3-Hydroxybutyrate Oligomer Hydrolase and 3-Hydroxybutyrate Dehydrogenase Participate in Intracellular Polyhydroxybutyrate and Polyhydroxyvalerate Degradation in *Paracoccus denitrificans*

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Genes encoding 3-hydroxybutyrate oligomer hydrolase (PhaZc) and 3-hydroxybutyrate dehydrogenase (Hbd) were isolated from *Paracoccus denitrificans*. PhaZc and Hbd were overproduced as His-tagged proteins in *Escherichia coli* and purified by affinity and gel filtration chromatography. Purified His-tagged proteins had molecular masses of 31 kDa and 120 kDa (a tetramer of 29-kDa subunits). The His-tagged PhaZc hydrolyzed not only 3-hydroxybutyrate oligomers but also 3-hydroxyvalerate oligomers. The His-tagged Hbd catalyzed the dehydrogenation of 3-hydroxyvalerate as well as 3-hydroxybutyrate. When both enzymes were included in the same enzymatic reaction system with 3-hydroxyvalerate dimer, sequential reactions occurred, suggesting that PhaZc and Hbd play an important role in the intracellular degradation of poly(3-hydroxyvalerate). When the *phaZc* gene was disrupted in *P. denitrificans* by insertional inactivation, the mutant strain lost PhaZc activity. When the *phaZc*-disrupted *P. denitrificans* was complemented with *phaZc*, PhaZc activity was restored. These results suggest that *P. denitrificans* carries a single *phaZc* gene. Disruption of the *phaZc* gene in *P. denitrificans* affected the degradation rate of PHA.

Poly(3-hydroxyalkanoates) (PHAs) are polyester compounds that are produced and degraded by many bacteria (1). Poly(3-hydroxybutyrate) (PHB) and poly(3-hydroxyvalerate) (PHV) homopolymers and poly(3-hydroxybutyrate-co-3-hydroxyvalerate) (PHB-co-PHV) copolymers are well-known examples. Many studies of microbial PHA production have been carried out. Because of the useful application of PHAs as biodegradable polymeric materials, PHA degradation has also been studied (2–5).

PHAs serve as carbon and energy reserve materials for PHA-producing bacteria. PHB has been widely used to investigate intracellular PHA metabolism. PHB is first degraded to 3-hydroxybutyrate (3HB) monomers or 3HB oligomers (e.g., 3HB dimers, 3HB trimers, and 3HB tetramers) by PHB depolymerase (PhaZ). The 3HB oligomers are then hydrolyzed to 3HB by 3HB oligomer hydrolase, followed by 3HB dehydrogenation to acetoacetate. PhaZ proteins from several bacteria have been characterized (6–11). Enzymatic studies of intracellular 3HB oligomer hydrolases have been reported from *Zoogloea ramigera* I-16-M (12), *Paucimonas lemoignei* (formerly *Pseudomonas lemoignei*) (13), *Ralstonia eutropha* (other names, *Wautersia eutropha* and *Cupriavidus necator*) H16 (14–16), *Acidovorax* sp. strain SA1 (5), and *Paracoccus denitrificans* (17). Genes encoding 3HB oligomer hydrolase were isolated from a few strains (5, 13, 14, 16). These enzymes have been classified as PhaZb and PhaZc. PhaZb from *R. eutropha* H16 is a relatively large protein (78 kDa) and hydrolyzes 3HB oligomers. The enzyme also degrades PHB into 3HB monomers. The molecular masses of PhaZc ranged from 28 to 32 kDa. It was reported that PhaZc from *R. eutropha* H16 did not degrade native PHB granules and semicrystalline PHB but degraded various 3HB oligomers at a high specific activity. PhaZc, with a lower molecular mass, exhibited stronger 3HB oligomer hydrolase activity than PHB depolymerases and PhaZb (16).

P. denitrificans is classified as a facultatively methylotrophic bacterium (18, 19), and it can synthesize PHA from methanol, ethanol, and *n*-pentanol (20, 21). When *n*-pentanol was used as a

sole carbon source, *P. denitrificans* ATCC 17741 produced PHV or 3-hydroxyvalerate (3HV)-rich P(3HB-co-3HV) (22). We previously studied the genetic and biotechnological basis of PHA synthesis and degradation by this strain (6, 23, 24). Although the 3HB oligomer hydrolase from this strain was characterized (17), its gene remained to be identified. To date, studies on intracellular PHA degradation have been focused widely on PHB. Therefore, neither PHV degradation nor the hydrolysis of 3HV oligomers by 3HB oligomer hydrolase and the dehydrogenation of 3HV monomers by 3HB dehydrogenase in PHA-producing bacteria has been reported.

In this study, we used *P. denitrificans* NBRC 13301 (*P. denitrificans* PD01). This study reports the identification of the gene encoding 3HB oligomer hydrolase from *P. denitrificans* PD01 and the characterization of the enzyme, including its activity with 3HV oligomers. The hydrolase was named PhaZc based on the classification of 3HB oligomer hydrolase. The effect of a *phaZc* disruption on PHA degradation is also described. In addition, characterization of 3HB dehydrogenase (Hbd) from *P. denitrificans* PD01 is reported.

MATERIALS AND METHODS

Bacterial strains, growth conditions, and plasmids. *P. denitrificans* PD01 was mainly used. *P. denitrificans* ATCC 17741 was used only for preparing native PHV granules (22). They were grown aerobically in an inorganic salt medium (PD medium [20]) at 30°C. In most cases, ethanol was used at 1% (vol/vol) as the carbon source. When specified, methanol (1%,

Received 12 October 2013 Accepted 18 November 2013

Published ahead of print 22 November 2013

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doi:10.1128/AEM.03396-13

TABLE 1 *P. denitrificans* PD01 strains and plasmids used in this study

Strain or plasmid	Description	Source or reference
Strains		
PD01	Wild type	NBRC 13301
PD01Km	PD01 derivative, Δ <i>phaZc</i> Km ^r	This study
PD01KmOH	PD01Km derivative harboring pBBR1MCS4- <i>phaZc</i> , Km ^r Amp ^r	This study
Plasmids		
pUC19	Cloning vector, Amp ^r	TaKaRa
pTAC-1	TA cloning vector, Amp ^r	BioDynamics
pQE30	Expression vector, Amp ^r	Qiagen
pUC4K	Source of Km ^r (Tn903) cassette, Amp ^r Km ^r	29
pRK2013	Helper plasmid, <i>traRK2</i> Km ^r	30
pSUP5011	Source of <i>mob</i> ; Amp ^r Km ^r Cm ^r Tn5- <i>mob</i>	31
pBBR1MCS-4	Broad-host-range plasmid vector, <i>mobRP4</i> Amp ^r	32
pUC19- <i>phaZc</i>	pUC19 carrying a 0.8-kb BamHI-HindIII fragment containing <i>phaZc</i>	This study
pQE30- <i>phaZc</i>	pQE30 carrying a 0.8-kb BamHI-HindIII fragment containing <i>phaZc</i>	This study
pUC19- <i>phaZc</i> -Km	pUC19 carrying <i>phaZc</i> ::Km ^r (a 1.2-kb Sall fragment of the Km ^r gene)	This study
pUC19- <i>phaZc</i> -Km- <i>mob</i>	Suicide vector carrying fragments of the <i>phaZc</i> ::Km ^r and <i>mob</i> genes	This study
pTAC1- <i>phaZc</i> -Km	pTAC-1 carrying an amplified PCR fragment containing <i>phaZc</i> ::Km ^r	This study
pBBR1MCS4- <i>phaZc</i>	pBBR1MCS4 carrying a 0.8-kb BamHI-HindIII fragment containing <i>phaZc</i>	This study
pTAC1- <i>hbd</i>	pTAC-1 carrying a 0.8-kb fragment amplified by PCR	This study

vol/vol) or *n*-pentanol (0.05%, vol/vol) was used as a carbon source. During cultivation under nitrogen-deficient conditions, (NH₄)₂SO₄ and yeast extract were eliminated from the PD medium. *P. lemoignei* ATCC 17989 was used to prepare extracellular PHB or PHV depolymerases (25, 26) and was cultivated aerobically at 30°C in a mineral salt medium (1 liter, pH 6.8) containing 2.3 g KH₂PO₄, 5.8 g K₂HPO₄, 1.0 g NH₄Cl, 0.5 g MgSO₄ · 7H₂O, 0.1 g FeCl₃ · 6H₂O, and 4.0 g sodium succinate (or 2.0 g sodium valerate). Sodium valerate was prepared from valeric acid as described by Ch'ng et al. (27). *Sinorhizobium fredii* NBRC14780 synthesizes PHB in a growth-associated fashion (unpublished results) and was used to prepare PHB granules. The bacterium was cultivated aerobically at 30°C in a medium (1 liter, pH 7.0) containing 0.1 g KH₂PO₄, 0.7 g K₂HPO₄, 0.5 g MgSO₄ · 7H₂O, 1.0 g yeast extract, and 10 g mannitol. *Escherichia coli* strains were grown aerobically at 37°C in Luria-Bertani (LB) medium (28). Ampicillin (Amp) and kanamycin (Km) were used at 100 µg/ml and 15 µg/ml, respectively, for both *P. denitrificans* PD01 derivatives and *E. coli* cells. Bacterial growth was monitored at 660 nm. Dry cell weight (DCW) was determined by drying cells from the culture at 60°C overnight. The plasmids used in this study are listed in Table 1.

DNA manipulations. Restriction enzymes (New England BioLabs) and *E. coli* JM109 competent cells (TaKaRa, Japan) were used as recommended by the suppliers. Plasmid DNA was isolated from *E. coli* cells by using a NucleoSpin Plasmid QuickPure kit (Macherey-Nagel, Germany). A Qiagen Genomic-tip kit (Qiagen, Germany) was used to prepare genomic DNA. NucleoSpin Extract II (Macherey-Nagel, Germany) was used to recover DNA fragments from agarose gels. DNA concentrations were measured fluorometrically by using a Qubit double-stranded DNA (dsDNA) BR assay kit and a Qubit fluorometer (Invitrogen). A KAPA Taq Extra PCR kit with deoxynucleoside triphosphates (dNTPs) (KAPA Biosystems) was used for PCR. Typical PCR conditions were 30 cycles of 95°C for 1 min, 54°C for 1 min, and 72°C for 1 min. A DynaExpress TA PCR cloning kit with pTAC-1 (BioDynamics Laboratory, Japan) was used for TA cloning of PCR fragments into *E. coli* DH5α. A DynaExpress DNA ligation kit (BioDynamics Laboratory, Japan) was used for DNA ligations. A DIG High Prime DNA Labeling and Detection Starter kit 1 (Roche Applied Science, Germany) was used for Southern blotting. A 1.2-kb Sall fragment of pUC4K (29) was used to prepare a digoxigenin (DIG)-labeled DNA probe. All kits were used according to the manufacturer's instructions.

Conjugative transfer. Conjugative transfers of plasmids from *E. coli* JM109 (donor strain) to *P. denitrificans* PD01 and its derivatives (recipient

strains) were carried out by filter mating. *E. coli* HB101 harboring pRK2013 (30) was used as a helper strain. After cultivating donor, helper, and recipient strains to late exponential growth phase, cells were washed and resuspended 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% (vol/vol) LB medium. After mating, cells were washed from the membranes and the cell suspension was plated on PD medium agar plates containing a suitable selective marker. The plates were incubated for 2 to 3 days at 30°C.

Cloning of the *phaZc* and *hbd* genes from *P. denitrificans* PD01. In order to obtain the *phaZc* gene, primers PDOHF2 (forward, 5'-CGAAGCTTGATGCGCCGTCCAGTTCCTCC-3') and PDOHR2 (reverse, 5'-CGGGATCCGTCCTTCAGGTGCTTCCGC-3') were designed based on a nucleotide sequence from *P. denitrificans* Pd1222, which was annotated to encode a hydrolase/acyltransferase (nucleotides [nt] 1633350 to 1634177 in the whole genomic sequence, GenBank accession no. NC_008686.1). The start codon in PDOHF2 is underlined. The introduced restriction enzyme sites are in italic. PCR was performed using the *P. denitrificans* PD01 genomic DNA as a template. A 0.8-kb DNA fragment was amplified by PCR. The fragment was digested with HindIII and BamHI and cloned in pUC19 to produce pUC19-*phaZc*. *PhaZc* activity was confirmed using *E. coli* JM109(pUC19-*phaZc*).

To obtain the *hbd* gene from *P. denitrificans* PD01, primers PD3HBF1 (forward, 5'-CGGGATCCCTTCGAGAAATTCCTCAGCGG-3') and PD3HBR (reverse, 5'-CGAAGCTTAGGTCTCGACCGAATATTCC-3') were designed according to the nucleotide sequence of *P. denitrificans* Pd1222 *hbd*. The introduced restriction enzyme sites are in italic. An amplified DNA fragment (0.8 kb) was introduced into pTAC-1 by TA cloning. A recombinant plasmid with *Hbd* activity in *E. coli* DH5α was named pTAC1-*hbd*.

The nucleotide sequences of the *phaZc* and the *hbd* genes were determined by Solgent (Daejeon, South Korea) using an ABI 3730XL sequencer.

Construction of a *phaZc*-disrupted strain of *P. denitrificans* PD01. A suicide plasmid, pUC19-*phaZc*-Km-*mob*, was constructed with pUC19, a 1.7-kb BamHI-BamHI fragment containing the *mob* gene from pSUP5011 (31), and a 2.1-kb BamHI-HindIII fragment containing the *phaZc*::Km^r gene. This suicide plasmid was transferred by triparental mating. Transconjugants of *P. denitrificans* PD01 displaying Km resistance were selected on PD medium agar plates containing methanol and Km. One of the transconjugants was chosen and named *P. denitrificans*

PD01Km. Disruption of the *phaZc* gene in this strain was verified by PCR with primers PDOHF2 and PDOHR2 and Southern blotting with a 1.2-kb *Sall* fragment (*Km^r* gene) from pUC4K.

Complementation of the *phaZc* mutation in *P. denitrificans* PD01Km. The 0.8-kb *Hind*III-*Bam*HI fragment was isolated from pUC19-*phaZc* and inserted into the same restriction sites of the broad-host-range vector pBBR1MCS-4 (32) to produce pBBR1MCS4-*phaZc*. *E. coli* JM109(pBBR1MCS4-*phaZc*) was used as a donor strain in a triparental mating with *P. denitrificans* PD01Km as the recipient. Transconjugants were selected on PD medium containing Amp and Km. *PhaZc* activity was measured as described below. One transconjugant was chosen and named *P. denitrificans* PD01KmOH.

Preparation of reaction substrates. The 3HB used as a substrate for enzymatic reactions was (*R*)-3-hydroxybutyric acid (Sigma-Aldrich). Solutions of 3HV were prepared from methyl-(*R*)-3-hydroxyvalerate (Sigma-Aldrich) by hydrolyzation of 10 mM in 0.1 M NaOH at 30°C for 40 min as described by Hesselmann et al. (33) with a slight modification. Concentrations of 3HB and 3HV were determined with a water-soluble tetrazolium salt, WST-1 [a sodium salt of 2-(4-iodophenyl)-3-(4-nitrophenyl)-5-(2,4-disulfonylphenyl)-2*H*-tetrazolium] (Dojindo, Japan) as described by Lam et al. (34).

Native PHA (PHB and PHV) granules were isolated from PHB-rich *S. fredii* NBRC14780 grown on mannitol and from PHV-rich *P. denitrificans* ATCC 17741 grown on *n*-pentanol (35, 36), respectively. The isolated PHA granules were treated with protease (0.1% [vol/vol] Alcalase 2.4L; Novo Nordisk A.S.) in the presence of sodium oleate as a protective agent (6).

3HB and 3HV oligomers were prepared by degrading the protease-treated PHA granules with extracellular *PhaZs* prepared from *P. lemoignei* (25, 26). After the degradation reactions, degradation products were recovered from reaction mixtures by ether extraction at pH 2.0 and separated by high-pressure liquid chromatography (HPLC) using an Aminex ion exclusion HPX-87H column (0.75 by 30 cm; Bio-Rad) with 5 mM H₂SO₄ as the mobile phase. The flow rate and temperature were 0.6 ml/min and 60°C, respectively. Detection was performed at 210 nm. Under these conditions, the 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. The monomer, dimer, and trimer (usually a small amount) of 3HB were products from the PHB granules. The monomer and dimer of 3HV were products from the PHV granules. 3HB dimer and 3HV dimer fractions in the HPLC were recovered, pooled, and used as substrates for 3HB oligomer hydrolase reactions.

Concentrations of the oligomers were determined as water-soluble ester compounds by the alkaline hydroxylamine procedure described by Hestrin (37) 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. The optical density at 540 nm (OD₅₄₀) of the mixture was measured. In order to obtain the correlation between the peak areas of the oligomers in HPLC analysis with the Aminex HPX-87H column and the OD₅₄₀ values 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, which was previously obtained from T. Saito (Kanagawa University) and has been stored in our laboratory, was also used for this purpose. A significant correlation was found (Fig. 1). Since spectrophotometric assay was a convenient procedure, the amount of 3HB or 3HV dimers in enzymatic reactions was routinely measured by OD₅₄₀.

Overproduction of *PhaZc* and *Hbd* in *E. coli*. To overproduce *PhaZc* in *E. coli*, pQE30-*phaZc* was constructed. PCR was performed with primers PDOHF2B (forward, 5'-CGGGATCCATGGCCGTCCAGTTCTTCC-3') and PDHR2H (reverse, 5'-CGAAGCTTGTCTCTTCAGGTGCTTCCGC-3'). The introduced restriction enzyme sites are in italic. The 0.8-kb amplified DNA fragment was inserted into pQE30. For *Hbd*, the 0.8-kb

FIG 1 Correlation between OD₅₄₀ and peak areas in the HPLC analysis with the Aminex HPX-75H column for 3HB dimer (closed circles, solid line), 3HB trimer (closed triangles, solid line), and 3HV dimer (open circles, dashed line). The data are means for three independent measurements. The coefficients of determination (*r*²) were 0.997 or higher.

*Bam*HI-*Hind*III DNA fragment was excised from pTAC1-hbd and inserted into pQE30 to produce pQE30-hbd. pQE30-*phaZc* and pQE30-hbd were transferred into *E. coli* M15 harboring pREP4. Both the *phaZc* and *hbd* genes were expressed with isopropyl-β-thiogalactopyranoside (IPTG).

Purification of His-tagged *PhaZc* and His-tagged *Hbd* from *E. coli*.

All purification steps were carried out at 4°C. After cultivating *E. coli* harboring pQE30-*phaZc* or pQE30-hbd, cells were harvested by centrifugation at 11,000 × *g* for 5 min. The cells were disrupted by sonication and centrifuged at 12,000 × *g* for 10 min. The resulting supernatant was used to purify the His-tagged proteins by affinity chromatography on an Ni-nitrilotriacetic acid (NTA) Superflow column (1.5 by 6 cm; Qiagen) according to the manufacturer's instructions. Fractions displaying enzyme activities were collected, dialyzed against 20 mM Tris-HCl (pH 7.0), and concentrated by ultrafiltration with Vivaspin 20 (Sartorius, Germany). The concentrated samples were purified on a TSK gel G3000SW column (0.75 by 30 cm; Tosoh, Japan) preequilibrated with 50 mM phosphate buffer (pH 7.0). Active fractions were collected, concentrated, and stored at -20°C. The concentrations of the purified His-tagged *PhaZc* and *Hbd* were 0.93 mg/ml and 8.2 mg/ml, respectively.

Enzyme assays. To prepare cell extracts, cells were suspended in 50 mM Tris-HCl (pH 7.0) and disrupted by sonication at 4°C. After centrifugation at 11,000 × *g* for 10 min at 4°C, the supernatants were dialyzed against 20 mM Tris-HCl (pH 7.0) at 4°C. Cell extracts for purifying His-tagged proteins were prepared as described above.

PhaZc activity was routinely assayed based on the amount of 3HB or 3HV dimers consumed during reactions. The reaction mixture (1 ml) contained 50 mM Tris-HCl (pH 8.0), substrate solution, and enzyme solution (74 μg protein). After the oligomer hydrolase reactions at 30°C, the amount of substrate remaining was measured as described by Hestrin (37). When *p*-nitrophenyl esters of fatty acids were used as substrates, the release of *p*-nitrophenol was measured at 400 nm. The reaction mixture contained 10 mM Tris-HCl (pH 7.5), 0.4 mM substrate, and enzyme sample.

Hbd activity was assayed as follows. Purified His-tagged *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.

One unit of His-tagged *PhaZc* activity was defined as a decrease of 1 OD₅₄₀ unit per minute. One unit of His-tagged *Hbd* activity was defined as an increase of 1 OD₃₄₀ unit per minute.

Other methods. Protein concentrations were determined by the method of Lowry et al. (38) with bovine serum albumin as the standard. Sodium dodecyl sulfate (SDS)-12.5% polyacrylamide gel electrophoresis (PAGE) was carried out as described by Laemmli (39). Proteins were

stained with Imperial protein stain (Thermo Scientific). Native PAGE was done as described by Ornstein and Davis (40, 41) with a 6% slab gel. Hbd activity staining after native PAGE was performed by incubating the gel in the reaction mixture for measuring Hbd activity but with 0.2 mM 1-methoxy phenazine methosulfate and 0.2 mM nitroblue tetrazolium. Molecular mass standards used for native PAGE and SDS-PAGE were Daiichi molecular weight markers I (for the Davis method) and III (for the Laemmli method) (Daiichi Pure Chemicals, Japan).

Cellular PHA contents in dried cells were determined by gas chromatography (42). The conditions for gas chromatographic analysis were as described previously (22).

Nucleotide sequence accession numbers. The nucleotide sequences of the *phaZc* and the *hbd* genes have been deposited in the GenBank/EMBL/DBJ databases under accession numbers [AB839771](#) and [AB839358](#), respectively.

RESULTS

Identification of the *phaZc* and *hbd* genes in *P. denitrificans* PD01. In our preliminary experiments, PhaZc activity was detected in cell extracts of *P. denitrificans* PD01. However, the *phaZc* gene has not been isolated. *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 genome is available in the NCBI database (GenBank accession number [NC_008686.1](#)). Based on BLAST searching (blastp) with PhaZc from *R. eutropha* H16 (16), 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 PhaZc.

A 0.8-kb DNA fragment was obtained with primers PDOHF2 and PDOHR2 by PCR, and the nucleotide sequence was determined. 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. In pUC19-*phaZc*, the ORF was in frame with the *lacZ* gene of pUC19. Therefore, PhaZc activity was measured using *E. coli* JM109 harboring pUC19-*phaZc*. When 3HB dimer was used as the substrate, a decrease in the substrate concentration was clearly observed, indicating that the cloned ORF encoded PhaZc.

The 3HB dehydrogenase gene (*hbd*) from *P. denitrificans* PD01 was isolated by PCR with primers PD3HBF1 and PD3HBR. A 0.8-kb fragment was amplified and sequenced. An ORF (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. The ORF was expressed as a *lacZ*-ORF fusion in *E. coli* DH5 α harboring pTAC1-*hbd*. Hbd activity was detected. With cell extract from *E. coli* DH5 α harboring pTAC1, Hbd activity was not detected.

Purification of PhaZc and Hbd. To purify PhaZc and Hbd from recombinant *E. coli* cells, the enzymes were overproduced as His-tagged proteins using pQE30-*phaZc* and pQE30-*hbd*. Since His-PhaZc was mostly in inclusion bodies following induction with 0.1 mM IPTG, it was produced at a lower IPTG concentration (10 μ M). In contrast, His-Hbd was produced at 0.1 mM IPTG as a soluble protein in *E. coli* harboring pQE30-*hbd* (data not shown).

Two-step chromatography with affinity and gel filtration columns was performed in order to purify His-PhaZc and His-Hbd. His-PhaZc was purified to homogeneity. Its molecular size was 31

FIG 2 Native PAGE (A) and substrate specificity (B) of the purified His-Hbd. (A) Lane 1, molecular mass standards; lanes 2 and 3, 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. (B) Substrate specificity of His-Hbd for 3HB and 3HV. When the enzyme was omitted from the reaction mixture, the OD at 340 nm did not change. The mean and standard deviation were calculated from experiments run in triplicate.

kDa, which corresponds to the estimated molecular mass of the protein deduced from the amino acid sequence. His-Hbd was also purified to homogeneity as a 120-kDa protein. The Hbd activity was confirmed by activity staining after native PAGE (Fig. 2A). A protein band with a molecular mass of 29 kDa was detected on SDS-PAGE (data not shown), indicating that Hbd is a tetrameric protein.

Properties of His-PhaZc and His-Hbd produced by *E. coli*.

The optimum pH and temperature, substrate specificity, and inhibitors of His-PhaZc were examined. The highest enzyme activity was obtained at pH 8.0 and 30°C to 50°C (Fig. 3A and B). Several *p*-nitrophenyl esters of fatty acids were tested as substrates. Among the compounds tested, *p*-nitrophenyl esters of butyric and pentanoic acids were most efficient (Fig. 3C). The purified enzyme hydrolyzed not only 3HB dimer but also 3HV dimer (Fig. 4A). The specific activity of His-PhaZc was 0.59 U/mg for 3HB dimer and 0.43 U/mg for 3HV dimer. PHB and PHV granules were not depolymerized by His-PhaZc (data not shown). The enzyme activity was completely inhibited by 1 mM diisopropylfluorophosphate (DFP). Phenylmethylsulfonyl fluoride (PMSF) was also an inhibitor; however, its inhibitory effect was weaker than that of DFP. *N*-Ethylmaleimide and iodoacetamide were slightly inhibitory (Table 2). SDS and Triton X-100 have usually been used for examining the inhibitory effect on PHA depolymerase (PhaZ) activity. Their inhibitory effects on His-PhaZc activity at 0.05% (wt/vol) were 61.1% and 67.3%, respectively (data not shown).

Purified His-Hbd was examined only for substrate specificity. It dehydrogenated not only 3HB but also 3HV (Fig. 2B). The specific activity of His-Hbd was 0.67 U/mg for 3HB and 0.48 U/mg for 3HV. When both His-PhaZc and His-Hbd were included in enzyme reactions, 3HV dimer underwent sequential reactions (Fig. 4B).

Disruption of the *phaZc* gene in *P. denitrificans* PD01. To assess the effect of disrupting the *phaZc* gene on PhaZc activity in *P. denitrificans* PD01, the *phaZc* gene was mutated by insertional inactivation. Disruption of the *phaZc* gene was confirmed by Southern blotting and PCR with *P. denitrificans* PD01 and PD01Km genomic DNAs. The DIG probe (1.2-kb *S*all fragment containing the *Km^r* gene) hybridized with *P. denitrificans* PD01Km DNA. With primers PDOHF2 and PDOHR2 in PCR,

FIG 3 Optimum pH (A), optimum temperature (B), and substrate specificity (C) of His-PhaZc. For panels A and B, enzymatic reaction time was 10 min. The reaction temperature for panel A was 30°C. The buffers used (50 mM each) were as follows: acetate, pH 4.0 to 6.0; phosphate, pH 6.0 to 8.0; Tris-HCl, 8.0 to 9.0; glycine-NaOH, pH 9.0 to 10.0. The pH for panel B was 8.0. The activities referred to as 100% relative activity were 25 mU at pH 8.0 (A) and 30 mU at 50°C (B). For panel C, the liberation of *p*-nitrophenol during the enzyme reactions was monitored at 400 nm. *p*-Nitrophenyl fatty acid esters of acetic (C₂), propionic (C₃), butyric (C₄), pentanoic (C₅), hexanoic (C₆), and decanoic (C₁₀) acids were used as substrates. Three independent experiments were performed. Representative results are shown.

0.8-kb and a 2.1-kb DNA fragments were amplified with *P. denitrificans* PD01 DNA and PD01Km DNA, respectively. Cell extracts were prepared from *P. denitrificans* PD01 and PD01Km grown on ethanol as a carbon source and were used to determine PhaZc activity. As shown in Fig. 5, no enzyme activity was detected in extracts of *P. denitrificans* PD01Km. These results indicate that the Km^r gene was successfully inserted in the *phaZc* gene. pBBR1MCS4-*phaZc* was introduced into *P. denitrificans* PD01Km by conjugative transfer. A transconjugant named *P. denitrificans* PD01KmOH restored PhaZc activity (Fig. 5).

Effect of disruption of the *phaZc* gene on intracellular PHA degradation in *P. denitrificans* PD01. To examine the effect of the *phaZc* mutation on the degradation of PHA, *P. denitrificans* PD01 and PD01Km were cultivated under the following conditions. The strains were first grown to late exponential growth phase (stage 1; 18 h). Cultivation was continued under carbon-sufficient and nitrogen-deficient conditions (stage 2; 24 h), and

subsequently carbon sources were omitted from the medium (stage 3; 24 h). The results are shown in Fig. 6. Both strains synthesized PHA as a copolymer composed of 3HB and 3HV units through the second stage of cultivation. The 3HV monomer composition in PHA remained at 60 to 65 mol%. In stage 2, under the conditions of PHA synthesis, the growth and PHA synthesis of *P. denitrificans* PD01Km were slightly affected. At the end of the stage 2, the PHA content in the cells of *P. denitrificans* PD01Km was 20% lower than that in *P. denitrificans* PD01. Cellular PHA contents decreased at stage 3. In particular, in the first 5 h of this stage, the PHA content in the *P. denitrificans* PD01 strain decreased by 40.5%, but it decreased by 26.3% in PD01Km.

DISCUSSION

This study focused mainly on intracellular 3HB oligomer hydrolase (PhaZc) from *P. denitrificans* PD01. To date, two types of 3HB oligomer hydrolase, PhaZb and PhaZc, whose substrate specificities were examined with 3HB oligomers, have been reported from several PHB-producing bacteria. Hence, PhaZc has been analyzed for catalytic activity toward 3HB oligomers. The hydrolysis of 3HV oligomers by extracellular and intracellular oligomer hydrolases has not been described. The experimental results described here demonstrated that PhaZc from *P. denitrificans* PD01 can hydrolyze 3HV oligomers.

The *phaZc* gene was identified from *P. denitrificans* in this study. When the nucleotide and the deduced amino acid sequences of *phaZb* from *R. eutropha* H16 and those of *phaZc* from *P. denitrificans* PD01 were compared, the similarities were very low. In addition, *phaZc* from *P. denitrificans* PD01 did not resemble the *phaZc* genes reported with *R. eutropha* H16 and *Acidovorax* sp. strain SA1 at the nucleotide sequence level. This is the reason why the *phaZc* regions from these strains could not hybridize with the *P. denitrificans* PD01 DNA. However, comparison of the deduced amino acid sequence of *P. denitrificans* PD01 *phaZc* with those reported for *R. eutropha* (PhaZc), *Acidovorax* sp. strain SA1 (i3HBOH), and *P. lemoignei* (PhaZc) revealed identities/similarities of 36%/48%, 34%/47%, and 32%/47%, respectively. The pentapeptide motif Gly-Thr-Ser-Arg-Gly is present in the deduced amino acid sequence of *P. denitrificans* PD01 PhaZc. This motif is known as a lipase box, which plays an essential role in lipase, esterase, and protease activities (43). The strong inhibitory effects of DFP and PMSF, which react with serine groups on a protein, support the importance of serine residue in the motif, indicating that the motif should serve as an active site of PhaZc. The sulfhydryl reagents tested in this study displayed little inhibitory effect on PhaZc activity, which implies that disulfide bonds are not important for the enzymatic activity.

Hbd is well known and has been characterized from many microbial sources. However, reports on the catalytic activity with 3HV are limited. In this study, purified His-tagged Hbd was used for examining its catalytic activity toward 3HV dehydrogenation. It was reported that purified Hbd from *Rhodospseudomonas sphaeroides* dehydrogenated 3HV about 20 times more slowly than 3HB (44). In contrast, Hbd from *P. denitrificans* PD01 catalyzed the dehydrogenation of 3HV and 3HB at similar rates. In the presence of both His-PhaZc and His-Hbd in an enzymatic reaction system, 3HV dimer underwent sequential reactions of hydrolysis and dehydrogenation (Fig. 4B). This result suggests that PhaZc and Hbd should contribute to the degradation of 3HV-containing PHA in *P. denitrificans* PD01.

FIG 4 Substrate specificity of His-PhaZc for 3HB and 3HV dimers (A) and two-step reaction with 3HV dimer as the substrate in the presence of His-PhaZc and His-Hbd (B). In panel A-1, the consumption of 3HB and 3HV dimers (OD₅₄₀) is shown by closed and open circles, respectively. Panels A-2 and A-3 show the chromatograms of HPLC with the Aminex HPX-75H column at 0 min (solid lines) and 30 min (dashed lines) in the enzymatic reactions (panel A-1). In panel B, closed and open circles show the consumption of 3HV dimer (OD₅₄₀) and the reduction of NAD (OD₃₄₀), respectively. When the enzymes were omitted from the reaction mixture, the ODs at 540 nm and at 340 nm did not change. For panels A-1 and B, the mean and standard deviation were calculated from experiments run in triplicate.

The *phaZc* and *hbd* 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. This strain is a derivative of *P. denitrificans* DSM413 (45). According to the classification of *P. denitrificans* strains, *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 (19). Therefore, it was not unexpected that the nucleotide sequences of the *phaZc* and *hbd* genes from the two strains are identical.

When the *phaZc* gene was disrupted in *P. denitrificans* PD01, the hydrolase activity was negative. After the gene was introduced into the mutant, the PhaZc activity was restored. Thus, *P. denitrificans* PD01 has a unique *phaZc* gene. Other candidate genes for PhaZc activity were not found in the nucleotide sequence (or amino acid sequence) of the *P. denitrificans* Pd1222 genome.

TABLE 2 Effects of chemical reagents on PhaZc activity^a

Reagent (1 mM)	Relative activity (%)
None	100
<i>N</i> -Ethylmaleimide	74.8
Iodoacetamide	74.8
Diisopropylfluorophosphate	0.0
Phenylmethanesulfonyl fluoride	27.4

^a Enzyme solution was incubated with each reagent for 10 min on ice and used in enzymatic reactions for 10 min. The activity in the absence of reagent, referred to 100% relative activity, was 27 mU.

It has been reported that the degradation of PHB in PHB-producing bacteria proceeds via PhaZ, PhaZc, and Hbd reactions. 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 its derivative strains, PHA should be degraded in these cells. In a previous study using *P. denitrificans* ATCC 17741, PhaZ catalyzed the degradation of PHB granules and the major product was 3HB dimer (6). If this is the case also for *P. denitrifi-*

FIG 5 PhaZc activity in *P. denitrificans* strains PD01 (open triangles), PD01Km (closed circles), and PD01KmOH (closed triangles). The same amount of protein (0.38 mg) was used in each enzymatic reaction. When the enzymes were omitted from the reaction mixture, the OD at 540 nm did not change. The mean and standard deviation were calculated from experiments run in triplicate.

FIG 6 Growth (A) and intracellular synthesis and degradation of PHA (B) in *P. denitrificans* strains PD01 (closed circles) and PD01Km (open circles). Cultivation was started with *n*-pentanol as a carbon source (stage 1). At 18 h, cells were collected and transferred into nitrogen-deficient PD medium (stage 2). At 42 h, cells were collected and transferred into carbon-deficient PD medium (stage 3). PHA contents were expressed as percent weight in the dried cells. The mean and standard deviation were calculated from experiments run in triplicate.

cans PD01, then PhaZc plays an important role in PHA metabolism via 3HB/3HV oligomers in *P. denitrificans* cells. In order to know the effect of PhaZc activity on intracellular PHA degradation, the growth and the PHA content during the cultivation were compared between the wild-type and the *phaZc*-disrupted mutant cells. Growth and PHA synthesis profiles at the PHA synthesis stage were similar in the two strains. However, a moderate difference in the PHA content profile was observed at the PHA degradation stage (i.e., a lower degree of decrease in PHA content was observed in the mutant cells), whereas the growth profile was almost the same during this stage. With this taken into consideration, PHA degradation in the mutant cells proceeded more slowly than that in the wild-type cells. Because the purified PhaZc from *P. denitrificans* PD01 was incapable of degrading PHA granules, the PHA degradation profile observed here suggests that the PHA depolymerization reaction should be affected by the PhaZc activity.

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