyme. pQE30-phaZc was successfully constructed.

3.3.2. IPTG concentrations for overproducing His-phaZc

With pQE30-phaZc, the *phaZc* was successfully expressed. To purify His-PhaZc from *E. coli* cells efficiently, the enzyme was tried to overproduce as a His-tagged protein using pQE30-phaZc. However, most His-PhaZc tended to be produced as an inclusion body at higher concentrations of IPTG (over 0.1 mM). Induction condition of His-PhaZc was examined. It was found in SDS-PAGE analysis that His-PhaZc was produced as a solubilized protein at a lower IPTG concentration (Fig. 10). The optimum conditions for His-PhaZc overproduction were 10 μ M of IPTG, 4.5 h of induction time, and 37°C of induction temperature.

As shown in Fig. 10, molecular mass of His-PhaZc was about 31 kDa in SDS-PAGE analysis, which was close to that obtained with the deduced amino acid sequence of the *phaZc*.

3.3.3. Purification of His-tagged PhaZc from *E. coli*

Two-step chromatography with affinity and gel filtration columns was performed to purify His-PhaZc. His-PhaZc was purified to homogeneity (Fig. 11). Molecular mass of the purified PhaZc was 31 kDa (as described in 3.3.2.).

3.3.4. Properties of His-PhaZc

The purified enzyme hydrolyzed not only 3HB dimer but also 3HV dimer (Fig. 12). Specific activity of His-PhaZc was 0.59 U/mg for 3HB dimer and 0.43 U/mg for 3HV dimer, respectively. Several *p*-nitrophenyl esters of fatty acids were tested as substrates. *p*-Nitrophenyl esters of propionic acid (C3) and butyric acid (C4) were most efficient substrates (Fig. 13).

The highest enzymatic activity was obtained at pH 8.0 and 30°C to 50°C (Fig. 13).

Effects of several chemical reagents commonly used in PHA depolymerase and 3HBOH assays on His-PhaZc activity were investigated. The enzyme activity was completely inhibited by 1 mM DFP. PMSF, SDS, and Triton X-100 were also inhibitors. However, their inhibitory effects were weaker than that of DFP. *N*-Ethylmaleimide and iodoacetamide were slightly inhibitory (Table 3).

3.4. Discussion

His-PhaZc was successfully expressed in *E.coli* in Chapter 2. In Chapter 3, His-PhaZc was purified to homogeneity and characterized.

Intracellular and extracellular 3HBOHs have been isolated from several bacterial strains. The terms of i3HBOH and e3HBOH have been used for intracellular and extracellular 3HBOHs, respectively. These 3HBOHs were classified into two groups based on their molecular mass: one group ranged from 70 to 78 kDa (*Pseudomonas* sp. strain A1, *A. faecalis* T1, and *R. eutropha*) and another group ranged from 30 to 32 kDa (*Z. ramigera* I-16-M, *Acidovorax* sp. strain SA1, *R. eutropha* H16, and *P. lemoignei*). In *R. eutropha* H16, 78-kDa and 32-kDa i3HBOHs were found and they were renamed PhaZb and PhaZc, respectively. According to the rule of terminology for 3HBOH,

31-kDa 3HBOH from *P. denitrificans* PD01 was named PhaZc.

Enzymatic properties of 3HBOHs have been investigated. Optimal pH and temperature for their enzymatic reactions are not drastically different when compared with the previous results (17). The strong inhibitory effect by DFP and PMSF, which react with serine groups on proteins, supports the importance of serine residue in the lipase box-like motif. The motif should serve as an active site of PhaZc. Sulfhydryl reagents tested in this study displayed little inhibitory effect on PhaZc activity, which implies that disulfide bond is not important for the enzymatic activity.

It was reported that substrate specificities of the i3HBOH were different from each other. i3HBOH from *Z. ramigera* I-16-M degraded 3HB trimer faster than 3HB dimer. Whereas, i3HBOH from *Acidovorax sp.* strain SA1 hydrolyzed 3HB dimer faster than 3HB trimer. PhaZb from *R. eutropha* H16 hydrolyzed 3HB dimers and 3HB trimer at a similar rate. Interestingly, PhaZc from *R. eutropha* H16 could degrade artificial amorphous PHB at a low activity. Hence, substrate specificity of 3HBOHs has been investigated only toward the oligomers of 3HB. Catalytic activity of 3HBOH toward 3HV oligomers has not reported.

P. denitrificans is able to synthesize PHAs (homopolymer and copolymer) composed of 3HB and/or 3HV units depending on carbon sources added to the cultivation medium. As described in Chapter 4, PHA accumulated in the cells is degraded under carbon-deficient and nitrogen-sufficient conditions. It was of interest to examine whether PhaZc catalyzes the hydrolysis of 3HV oligomers. The PhaZc of *P. denitrificans* PD01 could catalyze not only 3HB oligomers but also 3HV oligomers with similar rate.

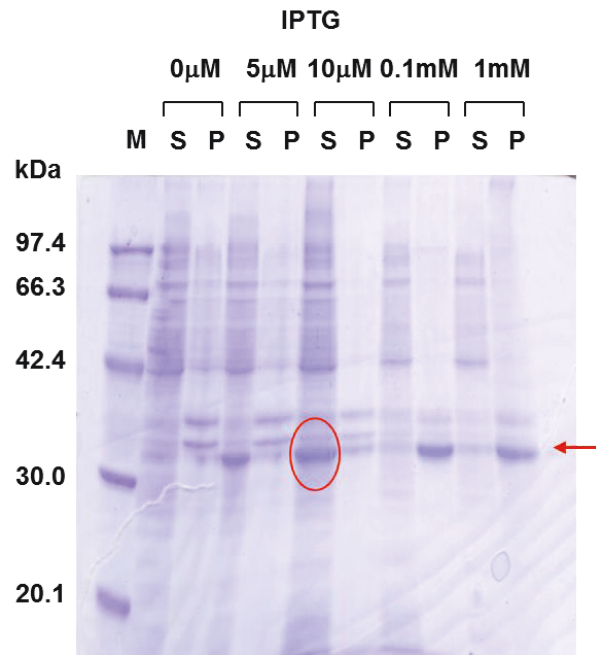


Fig. 10 SDS-PAGE analysis of the expression of His-PhaZc with IPTG in *E. coli* cells. M: molecular mass standards, S: supernatant fractions of cell-free extracts of *E. coli* carrying pQE30-phaZc, and P: precipitate fractions of cell-free extracts of *E. coli* carrying pQE30-phaZc.

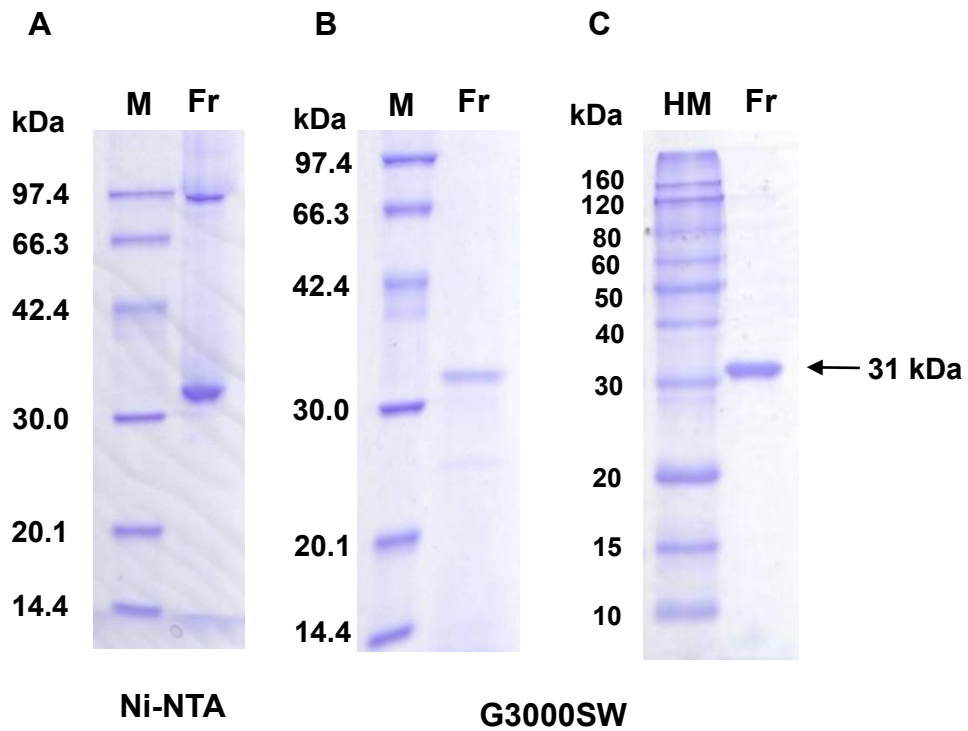


Fig. 11 SDS-PAGE of PhaZc after purification by affinity chromatography (A) and gel filtration chromatography (B and C). M: molecular mass standards, HM: His-tagged molecular mass standards, and Fr: PhaZc active fractions.

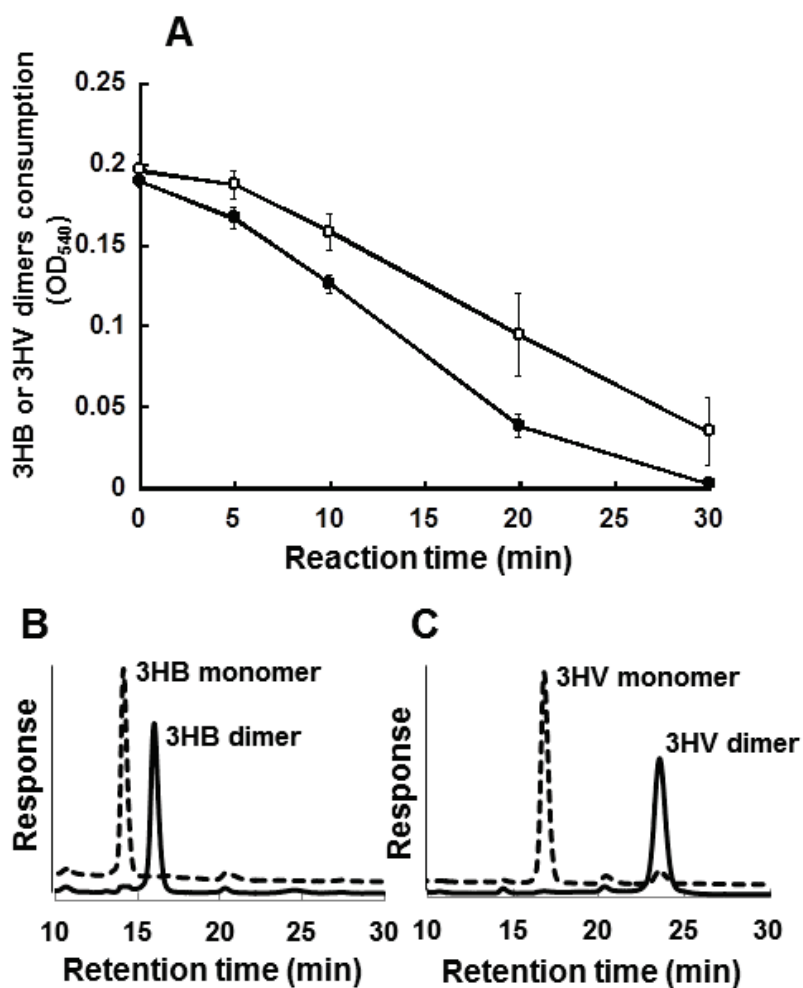


Fig. 12 Substrate specificity of His-PhaZc for 3HB and 3HV dimers. A: the consumption of 3HB and 3HV dimers (OD_{540}). The mean and standard deviation were calculated from the experiments run in triplicate. B and C: the chromatograms of HPLC with the Aminex HPX-75H column at 0 min and 30 min in the enzymatic reactions in A, respectively.

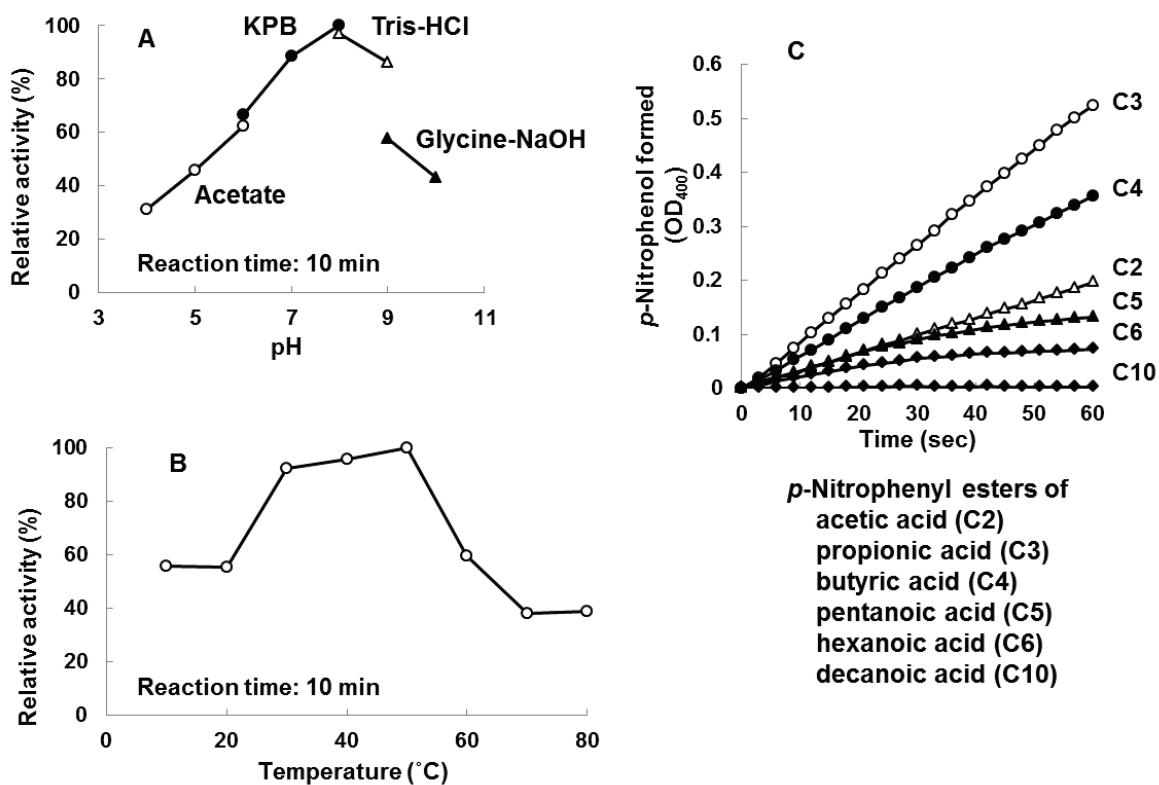


Fig. 13 Optimum pH (A), optimum temperature (B) and substrate specificity (C) of His-PhaZc. In A, reaction temperature was 30°C. In B, reaction pH was 8.0. In C, the formation of *p*-nitrophenol during the enzyme reactions was monitored at 400 nm. Three independent experiments were performed. Representative results are shown.

Table 3 Effect of chemical reagents on the PhaZc activity

Reagents	Relative activity (%)
None	100
<i>N</i> -Ethylmaleimide (1 mM)	74.8
Iodoacetamide (1 mM)	74.8
Diisopropylfluorophosphate (1 mM)	0.0
Phenylmethanesulfonyl fluoride (1 mM)	27.4
Sodium dodecyl sulfate (0.05%, w/v)	38.9
Triton X-100 (0.05%, w/v)	32.7

Enzyme solution was incubated with each reagent at the concentrations described for 10 min on ice and used for enzymatic reactions.

Chapter 4

Disruption of the *phaZc* gene in *P. denitrificans* PD01

4.1. Introduction

To complete PHB cycle, PHB synthesized in bacterial cells should be finally converted (depolymerized) to 3HB monomer. As described in Chapter 1, the depolymerization proceeds via five enzymatic reactions by PHB depolymerase, 3HB oligomer hydrolase, 3HB dehydrogenase, acetoacetyl-CoA synthase, and β -ketothiolase. Hence, 3HBOH has been recognized to be a key enzyme in PHB metabolism. Although reports on 3HBOH in PHB-producing bacteria are very limited, PHB might be directly depolymerized to 3HB monomer in some bacteria. Two oligomer hydrolases (PhaZb and PhaZc) have been reported from *R. eutropha* H16. In Chapters 3, it was described that the 3HBOH of *P. denitrificans* is a homologue of the *R. eutropha* H16 PhaZc, implying that PHB should be degraded to 3HB monomer via 3HBOH reaction in *P. denitrificans*. In order to know whether the PhaZc is involved in the mobilization, the effect of the *phaZc* disruption on PHA degradation in *P. denitrificans* was investigated in this chapter.

4.2. Materials and methods

4.2.1. Bacterial strains, growth conditions, and plasmids

P. denitrificans PD01 was aerobically grown at 30°C in PD medium, and growth was monitored by measuring the optical density at 660 nm with a spectrophotometer. *E. coli* strains were grown at 37°C in LB medium. When needed, the medium was supplemented with Amp (50 μ g/ml) and Km (15 μ g/ml). Plasmids of pUC19, pUC4K,

and pSUP5011 (Table 2 in Chapter 2) were used. Broad-host-range pBBR1MCS-4 (Table 2 in Chapter 2) was also used as a plasmid vector for *P. denitrificans*.

4.2.2. Disruption of the *phaZc* gene in *P. denitrificans* PD01

4.2.2.1. Construction of a suicide plasmid

A suicide plasmid, pUC19-*phaZc*-Km-mob, was constructed with pUC19, a 1.7-kb *Bam*HI-*Bam*HI fragment containing the *mob* gene from pSUP5011 (51), and a 2.1-kb *Bam*HI-*Hind*III fragment containing the *phaZc::Km'* gene (Fig. 14). This suicide plasmid was transferred by triparental mating.

4.2.2.2. Conjugative transfer

Conjugative transfer was performed by triparental filter mating. *E. coli* JM109/pUC19-*phaZc*-Km-mob, *P. denitrificans* PD01, and *E. coli* HB101/pRK2013 (52) were used as a donor, a recipient, and a helper strain, respectively. After cultivating donor, helper, and recipient strains to late exponential growth phase, cells were washed and suspended in sterile saline. The cell suspensions were mixed at a volumetric ratio of 1:1:8 and incubated overnight at 30°C on filter membranes (0.2 µm pore size) on PD medium agar plates containing 5% (v/v) LB medium. After mating, cells were washed from the membranes, and the cell suspension was plated on PD medium agar plates containing Km. The plates were incubated for 2 to 3 days at 30°C. One of transconjugants was chosen and named *P. denitrificans* PD01Km.

4.2.2.3. Southern blotting

A DIG High Prime DNA Labeling and Detection Starter kit I (Roche Applied

Science) was used for Southern blotting.

(1) Preparation of DNA

The genomic DNAs of *P. denitrificans* PD01 and PD01Km were prepared as described in Chapter 2 (2.2.3.). They were digested with *Pst*I and subjected to agarose gel electrophoresis.

(2) Preparation of DIG-labeled DNA probe

A 1.2-kb *Sal*I fragment (*Km^r* gene) from pUC4K (53) was used. The DNA (1 µg/16 µl of sterile water) in a microcentrifuge tube was denatured by heating in a boiling water bath and quickly chilled on ice. A DIG-High Prime solution (4 µl) was added to the tube. After incubating at 37°C for 16 h, the reaction was stopped by adding 2 µl of 0.2 M EDTA.

(3) Transfer

After electrophoresis of the *Pst*I-digested template DNA, the gel was acidified (depurinated) with 0.25 M HCl for 15 min, denatured with a mixture of 0.5 M NaOH/1.5 M NaCl for 30 min, and neutralized with a mixture of 0.5 M Tris-HCl (pH 7.4)/1.5 M NaCl for 30 min. The gel was shaken gently during these treatments. DNA fragments in the gel was transferred to a positively charged nylon membrane (Roche Applied Science) by capillary transfer method with 10× SSC buffer (1.5 M NaCl/0.15 M sodium citrate, pH 7.0) overnight. The membrane was performed by UV-crosslink without prior washing. After the UV-crosslinking, the membrane was rinsed briefly in Milli-Q water and allowed to air-dry.

(4) Hybridization

The membrane was put into a hybridization bottle containing appropriate volume of hybridization buffer (2 ml/10 cm²) which was pre-warmed to 42°C and incubated for 30

min. The DIG-labeled probe was boiled for 5 min, rapidly cooled on ice water, and mixed with pre-warmed hybridization buffer (0.2 ml/10 cm²). After the hybridization buffer in the bottle was poured off, the mixture was added to the bottle. The bottle was incubated under constant rotation at 42°C overnight. The membrane was washed twice for 5 min in 2× SSC/0.1% SDS at room temperature under gentle shaking and washed twice for 15 min in 0.5× SSC/0.1% SDS at 65°C under gentle shaking. The membrane was rinsed briefly in washing buffer (0.1 M maleic acid, 0.15 M NaCl, and 0.3% (w/v) Tween 20, pH 7.5) and then incubated for 30 min in 100 ml of blocking solution (prepared by diluting 10× Blocking solution with maleic acid buffer (0.1 M maleic acid and 0.15 M NaCl, pH 7.5)). The membrane was incubated for 30 min in 20 ml of antibody solution (diluted anti-DIG-AP conjugate to 150 mU/ml (1:5000) with blocking solution) and washed twice for 15 min in 100 ml of washing buffer.

(5) Detection

The membrane was equilibrated in 20 ml of detection buffer (0.1 M Tris-HCl and 1 mM EDTA, pH 8.0) for 3 min and incubated in 10 ml of freshly prepared color substrate (nitro-blue tetrazolium and 5-bromo-4-chloro-3-indoly phosphate) solution for chromogenic detection.

4.2.2.4. PCR

PCR was performed to confirm the disruption of *phaZc* in *P. denitrificans* PD01. The genomic DNAs of *P. denitrificans* PD01 and PD01Km were used as templates. PCR primers were PDOHF2 and PDOHR2. PCR conditions were as described in Chapter 2.

4.2.3. Complementation of the *phaZc* mutation in *P. denitrificans* PD01Km

4.2.3.1. Plasmid construction

A plasmid, pBBR1MCS4-*phaZc*, was constructed. A 0.8-kb *Hind*III-*Bam*HI fragment from pUC19-*phaZc* was inserted into the same restriction sites of the broad-host-range pBBR1MCS-4 (54) to produce pBBR1MCS4-*phaZc*. *E. coli* JM109 was transformed with pBBR1MCS4-*phaZc*.

4.2.3.2. Conjugative transfer

E. coli JM109 harboring pBBR1MCS4-*phaZc*, *P. denitrificans* PD01Km, and *E. coli* HB101 harboring pRK2013 (52) were used as a donor, a recipient, and a helper strain, respectively. Conjugative transfer was performed by filter mating as described in this chapter (4.2.2.2.). Transconjugants were selected against Amp^r and Km^r. One of transconjugants was chosen and named *P. denitrificans* PD01KmOH.

4.2.3.3. *PhaZc* assay of *P. denitrificans* PD01 and its mutants

In order to measure the *PhaZc* activity in *P. denitrificans* PD01 and its mutants, cell-free extracts were prepared from *P. denitrificans* PD01, PD01Km, and PD01KmOH. The protein concentrations of the cell-free extracts were measured by the method of Lowry et al. (40) with bovine serum albumin as a standard. *PhaZc* activity was assayed based on the amount of 3HB oligomers consumed during enzymatic reactions. The reaction mixture (1 mL) contained 50 mM Tris-HCl (pH 8.0), 3HB dimer, and cell-free extracts. The reaction temperature was 30°C and the reaction time ranged from 0.5 to 4 h. The amount of substrate remaining was measured as described by Hestrin (39).

4.2.4. Gas chromatographic analysis of PHA

Cellular PHA contents were determined by gas chromatography after methanolysis as described by Braunegg (55). The conditions for gas chromatographic analysis were as described previously (21). Cultures were centrifuged at 11,000 $\times g$ for 10 min. Precipitates (wet cells) were dried overnight at 60°C to constant weight. To screw-capped glass tubes containing dry cells (ca. 10 mg), 1 ml of chloroform and 1 ml of a mixture composed of 184 mg of benzoic acid (internal standard), 3.5 ml of sulfuric acid, and 96.5 ml of methanol were added. The tubes were incubated at 120°C for 90 min. Water (0.5 ml) was added, vortexed for 2 min, and centrifuged briefly at 2,000 $\times g$. The lower layer (chloroform layer) was used for gas chromatographic analysis. A gas chromatograph (model GC-4000, GL Sciences) equipped with a flame deionization detector (FID) was used. The column was a Reoplex 400 10% Chromosorb W AW-DMCS (60-80 mesh) (GL Sciences). Temperatures of injector, oven, and detector were 250, 160, and 200°C, respectively. Carrier gas was N₂.

4.3. Results

4.3.1. Disruption of *phaZc* gene in *P. denitrificans* PD01

The suicide plasmid was constructed according to design. After conjugative transformation was carried out, *phaZc* gene was disrupted in *P. denitrificans* PD01. The result was confirmed by Southern blotting and PCR (Fig. 15). A 1.2-kb *SaII* fragment (Km^r gene) from pUC4K was used as a probe for hybridization. From Fig. 15, the probe hybridized with the mutant genomic DNA, but did not hybridized with the wide type. When PCR was performed, the size of PCR product from the mutant was about 1.3 kb larger than from the wild type. These results indicate that Km^r gene was successfully inserted into the mutant genomic gene by homologous recombination. In order to know

whether the *phaZc* gene was inactivated, the PhaZc activity was also measured. Cell-free extracts were prepared from the cultures of *P. denitrificans* PD01 and PD01Km, PhaZc activity was measured. PhaZc activity of *P. denitrificans* PD01Km was negative, which indicates that *phaZc* gene was disrupted.

4.3.2. Effect of the disruption of the *phaZc* gene on intracellular PHA degradation in *P. denitrificans* PD01

The effect of the *phaZc* mutation on PHA degradation was examined using *P. denitrificans* PD01 and PD01Km. After the strains were grown for 18 h to late exponential growth phase (stage 1), cultivation was continued for 24 h under carbon-sufficient and nitrogen-deficient conditions (stage 2). At the end of stage 2, carbon sources were removed from the medium and cultivation was continued for 24 h (stage 3). The results are shown in Fig. 16. Both strains synthesized 3HB-3HV copolymer through the second stage of cultivation. The composition of 3HV monomer unit in the 3HB-3HV copolymer was 60 to 65 mol%. In the stage 2, there was a little affect in the growth and PHA synthesis of *P. denitrificans* PD01Km. At the end of this stage, PHA content in *P. denitrificans* PD01Km was 20 % lower than that of *P. denitrificans* PD01. Cellular PHA contents decreased in the stage 3. Especially, in the first 5 h of this stage, PHA content in the *P. denitrificans* PD01 strain decreased by 40.5%, but it decreased by 26.3% in the *P. denitrificans* PD01Km.

4.3.3. PhaZc activity assay of *P. denitrificans* PD01 and its mutants

Cell-free extracts were prepared from *P. denitrificans* PD01 and its mutants were used to determine PhaZc activity. As shown in Fig. 17, enzyme activity was not detected

in extracts of *P. denitrificans* PD01Km. These results indicate that the *phaZc* gene was successfully disrupted by inserting the Km^r gene. pBBR1MCS4-*phaZc* was introduced into *P. denitrificans* PD01Km by conjugative transfer. *P. denitrificans* PD01KmOH harboring pBBR1MCS4-*phaZc* restored PhaZc activity (Fig. 17). When the enzymes were omitted from the reaction mixture, the OD at 540 nm did not change.

4.4. Discussion

When the *phaZc* gene was disrupted in *P. denitrificans* PD01, the hydrolase activity was not detected. The PhaZc activity was restored by introducing the gene to the mutant (PD01Km strain). Thus, *P. denitrificans* PD01 has a unique *phaZc* gene. There were no other candidate genes for PhaZc activity in the nucleotide sequence (or amino acid sequences) of the *P. denitrificans* Pd1222 genome.

Intracellular degradation of PHB proceeds via PhaZ (PHB depolymerase), PhaZc, and Hbd reactions in PHB-producing bacteria. Although the *phaZ* gene (GenBank/EMBL/DDBJ accession number AB839772) was cloned from *P. denitrificans* PD01 by us, it was not disrupted in this study. Since PhaZ is produced in *P. denitrificans* PD01 and PD01Km, it is easily expected that PHA should be degraded in these cells. We previously found that PhaZ from *P. denitrificans* ATCC17741 catalyzed the degradation of PHB granules and the major product was 3HB dimer (28). If this is the case also for *P. denitrificans* PD01, PhaZc plays an important role in PHA metabolism via 3HB and 3HV oligomers in *P. denitrificans* cells. In order to know the effect of PhaZc activity on intracellular PHA degradation, growth and PHA content during the cultivation were compared between the wild type and the *phaZc*-disrupted mutant cells. Profiles of growth and PHA synthesis in stage 2 were similar between

them. However, the profile of PHA content was moderately different at the PHA degradation stage: the decrease in PHA content was lower in the mutant cells, whereas the growth profile was almost the same during this stage. Considering these results, PHA degradation in the mutant cells proceeded more slowly than in the wild type cells. Therefore, PHA depolymerization reaction should be affected by the PhaZc activity in *P. denitrificans* PD01. In *R. eutropha* H16, the effects of 3HBOH activity on PHB mobilization has been investigated by using *phaZc* or *phaZb* deletion mutants and a *phaZc-phaZb* double mutant. Cellular PHB contents of all the mutants were higher than wild type when grown under PHB accumulation conditions. The result indicates that PhaZc at least contribute to the mobilization of PHB.

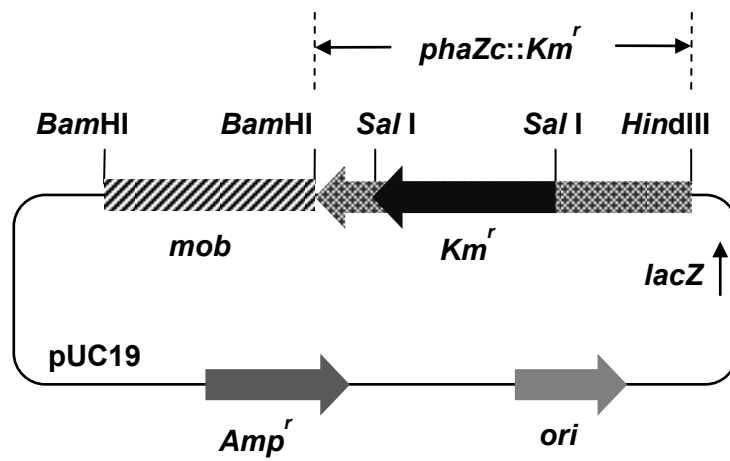


Fig. 14 Physical map of the suicide plasmid pUC19-phaZc-Km-mob

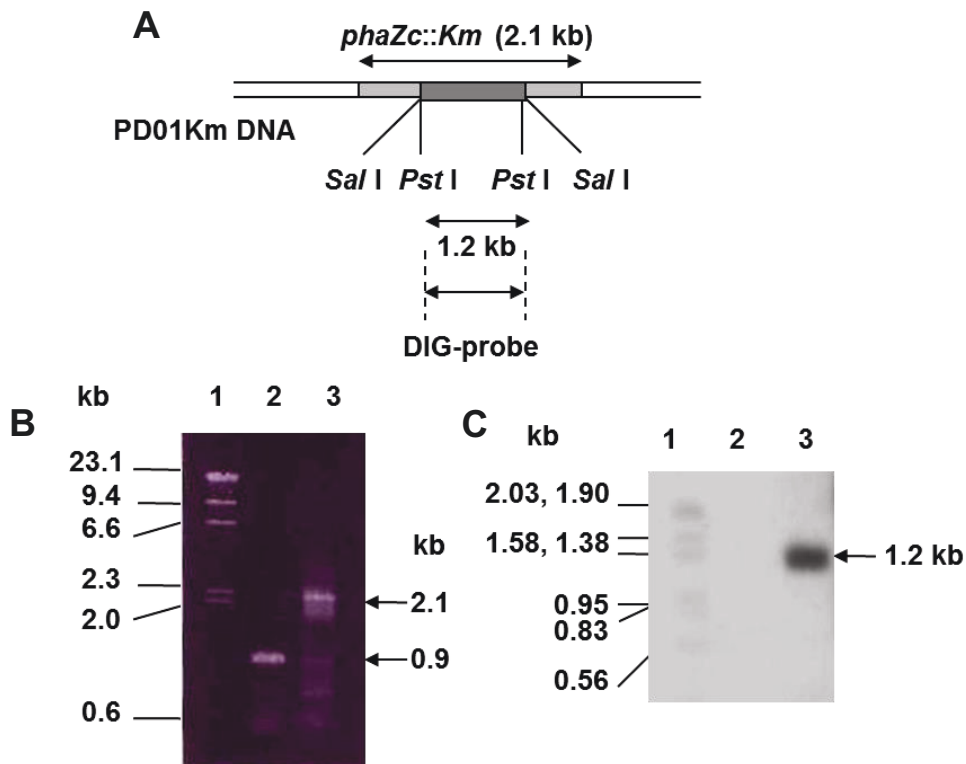


Fig. 15 Confirmation of the *phaZc* gene-disruption in *P. denitrificans* PD01. **A:** Physical map of *P. denitrificans* PD01Km DNA. **B:** Agarose gel electrophoresis. Lane 1: molecular size standards, lane 2: the DNA fragment amplified by PCR using *P. denitrificans* PD01DNA as a template, and lane 3: the DNA fragment amplified by PCR using *P. denitrificans* PD01Km DNA as a template. PCR primers PD01OHF2 and PD01OHR2 were used. **C:** Southern blotting. Lane 1: molecular size standards, lane 2: *P. denitrificans* PD01 DNA, and lane 3: *P. denitrificans* PD01Km DNA. In lanes 2 and 3, *P. denitrificans* DNAs were digested with *Pst*I. A 1.2-kb *Sal*I fragment (*Km^r* gene) of pUC4K was used as a probe for hybridization.

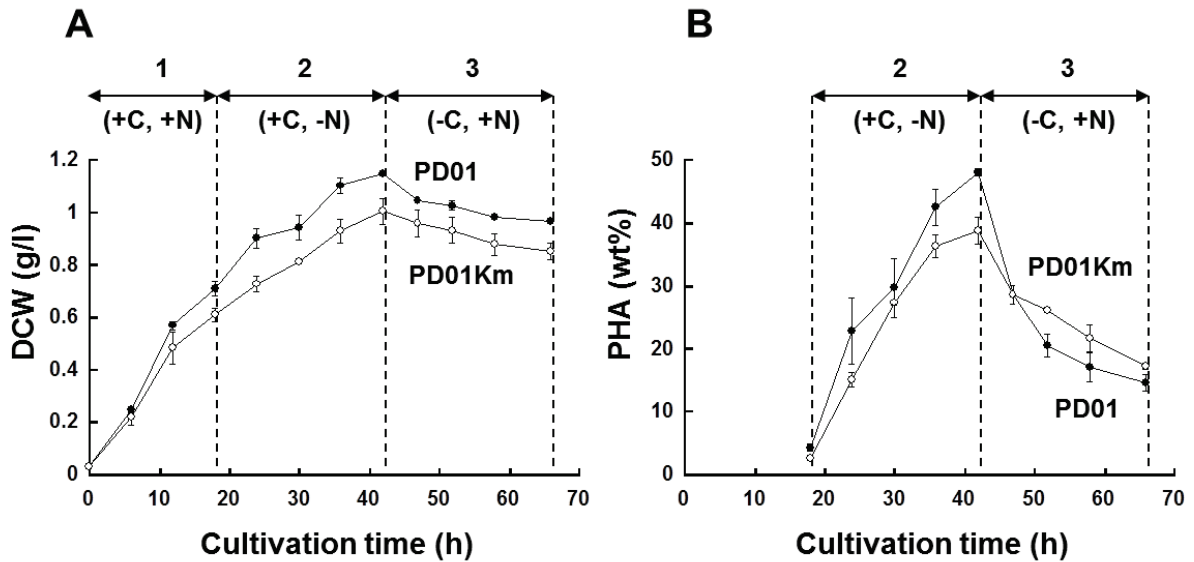


Fig. 16 Growth (A) and intracellular synthesis and degradation of PHA (B) in *P. denitrificans* strains PD01 and PD01Km. Cultivation was started with *n*-pentanol as carbon source (stage 1: +C, +N). At 18 h, cells were harvested and transferred into nitrogen-deficient PD medium (stage 2: +C, -N). At 42 h, cells were harvested and transferred into carbon-deficient PD medium (stage 3: -C, +N). PHA contents were expressed as wt% in the dried cells. In A and B, the mean and standard deviation were calculated from the experiments run in triplicate.

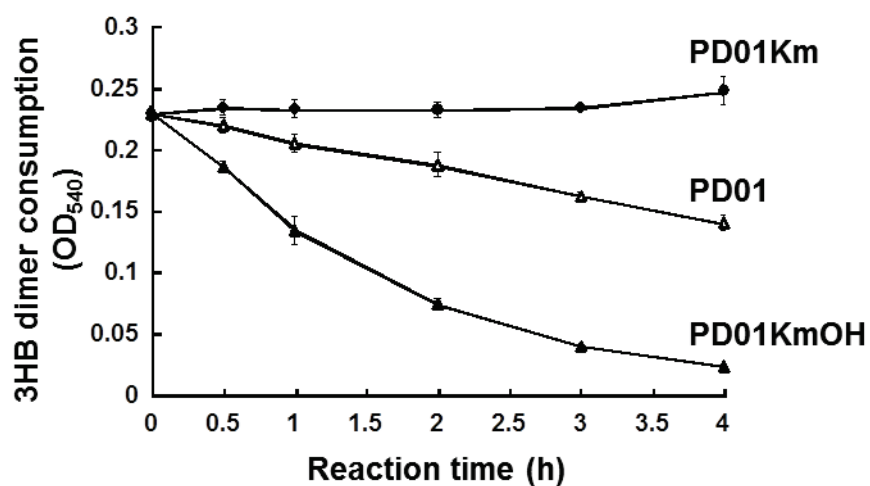


Fig. 17 PhaZc activity in *P. denitrificans* strains PD01, PD01Km, and PD01KmOH. Cell-free extracts (0.38 mg of protein) from each strain were used in each enzymatic reaction. The mean and standard deviation were calculated from the experiments run in triplicate.

Chapter 5

3HB dehydrogenase in *P. denitrificans* PD01

5.1. Introduction

3HB dehydrogenases (3HBDH) have been investigated with sources from microorganisms and mammals. A number of bacterial 3HBDHs have been purified and biochemically characterized. The enzyme plays a key role in the redox balance and in the energetic metabolism in PHB-producing bacteria. The genes encoding 3HBDH activity have been reported in several PHB-related bacterial strains (*R. pickettii* T1, *P. aeruginosa*, *Sinorhizobium* sp. strain NGR234, *R. meliloti*, *P. fragi*, and *Acidovorax* sp. strain SA1). The 3HBDH gene (*hbd*) has not been isolated from *P. denitrificans*. In this chapter, the gene was isolated to purify 3HBDH efficiently. The purified 3HBDH was mainly used for determining the substrate specificity toward 3HV monomer.

5.2. Materials and methods

5.2.1. Bacterial strains, growth conditions, and plasmids

E. coli M15 and DH5 α (Table 1 in Chapter 2) were grown at 37°C in LB medium. When needed, the medium was supplemented with Amp (50 μ g/ml), Km (15 μ g/ml), and IPTG (0.1 mM). pTAC-1 and pQE30 (Table 2 in Chapter 2) were used as plasmid vectors for *E. coli*.

5.2.2. Cloning of the *hbd* gene

In order to clone the *hbd* gene from *P. denitrificans* PD01, PCR was performed and an amplified DNA fragment was introduced into pTAC-1 by TA-cloning.

5.2.2.1. PCR primers

PCR primers were designed by referring the nucleotide sequence of *P. denitrificans* Pd1222 *hbd* as follows.

PD3HBF1 (forward): 5'-CGGGATCCTTCGAGAAATTCCTCAGCGG-3'
*Bam*HI

PD3HBR (reverse): 5'-CGAAGCTTAGGTCTCGACCGAATATTCC-3'
*Hind*III

5.2.2.2. PCR conditions

PCR was performed with a KAPA Taq EXtra PCR with dNTPs kit (KAPA Biodynamics) as follows.

PCR mixture (in 50 μ l)	
Sterilized Milli-Q water	33.25 μ l
5 \times KAPATaq EXtra Buffer	10 μ l
dNTPs (10 mM each)	1.5 μ l
MgCl ₂ (25 mM)	3.5 μ l
PD3HBF1 (20 mM)	1.25 μ l
PD3HBR (20 mM)	1.25 μ l
PD01 genomic DNA (46 μ g/ml)	1.0 μ l
KAPATaq EXtra DNA Polymerase	0.25 μ l

PCR conditions

Initial denaturing	95°C, 4 min	
Denaturing	95°C, 1 min	} 30 cycles
Annealing	51°C, 1 min	
Extension	72°C, 1 min	
Final extension	72°C, 5 min	

5.2.2.3. TA cloning

A 0.8-kb DNA fragment was amplified by PCR and named HbdF1R. The DNA fragment was inserted into pTAC-1 by TA-cloning.

TA cloning was performed by using a DynaExpress TA PCR Cloning kit (BioDynamics Laboratory).

Ligation reaction mixture (in 10 μ l)

pTAC-1 vector (50 μ g/ml)	1 μ l
HbdF1R (48 μ g/ml)	3 μ l
2 \times Ligation Buffer	5 μ l
Ligase Mixture	1 μ l

After the ligation reaction at 16°C for 30 min, 2 μ l of the mixture were used for transforming Jet competent cells (*E. coli* DH5 α). The ligation reaction mixture (2 μ l) was mixed with 50 μ l of the competent cells. The mixture was incubated on ice for 5 min and transferred to a new 1.5-ml sterilized tube containing 0.45 ml of Recovery

Medium. After vortexed briefly, the tube was incubated at room temperature for 5 min. Aliquots (50 μ l) of the cell suspension in the tube were spread onto LB agar plates containing Amp and X-Gal. The plates were incubated at 37°C overnight. Colonies grown on the LB agar plates were screened for plasmid isolation.

A recombinant plasmid was obtained and named pTAC1-hbd. It was used for nucleotide sequencing with primers of T7 (5'-AATACGACTCACTATAG-3') and M13R (-20) (5'-GCGGATAACAATTTCACACAGG-3'). Nucleotide sequence of the *hbd* was deposited in the GenBank/EMBL/DDBJ databases under accession no. AB839358.

Hbd activity was detected in *E. coli* DH5 α harboring pTAC1-hbd when assayed as described in this chapter (5.2.4.).

5.2.3. Preparation of reaction substrates

(*R*)-3-hydroxybutyric acid (Sigma-Aldrich) was used as a substrate for Hbd reaction. 3HV was prepared from methyl (*R*)-3-hydroxyvalerate (Sigma-Aldrich) by hydrolyzation of 10 mM in 0.1 M NaOH at 30°C for 40 min according to Hesselmann et al. (56) with a slight modification. 3HB and 3HV concentrations were determined with a water-soluble tetrazolium salt, WST-1 (a sodium salt of 2-(4-iodophenyl)-3-(4-nitrophenyl)-5-(2,4-disulfophenyl)-2*H*-tetrazolium) (Dojindo, Japan) as described by Lam et al. (57).

5.2.4. Hbd assay

To prepare cell-free extracts, cells were suspended in 50 mM Tris-HCl (pH 7.0) and disrupted by sonication at 4°C for 3 min (Bioruptor UCD-200T, Tosho Denki). After

centrifugation at $11,000 \times g$ and 4°C for 10 min, the supernatant was dialyzed against 20 mM Tris-HCl (pH 7.0) at 4°C overnight.

Hbd activity was assayed as follows. Cell-free extracts were added to a reaction mixture (3 ml) composed of 25 mM Tris-HCl (pH 8.5), 2.5 mM nicotinamide adenine dinucleotide (NAD), and 0.8 mM 3HB. The conversion of NAD to NADH was measured by monitoring the change in OD at 340 nm.

5.2.5. Overproduction of His-Hbd in *E. coli*

To purify Hbd from *E. coli* cells, pQE30-hbd was constructed. A 0.8-kb *Bam*HI-*Hind*III DNA fragment (HbdF1R) was excised from pTAC1-hbd and inserted into pQE30.

5.2.5.1. Construction of pQE30-hbd

pQE30 and pTAC1-hbd were digested with restriction enzymes as follows.

Restriction enzyme reaction mixture (in 20 μl)	
Sterilized Milli-Q water	6 μl
<i>Bam</i> HI	1 μl
<i>Hind</i> III	1 μl
10 \times Buffer 2	2 μl
pQE30	10 μl
or pTAC1-hbd	

The mixture was incubated at 37°C for 3 h. After the restriction enzyme reaction, agarose gel electrophoresis was performed. DNAs were recovered from agarose gel with a NucleoSpin Extract II kit as described in Chapter 2 (2.2.2.3).

Ligation of pQE30 and HbdF1R fragment was performed by using a DynaExpress DNA Ligation kit at 16°C for 30 min.

Ligation reaction mixture (in 20 µl)	
<i>Bam</i> HI/ <i>Hind</i> II-digested pQE30 (11.2 µg/ml)	2.5 µl
<i>Bam</i> HI/ <i>Hind</i> II-digested HbdF1R (9.1 µg/ml)	7.5 µl
2 × Ligation buffer	10 µl
Ligase mixture	1 µl

5.2.5.2. Preparation of *E. coli* M15/pREP4 competent cells

Competent cells of *E. coli* M15/pREP4 were prepared as described in Chapter 3 (3.2.2.5.).

5.2.5.3. Transformation

Transformation of *E. coli* M15/pREP4 with the ligation mixture was performed as described in Chapter 3 (3.2.2.6.).

5.2.5.4. Overproduction of His-Hbd in *E. coli* cells

E. coli M15/pREP4 was cultivated as described for His-PhaZc in Chapter 3 (3.2.3.). His-Hbd production was induced with 0.1 mM IPTG.

5.2.6. Purification of His-Hbd from *E. coli*

His-Hbd was purified from *E. coli* cells as described for His-PhaZc in Chapter 3 (3.2.4.).

5.2.7. Native PAGE

Native PAGE was done as described by Ornstein and Davis (58, 59).

5.2.7.1. Composition of polyacrylamide gel

Running gel (6%, 15 ml):

Acrylamide-Bis mixture	2 ml
1.5 M Tris-HCl (pH 8.8)	3.75 ml
Milli-Q water	9.1 ml
APS (0.1 g/ml)	0.15 ml
TEMED	5 μ l

Stacking gel (5%, 5 ml):

Acrylamide-Bis mixture	0.55 ml
1.5 M Tris-HCl (pH 6.8)	0.625 ml
Milli-Q water	3.725 ml
APS (0.1 g/ml)	0.05 ml
TEMED	5 μ l

5.2.7.2. Electrophoresis

Electrophoresis was performed at 6°C and 20 mA with a running buffer (3.03 g of Tris-HCl and 14.4 g of glycine, pH 8.3, 1 L). Sample buffer (0.8 ml) composed of 0.05 ml of 1 M Tris-HCl (pH 6.8), 0.2 ml of glycerol, 0.02 ml of 0.5% (w/v) bromophenol blue, and 0.53 ml of Milli-Q water. An equal volume mixture of enzyme solution and sample buffer was mixed and subjected to electrophoresis.

5.2.7.3. Activity staining

Activity staining of Hbd after native PAGE was performed by incubating the gel in the reaction mixture with gentle shaking. Reaction mixture (20 ml) consisted of 25 mM Tris-HCl (pH 8.5), 2.5 mM NAD, 0.8 mM DL-3-hydroxybutyrate (Na salt), 0.2 mM 1-methoxy phenazine methosulfate, and 0.2 mM nitroblue tetrazolium. A M.W. Marker of “Daiichi” I (for Davis’ method) (Daiichi Pure Chemicals) was used as a molecular mass standard.

5.2.8. Substrate specificity of His-Hbd

Substrate specificity was determined as follows. Purified His-Hbd was added to a reaction mixture (3 ml) consisting of 25 mM Tris-HCl (pH 8.5), 2.5 mM NAD, and 0.8 mM 3HB or 3HV. The conversion of NAD to NADH was measured by monitoring the change in OD at 340 nm.

5.2.9. Enzymatic reaction with His-PhaZc and His-Hbd

Purified His-PhaZc and His-Hbd were added to a reaction mixture (3 ml) consisting of 25 mM Tris-HCl (pH 8.5), 2.5 mM NAD, and 3HV dimer. Consumption of the dimer

was determined by the alkaline hydroxylamine procedure described by Hestrin's. The conversion of NAD to NADH was measured by monitoring the change in OD at 340 nm.

5.3. Results

5.3.1. Cloning and analysis of the *hbd* gene

Complete nucleotide sequence of the *P. denitrificans* Pd1222 genome is available in the NCBI database. An ORF annotated as “3-hydroxybutyrate dehydrogenase” was found (gene id: 4579406, protein id: YP 914739.1). Although not demonstrated experimentally, it was possible that the ORF encodes Hbd activity. According to the nucleotide sequence of the ORF region, PCR primers for Hbd were designed (Fig. 18) and PCR was performed. A 0.8 kb DNA fragment (HbdF1R) was obtained with the primers. After pTAC1-hbd was constructed, nucleotide sequence of the fragment was determined. An ORF composed of 780 nucleotides was found in the 789-bp fragment. The nucleotide sequence of the ORF was identical to that of *P. denitrificans* Pd1222. The ORF encoded a 27.2-kDa protein with 259 amino acids.

5.3.2. Expression and overproduction of His-Hbd

Hbd expression was examined with *E. coli* cells harboring pTAC1-hbd. A protein with predicted molecular mass was produced. As expected, the protein catalyzed the dehydrogenation of 3HB in the presence of NAD. NAD could not be replaced by NADP in the enzymatic reaction (data not shown).

In order to overproduce the enzyme for purification, it was expressed with pQE30 as a His-tagged protein. A large amount of protein band (His-Hbd) with a molecular mass

of 29 kDa was detected on SDS-PAGE (Fig. 19A). His-Hbd was purified to homogeneity as a 120-kDa protein. The Hbd activity was confirmed by activity staining after native PAGE (Fig. 19B), indicating that Hbd is a tetrameric protein.

5.3.3. Substrate specificity of His-Hbd

Purified His-Hbd was examined only for substrate specificity. It dehydrogenated not only 3HB but also 3HV (Fig. 20). Specific activity of His-Hbd was 0.67 U/mg for 3HB and 0.48 U/mg for 3HV, respectively. When the enzyme was omitted from the reaction mixture, the OD at 340 nm did not change.

5.3.4. Sequential His-PhaZc and His-Hbd reactions

Two-step reaction with two enzymes of His-PhaZc and His-Hbd was carried out by using 3HV dimer as a substrate. When both enzymes were included in enzymatic reactions, 3HV dimer underwent sequential reactions of hydration and dehydrogenation (Fig. 21). When the enzymes were omitted from the reaction mixture, the OD at 540 nm and at 340 nm did not change. The result implies that the two-step reaction should proceed also in *P. denitrificans* PD01 cells. Therefore, these two enzymes are important for intracellular PHV degradation.

5.4. Discussion

3-Hydroxybutyrate dehydrogenase (3HBDH) exists in a wide variety of organisms. The enzyme has been investigated with PHB-producing bacteria. Although 3HBDH has been considered as a key enzyme in PHB metabolism, investigations concerning it seem to be a very few. In Chapter 5, 3HBDH of *P. denitrificans* PD01 (Hbd) was investigated

and intensely focused on 3HV as a substrate for Hbd reaction.

The *hbd* gene of *P. denitrificans* PD01 was cloned in *E. coli* and its nucleotide sequence was determined. The nucleotide sequences of 3HBDH genes were compared among *P. denitrificans* PD01 and other PHB-relating bacteria (*R. meliloti* Rm11107 and *R. pickkettii* T1) (Fig. 22). *R. meliloti* Rm11107 is a PHB-producing bacterium and used to investigate the relation between PHB cycle and symbiosis. The *bdhA* gene has been reported in *R. meliloti* Rm11107. *R. pickkettii* T1 is a PHB-degradable bacterium and extracellularly produces PHB depolymerase. Two genes of *bdh1* and *bdh2* have been isolated from *R. pickkettii* T1. Identities of *P. denitrificans* PD01 *hbd* to *bdh2* and *bdhA* were 78% and 69%, whereas was too low to *bdh1*. In the deduced amino acid sequences, identities and similarities of *P. denitrificans* PD01 Hbd to BdhA, BDH1, and BDH2 were 58%/72%, 57%/70%, and 41%/56%, respectively (Fig. 23).

In investigations of 3HBDH including BdhA from *R. meliloti* and BDH1/BDH2 from *R. pickkettii*, 3HB has been used for examining their catalytic activity. 3HV has not been used as a reaction substrate with an exception of 3HBDH from *R. sphaeroides*. A purpose of this study is to investigate intracellular degradation of PHA containing 3HV unit (PHV and/or PHB-PHV). Therefore, in Chapter 3, 3HBOH was purified as His-PhaZc and examined its catalytic activity toward 3HV oligomers. In Chapter 5, the *hbd* gene of *P. denitrificans* PD01 was isolated and characterized. The gene was expressed in *E. coli*. His-Hbd was purified and used for examining its catalytic activity toward 3HV. It was reported that 3HBDH purified from *R. sphaeroides* dehydrogenated 3HV about 20 times more slowly than 3HB (60). In contrast, His-Hbd from *P. denitrificans* PD01 catalyzed the dehydrogenation of 3HV and 3HB at similar rates.

When both His-PhaZc and His-Hbd were included in an enzymatic reaction system,

3HV dimer underwent sequential reaction of hydrolysis and dehydrogenation. This result suggests that PhaZc and Hbd should contribute to the degradation of 3HV-containing PHA in *P. denitrificans* PD01.

Acetoacetate (3-oxobutanoate) is formed from 3HB in dehydrogenation reaction by Hbd. PHB cycle is completed by converting acetoacetate to acetoacetyl-CoA. On the other hand, the reaction product from 3HV in Hbd reaction is 3-oxovalerate (3-oxopentanoate). It is interesting to make clear how 3-oxovalerate is metabolized in *P. denitrificans* cells.

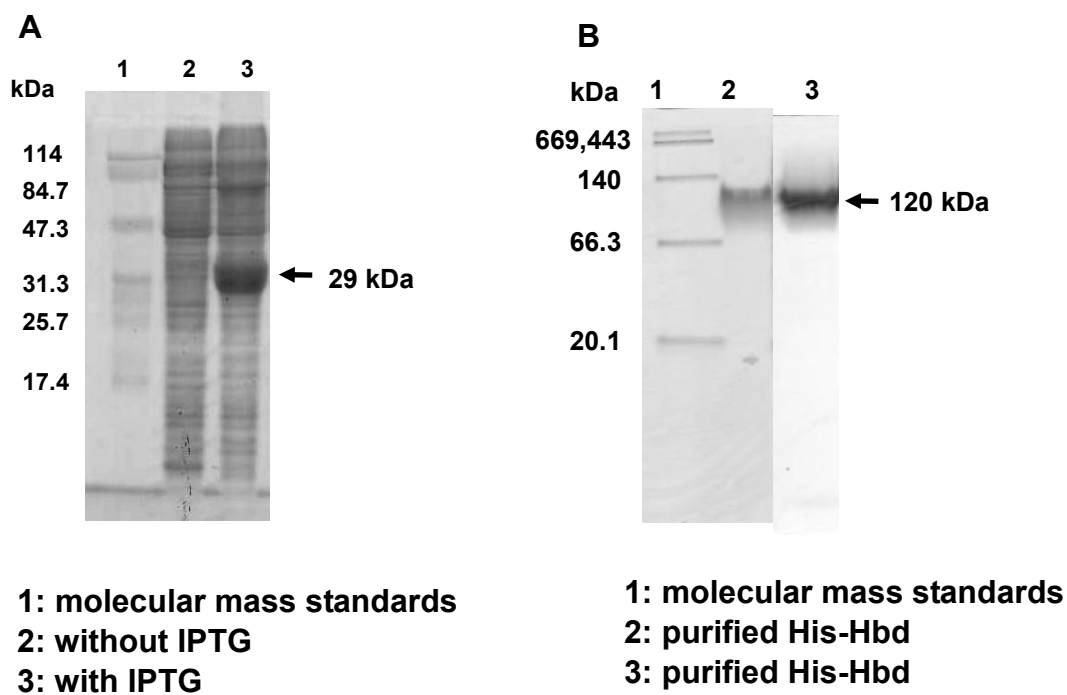


Fig. 19 Expression and purification of His-Hbd. **A:** SDS-PAGE of cell-free extracts from *E. coli* carrying pQE30-hbd with or without IPTG. **B:** Native-PAGE of purified His-Hbd. Proteins in lanes 1 and 2 were stained with Imperial Protein Stain. His-Hbd in lane 3 was visualized by activity staining as described in Materials and Methods.

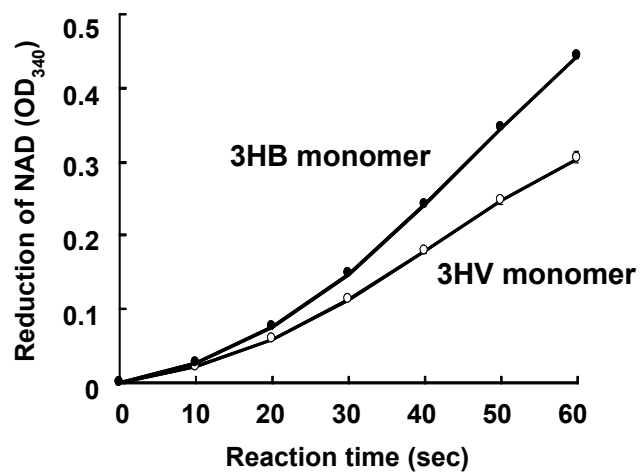


Fig. 20 Substrate specificity of the His-Hbd for 3HB and 3HV monomers. The mean and standard deviation were calculated from the experiments run in triplicate.

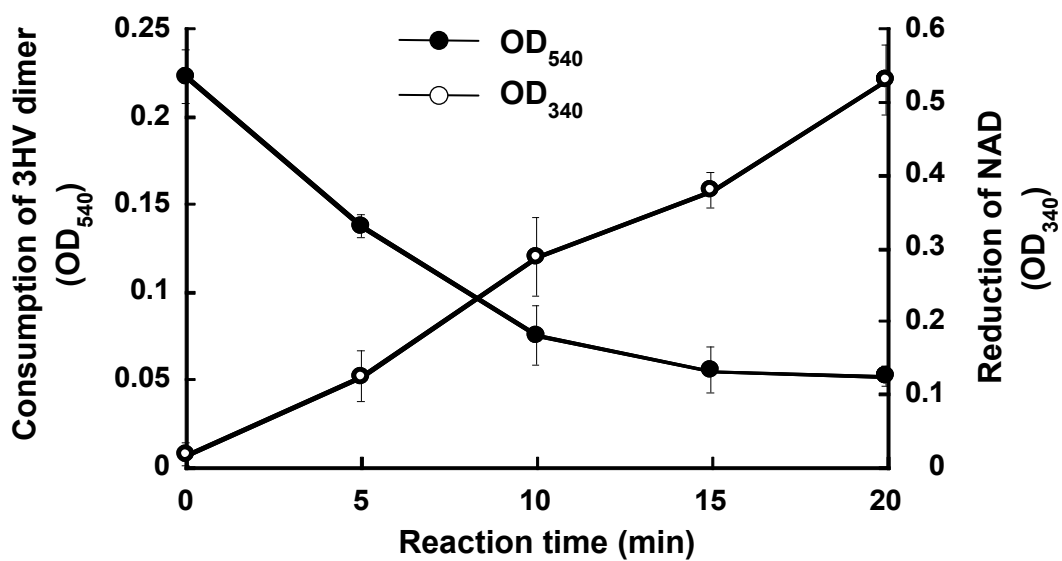


Fig. 21 Two-step reaction with 3HV dimer as a substrate in the presence of His-PhaZc and His-Hbd. The mean and standard deviation were calculated from the experiments run in triplicate.

```

hbd      TTCGAGAAATTCCTCAGCGGCAAGACGGCGGTGGTGACGGGCTCCAATTGCGGGATCGGG
bdh1     -----ATGCAGCTCAAAGGAAAGTCCGGCCATCGTGACCGGTGCCGCCAGCGGTATCGGC
bdhA     -----ATGACCAAGACTCGGGTGATAACGGGTTCACCAGCGGCGCATCGGA
bdh2     -----ATGCTTCAAGGCAAGACGGCGGTGGTGACGGGTTCACCCTGCGGCATCGGA
          . . . . . * * * * * * * * * * * * * * * * * * * * * * * * * * * * * *
hbd      CTCGGGATCGCGCATGAACTGGCGCGCGGGCGGTGATCTCGTGCTGAACAGCTTTACC
bdh1     AAGGCCATCGCCGAACCTGCTGGCCAAAGGAGGGCGCGCGGTGCGCATCGCCGACCTGAAC
bdhA     TTGGCGATCGCCCGGACCTTGGCCAAAGGCGGTGCGCATATCGTCTGACCGG-----C
bdh2     CTCGGCATCGCCAGGCGCTTGGCGCGCAAGGCGCCAACTCATCGTCAACGGT-----T
          . * * * * * * * * * * * * * * * * * * * * * * * * * * * * * * * * *
hbd      GACATGCCCGAGGACCACGCCCTTGGCGAAAGCCTTGGCGCGG--AGCATGG--CGTCG--
bdh1     CTGGAGGCGCGCGCGCGCGCGCTGCCGGCATCGAGGCGCGCGCGGCAAGGCCATCG--
bdhA     TTCGGTGC CGCGGACGAGATCAGGACCGTACGGATGAACTGCGAGGCTGAGCTCGGT
bdh2     TTCGGGAGCGCGCGCGCGCGGACGAGATCG---CGGCGCGCGCGCAAGTCATCGCG
          . . . . . * * * * * * * * * * * * * * * * * * * * * * * * * * * *
hbd      -AGGTGCGCTATGTCAGGCGGACATGTCCAAGGGCGCGACTGCCGCGCCTGATCG--
bdh1     -CGGTGGCATGGACGTCACCGAGCGGCGTCCGTCAACAGGCGACCGACGAGGTCG--
bdhA     ACGGTGCTTCATCACC CGCGGACATGACCAAGCCCTCCGAAATCGCCGACATGATGGG
bdh2     CTGGG---CTACCAGGTGCCGACATGAGCAAGGCATCGGAGATCGAGCAGATG---
          ** * * * * * * * * * * * * * * * * * * * * * * * * * * * * * *
hbd      -----AAAAGCCGCGCGCTG---CGACATCCTGGTGAACAATGCCGGCATCCAGCAT
bdh1     -----CGCAGGCCTTCGGCAATATCGATATCCTGGTGTGCAACCGCGCATCCAGATC
bdhA     ATGGTTCGCGATCGCTTCGGCGCGCGCGCATATCCTCGTCAACAATGCCGCGCTGCGATTC
bdh2     CGCTATGCGAAGCGGAATTCGGCGCGGACATCCTCGTCAACAATGCCGGCATCCAGCAC
          . * * * * * * * * * * * * * * * * * * * * * * * * * * * * * * *
hbd      GTCGCACCGATCCCGGATTTCCCGGGGAGAAATGGGATGCGATCATCGCCATCAACCTG
bdh1     GTCACCCGATCCAGACTACGCTTCTCCGACTGGAAGAAGATCAGGCCATCCAGATC
bdhA     GTTGAAAAGATCGAGGATTTCCCGTTCGAGCATGGGACCGGATCATCGCCGTCATCTC
bdh2     GTCGCGCAGCATCGAGGACTTCCCGCGCGGACGCTGGGACCGCATCATCGCCATCAACCTG
          * * * * * * * * * * * * * * * * * * * * * * * * * * * * * * * * *
hbd      AGTTCGCGCTTTACACCCAGCGCGCGCGCT---GCCCTGATGCGCAAGGCGAGGCTGG
bdh1     GACGGCGCCTTCTCAACCACCAAGCGCGCGCTCAAATACATGTCACCGGACAGCGCGCG
bdhA     TCCTCTCTCTTTCACACCATTCGTGGCGCAT---TCCGCGATGAAGAAGAGGCTGG
bdh2     ACCTCGCGCTTCCATACCACGCGGCTGGCCCT---GCCCGCATGCGCGAGAGACTGG
          * * * * * * * * * * * * * * * * * * * * * * * * * * * * * *
hbd      GGGCGGTGATCAACATCGCCTCGGCGCACGGGCTGACGGCCAGGCAATACAATCGGCC
bdh1     GGCACGGTATCTACATGGGCTCGGTGCATTCACCAGGCGCTCGCCGCTGAAGTCGGCC
bdhA     GGCCGGATCATCAATATCGCGTCCGCTCATGGCCTCGTGGCCTCCCGCTTCAAGTCGGC
bdh2     GGGCGGTGATCAATATCGCCTCCACGCGCGGCTGGTCCGCTCGGACAGGACTCGCC
          * * * * * * * * * * * * * * * * * * * * * * * * * * * * * * * * *
hbd      TATGTCGCGGCCAAGCAGGCGCATCGTCGGCCTGACCAAGGTGACGGCGCTGGAGACCGG
bdh1     TATGTCGCGCCAAACATGCGCTGCTGGCCTGGCGCGGTGCTGGCCAAAGGAGGGGGG
bdhA     TATGTCGCGGCCAAGCATGGTATCATGGGTTGACCAAGACTGTGGCGTGGAGGTTGGG
bdh2     TACGTGGCGCCCAAGCAGGCGCATCGTCGGCCTGACCAAGGTGACCGCGCTGGAAACCGG
          * * * * * * * * * * * * * * * * * * * * * * * * * * * * * * * * *
hbd      AAGGAGCGGATCACCTGCAACGCCATCTGCCCCGGCTATGTGCTGACCCCGATCGTGAA
bdh1     GAGTTCACCGTGGCTCCACGCTGATCTGCCCCGGCTTCGTGCGCACGCGCTGGTGCAG
bdhA     GAGAGCGGTGTCACCGTGAACCTGATCTGCCCCGGCTACGTTCTGACGCGCTCGTTCGAA
bdh2     CAGAACCGGCTCACCGCAACGCGATCTGCCCCGGCTGGTGTGCTGACCGCTGGTGCAG
          * * * * * * * * * * * * * * * * * * * * * * * * * * * * * * * * *
hbd      AAGCAGATCCCCGACCGATGAAGACCCACAACATGAGCCGCGAGGACGTGAT--CGCCAA
bdh1     AAGCAGATTCCCGAGCAGGCAAGGAGCTGGGCGATCAGCGAGGAGGAGGTAGTTCGCGCG
bdhA     AAGCAGATACCGGATCAGGCGAGAACCGCGCGCATCACCGAGGAACAGGTGAT--CAACGA
bdh2     AAGCAGGTGACGCGCGCCAG---CGCACGGCATCAGGTCGAGCAGGCCAA--GCGCGA
          * * * * * * * * * * * * * * * * * * * * * * * * * * * * * * * * *
hbd      GGTCAATGCTG--CAGCGCCAGCCCTCGGGCAATTCGCCACGGTCGAGCAGATGGGGCGG
bdh1     G--TGATGCTGGGCGGTACCG--TCGACGGCGTCTTCAACAGGTTGACGACGTCGCGCGC
bdhA     GGTGATGCTCA--AGGACAGCGGACGAAAAAGTTCATCACCGTCAACAGGTTGCTCC
bdh2     GCTCGTCAATCG--AGAAGCAGCCCTCGGGCCAGTTCGTCACGCGCGCAACTGGGCGG
          * * * * * * * * * * * * * * * * * * * * * * * * * * * * * * * * *
hbd      ACGGCGGTCTTCTGTGCTCGCGCGCGGGAGCAGATCACCGGACGACCATCTCGGTG
bdh1     ACGGCGGTCTTCTGTGCTCGCGCGCGGGAGCAGATCACCGGACGACCATCTCGGTG
bdhA     CTGGCGCTCTATCTGAGGCGGACGATGCGCGCGGATCACCGGACGATGTTTCGATG
bdh2     CTGGCGGTCTTCTGCGCAGGAGCAGGCGCGGAGTGGCGCGGCGCATCTGGAACATG
          . * * * * * * * * * * * * * * * * * * * * * * * * * * * * * * * * *
hbd      GACGGGGGTGGACGGCGCTTTAG
bdh1     AGCCACGGCTGGTATATGCAGTAA
bdhA     GATGGCGGCTGGACGGCGAGTA-
bdh2     GCCGCGGCTGGTTCGCGCAATAG
          . * * * * * * * * * * * * * * * * * * * * * * * * * * * * * *

```

Fig. 22 Alignment of nucleotide sequences among *hbd* from *P. denitrificans* PD01, *bdhA* from *R. meliloti* Rm11107, and *bdh1/bdh2* from *R. pickettii* BCM18351.

```

Hbd      EKFLSGKTAVVTGSNSGIGLGLIAHELARAGADLVLNSFTDMPEDHALAESLAAEHGVEVR
BDH2     --MLQGKTALVTGSTCGIGLGLIAQALAAQGANIIVNGFR--RADGARQQIAAAGQVIRLG
Bdh      -----TKTAVITGSTSGIGLAIARTLAKAGANIVLNGFGAPDEIRTVTDEVAGLSSGTVL
BDH1     -MQLKGKSAIVTGAASGIGKAI AELLAKEGA AVAIADLNLEAARAAAAGIEAAG--GKAI
          *:>::*: .*** .** .** ** : : .: : * .

Hbd      YVQADMSKG--ADCRALIEKAG-ACDILVNNAGIQHVAPIPDFPGEKWDIA IAINLSSA
BDH2     YHGADMSKASEIEDMMRYAEAEF-AADILVNNAGIQHVASIEDFPPERWDIA IAINL TSA
Bdh      HHPADMTPKSEIADMMAMVADREFGGADILVNNAGVQFVEKIEDFPVEQWDRI IAVNLSSS
BDH1     AVAMDVTSEASVNQATDEVAQA FGNIDILVSNAGIQIVNPIQNYAFSDWKKMQAIHVDGA
          *: : . : ****.***:* * * : : . * . : *:: : .

Hbd      FHHTAAALPLMRK-AGWGRVINIASAHLTASEYKSA YVAAKHGIVGLTKVTALETAKEP
BDH2     FHTRLALPGMRQ-KNWGRVINIASTHGLVASAQKSA YVAAKHGIVGLTKVTALETAQNR
Bdh      FHTIRGAI PPMKK-KGWGRIINIASAHLVAS PFKSA YVAAKHGIMGLTKTVALEVAESG
BDH1     FLTTKAALKYMYRDKRGGTVI YMGSVHSHEAS PLKSA YVAAKHALLGLARVLAKEGAEFN
          * * * : * : * : * : .*. ** *****.:*::. * * * :

Hbd      ITCNAICPGYVLTPIVEKQIPDQMKTHNMSREDVIAKVMLQRQPSGQFATVEQMGGTAVF
BDH2     VTANAI CPGWVLTPLVQKQVQARF-AHGISVEQAKRELVIEKQPSGQFVTPDELGALAVF
Bdh      VTVNSICPGYVLTPLVEKQIPDQARTRGITEEQVINEVMLKGQPTKFFITVEQVASLALY
BDH1     VRSHVICPGFVRTPLVDKQIPEQAKELGISEEEVRRVMLGGTVDGVFTTVDDVARTALF
          : : ***** * *:***: : .:: *:. .::: * * : : . *::

Hbd      LCSPA AEQITGTTISVDGGWTAL
BDH2     LASEAGRQVRGAIWNMAGGWF AQ
Bdh      LAGDDAAQITGTHVSMDGGWTAQ
BDH1     LCAFP SAALTGQSFIVSHGWYMQ
          *.. . : * : **

```

Fig. 23 Alignment of amino acid sequences among Hbd from *P. denitrificans* PD01, Bdh from *R. meliloti* Rm11107, and BDH1/BDH2 from *R. pickettii* BBCM18351.

Chapter 6

Conclusions

6.1. Cloning and expression of the *phaZc* of in *E. coli*

In Chapter 2, the gene was cloned by PCR and TA-cloning. For PCR, DNA primers were designed by referring an ORF annotated as “hydrolase or acetyltransferase” in the genomic DNA of *P. denitrificans* Pd1222. A 0.8-kb DNA fragment was introduced into pTAC-1 to produce pTAC1-OHF2R2. Nucleotide sequence of the amplified DNA fragment was determined, which was identical to that of *P. denitrificans* Pd1222. One ORF was found and compared with 3HBOHs from *R. eutropha* H16 and *Acidovorax* sp. strain SA1. Although nucleotide sequences were not resemble each other, relatively higher identity was observed in the deduced amino acid sequences. The fragment was introduced into pUC19 to produce pUC19-*phaZc*. 3HBOH activity was detected in *E. coli* harboring pUC19-*phaZc*. It was concluded that 3HBOH gene of *P. denitrificans* PD01 was successfully cloned in *E. coli*. The ORF was named *phaZc* according to the classification of 3HBOH.

6.2. Purification and properties of His-PhaZc

In Chapter 3, pQE30-*phaZc* was constructed and expressed in *E. coli* to purify PhaZc as a His-tagged protein. The protein expression was induced with a relatively lower concentration of IPTG (10 μ M) to avoid the formation of inclusion body. His-PhaZc was successfully purified to homogeneity by using affinity and gel filtration chromatography.

Some enzymatic properties were clarified with the purified His-PhaZc. Optimum pH

for enzymatic reaction was 8.0. The enzyme revealed the highest activity at a broad range of temperature from 30°C to 50°C.

Substrate specificity of the His-PhaZc was determined. Here, the catalytic activity of PhaZc toward 3HV oligomers was especially noted. 3HB and 3HV dimers were prepared from PHB and PHV granules by extracellular PHB and PHV depolymerase reactions. It was demonstrated that His-PhaZc can hydrolyze not only 3HB dimer but also 3HV dimer. Specific activity of the prepared His-PhaZc was similar. It was the first report showing the reactivity of 3HBOH toward 3HV oligomers.

p-Nitrophenyl fatty acid esters with several carbon atoms ranging from 2 to 10 were examined for PhaZc activity. *p*-Nitrophenyl esters of propionic (C3) and butyric (C4) acids were efficiently hydrolyzed, which was reasonable. Among chemical reagents tested, His-PhaZc was strongly inhibited by DFP. It suggests that the active site of PhaZc contains a serine residue. The presence of lipase box-like motif in PhaZc supports the suggestion.

6.3. Effect of mutation of the *phaZc* gene on cellular PHA content

In Chapter 4, the *phaZc* gene was disrupted in *P. denitrificans* PD01 to examine how the gene affects the PHA synthesis and degradation in the cells. Disruption of the gene was successfully performed, judging from the results of Southern blotting, PCR, and enzymatic activity.

Growth was not affected by disrupting the *phaZc* gene. Also, PHA synthesis profile was similar between *P. denitrificans* wild type (PD01) and mutant (PD01Km) strains. However, under PHA degradation conditions (carbon-sufficient and nitrogen-deficient conditions), PHA degradation profile was different between these strains. Cellular PHA

contents were decreased by 40.5 % (w/w) and 26.3% (w/w) in the wild type and mutant strains, respectively. It was concluded that PhaZc activity at least affects the degradation rate of PHA.

In order to confirm whether only one 3HBOH gene exists in *P. denitrificans* PD01, another mutant was constructed. To do it, pBBR1MCS4-phaZc was introduced into *P. denitrificans* PD01Km. A transconjugant was named *P. denitrificans* PD01KmOH. Oligomer hydrolase activity of cell-free extracts from *P. denitrificans* PD01 and derivatives was determined. Cell-free extracts from *P. denitrificans* PD01 and PD01KmOH had oligomers hydrolase activity, but cell-free extracts of *P. denitrificans* PD01Km had no enzyme activity. The result indicates that *P. denitrificans* PD01 has a unique oligomer hydrolase gene.

6.4. Cloning, expression, and properties of Hbd

In Chapter 5, the *hbd* gene encoding 3HBDH activity was cloned from *P. denitrificans* PD01. The cloning strategy was the same as that for PhaZc. Nucleotide sequence of the *hbd* gene was determined and identical to that of *P. denitrificans* Pd1222. *E. coli* cells containing the *hbd* gene successfully displayed 3HBDH activity. 3HBDH was named Hbd.

Hbd was overproduced as His-tagged protein in *E. coli* and used for purification. His-Hbd was purified to homogeneity as a 120-kDa protein and found to be tetrameric.

As in PhaZc, it was of interest whether 3HV can be dehydrogenated by Hbd. Substrate specificities of His-Hbd were determined with 3HV and 3HB as substrates. It was concluded that both 3HB and 3HV are dehydrogenated at a similar rate by Hbd. Two-step reaction by PhaZc and Hbd was examined by using 3HV dimer as a reaction

substrate. It was concluded that 3HV oligomers also can be underwent the sequential reactions by PhaZc and Hbd.

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