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 acetooacetate. 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), Pseudomonas 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%,...
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. E. coli ATCC 17989 was used to prepare extracellular PHB or PHV depolymerases (25, 26) and another strain of 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 thephaZc and hbd genes from P. denitrificans PD01.** In order to obtain thephaZc gene, primers PDOHF2 (forward, 5'-CGAGAAGGGATCCGATTCGACCG-3') and PDOHR2 (reverse, 5'-CGGATCCCTTGCGATTTCTC-3') were designed based on a nucleotide sequence from P. denitrificans Pd1222, which was annotated to encode a hydrolyase/acyltransferase (nucleotides [nt] 1633350 to 1634177 in the whole genomic sequence, GenBank accession no. NC_008686.1). The start codon in PDOHR2 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 into pUC19. PhaZc activity was confirmed using a NucloSpin Plasmid QuickPure kit (Macherey-Nagel, Germany) was used to prepare genomic 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 Qapa Taq Extra PCR kit with deoxynucleoside triphosphates (dNTPs) (KAPA Bio-systems) 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 cloning kit cloning with pTAC-1 (Biodynamics Laboratory, Japan) was used for TA cloning of PCR fragments into E. coli DH5a. A DynaExpress DNA ligation kit (Biodynamics Laboratory, Japan) was used for DNA ligations. A DIG High Prime DNA Labeling and Detection Starter kit I (Roche Diagnostics, Germany) was used for Southern blotting. A 1.2-kb SalI 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.

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

<table>
<thead>
<tr>
<th>Strain or plasmid</th>
<th>Description</th>
<th>Source or reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>PD01</td>
<td>Wild type</td>
<td>NBRC 13301</td>
</tr>
<tr>
<td>PD01Km</td>
<td>PD01 derivative, ΔphaZc Km'</td>
<td>This study</td>
</tr>
<tr>
<td>PD01KmOH</td>
<td>PD01Km derivative harboring pBRR1MCS4-phaZc, Km' Amp'</td>
<td>This study</td>
</tr>
<tr>
<td>pUC19</td>
<td>Cloning vector, Amp'</td>
<td>TaKaRa</td>
</tr>
<tr>
<td>pTAC-1</td>
<td>TA cloning vector, Amp'</td>
<td>BioDynamics</td>
</tr>
<tr>
<td>pQE30</td>
<td>Expression vector, Amp'</td>
<td>Qiagen</td>
</tr>
<tr>
<td>pUC4K</td>
<td>Source of Km' (Tn903) cassette, Amp' Km'</td>
<td>29</td>
</tr>
<tr>
<td>pRK2013</td>
<td>Helper plasmid, traRK2 Km'</td>
<td>30</td>
</tr>
<tr>
<td>pSUP5011</td>
<td>Source of mob; Amp' Km' Cm' Tn5-mob</td>
<td>31</td>
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<tr>
<td>pBRR1MCS-4</td>
<td>Broad-host-range plasmid vector, mobRP4 Amp'</td>
<td>32</td>
</tr>
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<td>pUC19-phaZc</td>
<td>pUC19 carrying a 0.8-kb BamHI-HindIII fragment containing phaZc</td>
<td>This study</td>
</tr>
<tr>
<td>pQE30-phaZc</td>
<td>pQE30 carrying a 0.8-kb BamHI-HindIII fragment containing phaZc</td>
<td>This study</td>
</tr>
<tr>
<td>pUC19-phaZc-Km</td>
<td>pUC19 carrying phaZc::Kmr (a 1.2-kb Sall fragment of the Km' gene)</td>
<td>This study</td>
</tr>
<tr>
<td>pUC19-phaZc-Km-mob</td>
<td>Suicide vector carrying fragments of the phaZc::Kmr and mob genes</td>
<td>This study</td>
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<tr>
<td>pTAC1-phaZc-Km</td>
<td>pTAC-1 carrying an amplified PCR fragment containing phaZc::Kmr</td>
<td>This study</td>
</tr>
<tr>
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<td>pBRR1MCS4 carrying a 0.8-kb BamHI-HindIII fragment containing phaZc</td>
<td>This study</td>
</tr>
<tr>
<td>pTAC1-hbd</td>
<td>pTAC-1 carrying a 0.8-kb fragment amplified by PCR</td>
<td>This study</td>
</tr>
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</table>
PD01Km. Disruption of the *phaZc* gene in this strain was verified by PCR with primers PD0HF2 and PD0HR2 and Southern blotting with a 1.2-kb SalI fragment (Km' gene) from pUC4K.

**Complementation of the *phaZc* mutation in *P. denitrificans* PD01Km.** The 0.8-kb HindIII-BamHI fragment was isolated from pUC19-phaZc and inserted into the same restriction sites of the broad-host-range vector pBRR1MCS-4 (32) to produce pBRR1MCS4-phaZc. *E. coli* JM1109 (pBRR1MCS4-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 as a water-soluble tetrazolium salt, WST-1 [a sodium salt of 2-(4-iodophenyl)-3-(4-nitrophenyl)-5-(2,4-disulfophenyl)-2H-tetrazolium] (Dojindo, Japan) as described by Lam et al. (34).

Native PHA (PHB and PHV) granules were isolated from PHB-rich *S. fredii* NBR1C14780 grown on mannitol and from PHV-rich *P. denitrificans* ATCC 17741 grown on p-npentanol (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 catalyst (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-75H column for 3HB dimer (closed circles, solid line), 3HB monomer (14.1 min), 3HB trimer (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 determined with a water-soluble tetrazolium salt, WST-1 [a sodium salt of 2-(4-iodophenyl)-3-(4-nitrophenyl)-5-(2,4-disulfophenyl)-2H-tetrazolium] (Dojindo, Japan) as described by Lam et al. (34).

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-75H 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 enzymic 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 PD0HFB2 (forward, 5'-GGGAATCTCATGCGGTGCTGTTCTCC-3') and PDHR2H (reverse, 5'-GGGAATCTCATGCGGTGCTGTTCTCCGC-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 BamHI-HindIII DNA fragment was excised from pTA1-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-nitritolactiatic 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 hydrolysis 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 OD540 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 Daichi molecular weight markers I (for the Davis method) and III (for the Laemmli method) (Daichi 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/DDJB 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 DH5α harboring pTAC1-hbd. Hbd activity was detected. With cell extract from E. coli DH5α 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 μM). 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 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 Sall fragment containing the Km’ gene) hybridized with P. denitrificans PD01Km DNA. With primers PDOHF2 and PDOHR2 in PCR,
sufficient and nitrogen-deficient conditions (stage 2; 24 h), and the strains were first grown to late exponential growth. The strains were then grown under aerobic conditions in the presence of PHA and the appropriate carbon source (Table 1). The PHA content in the PD01 strain decreased by 20% lower than that in PD01Km. The PHA content in the 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 hydrolases, 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 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 (PhaZc), Acidovorax sp. strain SA1 at the nucleotide sequence level. This is the reason why the phaZc regions from these strains could not hybridize with 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 (13HBOH), 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 Rhodopseudomonas palis-roides 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.
The \(\text{phaZc}\) and \(\text{hbd}\) 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 \(\text{phaZc}\) and \(\text{hbd}\) genes from the two strains are identical.

When the \(\text{phaZc}\) 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 \(\text{phaZc}\) gene. Other candidate genes for PhaZc activity were not found in the nucleotide sequence (or amino acid sequence) of the \(P.\) denitrificans Pd1222 genome.

It has been reported that the degradation of PHB in PHB-producing bacteria proceeds via PhaZ, PhaZc, and Hbd reactions. Although the \(\text{phaZ}\) 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 \(P.\) 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 \(\text{phaZc}\) 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

<table>
<thead>
<tr>
<th>Reagent (1 mM)</th>
<th>Relative activity (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>None</td>
<td>100</td>
</tr>
<tr>
<td>(N)-Ethylmaleimide</td>
<td>74.8</td>
</tr>
<tr>
<td>Iodoacetamide</td>
<td>74.8</td>
</tr>
<tr>
<td>Diisopropylfluorophosphate</td>
<td>0.0</td>
</tr>
<tr>
<td>Phenylmethanesulfonyl fluoride</td>
<td>27.4</td>
</tr>
</tbody>
</table>

\(a\) 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.

**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 (OD\(_{540}\)) is showed 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 (OD\(_{540}\)) and the reduction of NAD (OD\(_{340}\)), 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.

**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.

REFERENCES


