Properties of a Novel Intracellular Poly(3-Hydroxybutyrate) Depolymerase with High Specific Activity (PhaZd) in *Wautersia eutropha* H16

Tomoko Abe,¹ Teruyuki Kobayashi,¹ and Terumi Saito^{1,2*}

Laboratory of Molecular Microbiology, Department of Biological Sciences, Faculty of Science, Kanagawa University, 2946 Tsuchiya, Hiratsuka, Kanagawa 259-1293, Japan,¹ and Research Institute for Integrated Science, Kanagawa University, 2946 Tsuchiya, Hiratsuka, Kanagawa 259-1293, Japan²

Received 14 May 2005/Accepted 27 July 2005

A novel intracellular poly(3-hydroxybutyrate) (PHB) depolymerase (PhaZd) of *Wautersia eutropha* (formerly *Ralstonia eutropha*) H16 which shows similarity with the catalytic domain of the extracellular PHB depolymerase in *Ralstonia pickettii* T1 was identified. The positions of the catalytic triad (Ser¹⁹⁰-Asp²⁶⁶-His³³⁰) and oxyanion hole (His¹⁰⁸) in the amino acid sequence of PhaZd deduced from the nucleotide sequence roughly accorded with those of the extracellular PHB depolymerase of *R. pickettii* T1, but a signal peptide, a linker domain, and a substrate binding domain were missing. The PhaZd gene was cloned and the gene product was purified from *Escherichia coli*. The specific activity of PhaZd toward artificial amorphous PHB granules was significantly greater than that of other known intracellular PHB depolymerase or 3-hydroxybutyrate (3HB) oligomer hydrolases of *W. eutropha* H16. The enzyme degraded artificial amorphous PHB granules and mainly released various 3-hydroxybutyrate oligomers. PhaZd distributed nearly equally between PHB inclusion bodies and the cytosolic fraction. The amount of PHB was greater in *phaZd* deletion mutant cells than the wild-type cells under various culture conditions. These results indicate that PhaZd is a novel intracellular PHB depolymerase which participates in the mobilization of PHB in *W. eutropha* H16 along with other PHB depolymerases.

Poly(3-hydroxybutyrate) (PHB), a homopolymer of *R*(-)-3-hydroxybutyrate (3HB), is a storage material produced by a wide variety of bacteria under certain conditions (5). Intracellular PHB, which is accumulated in an amorphous state, is degraded by several hydrolases and the degraded products are used as a source of carbon and energy (36). When PHB-accumulating bacteria die, the PHB released into the environment is hydrolyzed by various microorganisms which secrete extracellular PHB depolymerases (12). In the past few decades, the application of this biopolymer to the production of biodegradable polymers/plastics has been studied extensively (26). The extracellular metabolism of PHB has been clarified in many bacteria including *Ralstonia pickettii* T1 and *Paucimonas lemoignei* as well as in some fungi (12).

The intracellular PHB mobilization system is not as well understood as the extracellular PHB degradation system. The intracellular PHB depolymerase system was first reported in *Rhodospirillum rubrum* in 1964 (27). The system consists of a thermostable activator and a thermolabile esterase and recent reinvestigations have lead to considerable progress being made (7, 8). The amino acid sequence of the intracellular soluble PHB depolymerase in *R. rubrum* (PhaZ1_{Rru}) (8) shows similarity with the type II catalytic domain of the extracellular PHB depolymerases in bacteria such as *Acidovorax* sp. strain TP4 (17) which has a lipase box (G-X-S*-X-G) with an active res-

idue at the N-terminal side. Another type of catalytic domain (type I) whose lipase box is located near the middle of the domain has been reported in *R. pickettii* T1 and other bacteria (14).

In *Wautersia eutropha* (formerly *Ralstonia eutropha*) H16, three types of intracellular PHB depolymerase or 3HB oligomer hydrolase genes have been cloned and some features of the gene products have been reported (19, 20, 21, 31, 32). PhaZa1_{Weu} (formerly PhaZ1_{Reu}) exists in PHB inclusion bodies and degrades artificial amorphous PHB (20, 30). The existence of PhaZa1_{Weu} homologs has also been reported in *W. eutropha* H16 (29, 44). Additionally, enzymes with properties similar to PhaZa1_{Weu} have been studied in other bacteria (6, 18). PhaZb_{Weu} (formerly PhaZ2_{Reu}) (20, 32) and PhaZc_{Weu} (19) mainly degrade 3HB oligomers and the degradation product is 3HB monomer. Another PHB depolymerase or related hydrolase in *W. eutropha* H16 with different properties from these enzymes has been reported (35), but it has not been fully characterized. PhaZ1_{Rru} of *R. rubrum* has no similarity with any known intracellular PHB depolymerases or 3HB oligomer hydrolases in *W. eutropha* H16.

In this report, a fourth novel intracellular PHB depolymerase in *W. eutropha* H16, PhaZd, whose amino acid sequence is similar to that of the catalytic domain of the extracellular PHB depolymerase of *R. pickettii* T1 was identified, and some of the properties of the enzyme were examined.

* Corresponding author. Mailing address: Laboratory of Molecular Microbiology, Department of Biological Sciences, Faculty of Science, Kanagawa University, 2946 Tsuchiya, Hiratsuka, Kanagawa 259-1293, Japan. Phone and fax: 81-463-59-8673. E-mail: 43saito-bio@kanagawa-u.ac.jp.

MATERIALS AND METHODS

Bacterial strains, plasmids, and culture. The bacterial strains and plasmids used in the present study are listed in Table 1. All *Escherichia coli* strains were

TABLE 1. Bacterial strains and plasmids

Strain or plasmid	Description ^a	Source or reference
<i>E. coli</i>		
DH5	<i>supE44 hsdR17 recA1 endA1 gyrA96 thi-1 relA1</i>	Nippon Gene
S17-1/λpir	<i>Tra⁺ recA pro thi hsdR chr::RP4-2</i>	38
JM109	<i>recA1 endA1 gyrA96 thi hsdR17 supE44 relA1 Δ(lac-proAB)/F' [traD36 proAB⁺ lacI^a lacZΔM15]</i>	Takara
BLR (DE3)/pLysS	<i>F⁻ ompT hsdS_B(r_B⁻ m_B⁻) gal dcm Δ(srl-recA)306::Tn10 (Tc^r)(DE3)/pLysS (Cm^r)</i>	Novagen
<i>W. eutropha</i>		
H16	Wild type	ATCC 17699
H16DZd1	Δ <i>phaZd</i> strain derived from H16	This study
Plasmids		
Charomid9-36	Cosmid; Amp ^r	Nippon Gene
pBluescript II SK (+)	Cloning vector; Amp ^r	Stratagene
pET23b	Expression vector; Amp ^r	Novagen
pET15b	Expression vector; Kan ^r	Novagen
pE3ReZd1	pET23b carrying amplified NdeI-XhoI fragment containing <i>phaZd</i>	This study
pE5ReZd1NHIS	pET15b carrying amplified NdeI-XhoI fragment containing N-terminally His-tagged <i>phaZd</i>	This study
pLO3	Tc ^r <i>sacB</i> , RP4 <i>oriT</i> , ColE1 <i>ori</i>	23
pO3DZd1	pLO3 carrying N-terminal 239 bp and C-terminal 340 bp of <i>phaZd</i>	This study

^a Amp^r, ampicillin resistant; Cm^r, chloramphenicol resistant; Tc^r, tetracycline resistant; Kan^r, kanamycin resistant.

grown in Luria-Bertani (LB) medium. *E. coli* BLR(DE3)/pLysS was used as a host cell for the recombinant plasmids carrying *phaZd*. *E. coli* S17-1/λpir was used for mobilization of the suicide vector into *W. eutropha* H16. All *W. eutropha* strains were grown in a nutrient-rich medium at 30°C as described previously (31). To produce PHB, *W. eutropha* cells were cultured on a minimum salt medium containing 2% (wt/vol) fructose and 0.1% (wt/vol) ammonium sulfate as described previously (16) (PHB-accumulating conditions). To induce the degradation of PHB, *W. eutropha* cells grown under PHB-accumulating conditions for 60 h were transferred to a minimum salt medium containing 0.5% (wt/vol) ammonium sulfate (PHB-degrading conditions) (35).

Cloning of *phaZd* from *W. eutropha* H16. A candidate for the PHB depolymerase gene was identified in a BLAST search using the amino acid sequence of the catalytic domain of the extracellular PHB depolymerase of *Ralstonia pickettii* T1 (accession number P12625) (34) as a probe. DNA sequences of the putative intracellular PHB depolymerase genes in *W. eutropha* JMP134, *W. metallidurans* CH34, and *Rhizobium solanacearum* GMI1000 (accession numbers ZP_00166687, ZP_00276315, and NP_520270, respectively) were used to design a set of degenerate primers: ZdprobeF (5'-CACRCGCATGAAYGCGCTGGC CG-3') and ZdprobeR (5'-CAGGCSGTTGTAGGCCAGGAAGT-3'). All DNA manipulations were performed using standard procedures (37). Using chromosomal DNA of *W. eutropha* H16 as a template, these primers were employed to amplify a fragment of about 500 bp. The PCR product was labeled with ³²P and used as a probe in Southern hybridization. EcoRI-digested chromosomal DNA fragments of 8 or 12 kbp were ligated to an EcoRI-digested cosmid vector, charomid 9-36 (33), and then packaged. *E. coli* DH5α was infected by the packaged charomid. To select a positive clone, colony PCR was performed with the degenerate primers (ZdprobeF and ZdprobeR). The DNA fragment obtained was subcloned into pBluescript II SK(+) phagemid (STRATAGENE) and sequenced.

Construction of an expression vector and purification of PhaZd from *E. coli*. To express *phaZd*, the gene was amplified by PCR with a pair of primers: PhaZd1f (5'-AGAATTCCATATGACCAAAGCTTTGCCGCTGAC-3') and PhaZd1r (5'-TTCTCGAGTCAACGCGGCTGCTGGCTGAAG-3'). The product was digested with NdeI and XhoI, and inserted into the corresponding sites of pET23b (Novagen). The resulting plasmid was designated pE3ReZd1. To express N-terminally histidine-tagged *phaZd*, the gene was inserted into pET15b (Novagen) from pE3ReZd1 as described above. The resulting plasmid was designated pE3ReZd1NHIS. The integrity of the nucleotide sequence of all newly constructed plasmids was confirmed by DNA sequencing.

E. coli BLR(DE3)/pLysS was transformed with pE3ReZd1 or pE3ReZd1NHIS. The transformed cells were grown in LB medium with ampicillin (50 μg/ml), tetracycline (12.5 μg/ml), and chloramphenicol (34 μg/ml) at 37°C. At an optical density of 600 nm of 0.6, the culture temperature was reduced, and the gene expression was induced by the addition of isopropyl-β-D-thiogalactopyranoside (IPTG). The culture temperature and IPTG concentration were 22°C and 1 mM or 20°C and 10 μM for pE3ReZd1 and pE3ReZd1NHIS, respectively. The cultures were incubated overnight. Bacteria were harvested by centrifugation, and the cells were suspended in 20 mM Tris-

HCl (pH 8.0). The resuspended cells were sonicated on ice for 4 min (20 kHz, 30 W), and centrifuged at 10,000 × *g* for 30 min. The supernatant of *E. coli* harboring pE3ReZd1NHIS was applied to a Ni-chelating column (1 ml, Amersham Biosciences), and the column was washed with 20 mM sodium phosphate (pH 7.4) containing 0.5 M NaCl. PhaZd was eluted with a linear gradient of imidazole (0.04 to 0.4 M, 25 ml) in 20 mM sodium phosphate (pH 7.4) containing 0.5 M NaCl.

Construction of a *phaZd* deletion mutant. A part of the N-terminal (294 bp) and a part of the C-terminal end (340 bp) of *phaZd* were amplified by PCR. The two PCR products were connected, and then ligated into a suicide vector, pLO3 (575-bp deletion: from nucleotides 295 to 749 in *phaZd*) (23). The resulting plasmid was designated pO3DZd1. Using pO3DZd1, a *phaZd* deletion mutant was constructed as described previously (24, 38). Transconjugants were selected on minimum salt medium plates containing tetracycline (10 μg/ml) and subsequent LB plates containing 15% (wt/vol) sucrose. The mutant was confirmed based on antibiotic susceptibility, PCR, Southern hybridization, and immunological analysis. Strain H16DZd1, the *phaZd* deletion mutant, gave no immunostained band when the antisera against PhaZd were used.

Preparation of substrates. Semicrystalline PHB was prepared from PHB-rich *W. eutropha* H16 cells as described previously (39). Artificial amorphous PHB granules were prepared from the purified semicrystalline PHB granules as described by Horowitz and Sanders (11). Briefly, PHB was dissolved in chloroform, and then 0.1% (wt/vol) sodium deoxycholate was added to the dissolved PHB solution. The mixture was sonicated (20 kHz, 100 W) for 2 min. The emulsion was heated to remove chloroform and dialyzed for 24 h against 0.01% (wt/vol) sodium deoxycholate at room temperature. Native PHB was isolated from *W. eutropha* H16 and purified by glycerol density gradient (10). The 3HB oligomers were prepared as described previously (42). Poly(3-hydroxyoctanoate) was prepared according to previous reports (30).

Enzyme assays. PHB depolymerase activity and 3HB oligomer hydrolase activity were assayed by measuring the amount of 3HB released from PHB and 3HB oligomers, respectively. The reaction mixture (50 μl) was composed of 100 mM Tris-HCl (pH 8.5), PHB granules (0.5 mg/ml as a solid), and enzyme. The reaction was started by the addition of substrate at 30°C (20). To quantify the amount of 3HB oligomer released from PHB by the enzyme, the supernatant of the reaction mixture was treated with 1.1 U (3HB dimer-hydrolyzing activity) of 3HB oligomer hydrolase from *W. eutropha* H16 (PhaZb_{Wec}) (20, 32). The amount of 3HB was measured by an enzymatic method using 3HB dehydrogenase and hydrazine hydrate as described previously (20). Hydrolytic activity toward *p*-nitrophenyl esters was determined spectrophotometrically and lipase activity was assayed by titration of the acid released from olive oil as described previously (45). To investigate the effect of reagents, they were added to the reaction mixture containing the enzyme, and then the mixture was incubated for 10 min at room temperature. The reaction was started by adding a substrate. To clarify whether a thermostable activator exists (7, 27), the cytosolic fraction of *W. eutropha* H16 cells was heated at 100°C for 10 min, and the supernatant was mixed with the assay mixture.

Immunoblot analysis. Samples were subjected to immunoblot analysis according to standard procedures using rabbit antisera against PhaZd_{Weu} as the primary antiserum and alkaline phosphatase-conjugated goat anti-rabbit immunoglobulin G (SIGMA-ALDRICH) as the secondary antibody. The immunocomplex was visualized using nitroblue tetrazolium and 5-bromo-4-chloro-3-indolylphosphate *p*-toluidine. The rabbit antisera against PhaZd_{Weu} were generated by the purified histidine-tagged protein.

Other methods. The protein concentration was measured by the method of Lowry et al. (25) with bovine serum albumin as the standard. The purity and size of the purified proteins were estimated by sodium dodecyl sulfate (SDS)-polyacrylamide gel electrophoresis (PAGE) as described by Laemmli (22). Proteins on the gel were stained with Coomassie brilliant blue R250. Sucrose density gradient centrifugation was performed as reported previously (20). PHB content was quantitated as crotonic acid by high-pressure liquid chromatography as described by Karr et al. (15). The periplasmic fraction was separated by lysozyme-EDTA treatment modified for *W. eutropha* H16 cells (2, 3). PHB degradation activator (*apdA*) (accession number AAO00724) of *R. rubrum* (7) was cloned by PCR. *ApdA* was expressed as a C-terminally histidine-tagged protein in *E. coli* and purified by a Ni-chelating column.

RESULTS

Identification of a putative intracellular PHB depolymerase.

In a BLAST search using the amino acid sequence of PhaZ1_{Rru}, the periplasmic PHB depolymerase recently purified from *R. rubrum*, or some extracellular PHB depolymerases which have type II catalytic domain (*Acidovorax* sp. strain TP4 and *Comamonas* sp.) (8, 14) as references, no candidate for an intracellular PHB depolymerase from the genus *Wautersia* or *Ralstonia* was found. However, a BLAST search using the amino acid sequence of the type I catalytic domain of the extracellular PHB depolymerase in *R. pickettii* T1 as a probe resulted in several hits. Some bacteria of the genus *Burkholderia* (*B. cepacia* R1808, *B. cepacia* R18194, and *B. mallei* ATCC23344) (43) and both *W. eutropha* JMP134 and *W. metallidurans* CH34 contained two putative open reading frames (ORFs) each (accession numbers ZP_00166687 and ZP_00170022 [*W. eutropha* JMP134], and ZP_00276315 and ZP_00273737 [*W. metallidurans* CH34]). *R. solanacearum* GMI1000 (NP_520270) and some other bacteria contained a similar ORF.

Although the BLAST search revealed the putative intracellular PHB depolymerase, *phaZd*, in several bacteria, the incidence of two putative *phaZd* genes in a single bacterium was limited. The identity of the deduced amino acid sequences of these ORFs with the catalytic domain of the extracellular PHB depolymerase of *R. pickettii* T1 was 24.1% (similarity, 52.5%) (ZP_00166687) or 27.1% (55.8%) (ZP_00170022) in *W. eutropha* JMP134, 24.1% (54.2%) (ZP_00276315) or 24.6% (52.3%) (ZP_00273737) in *W. metallidurans* CH34, and 29.6% (63.4%) (NP_520270) in *R. solanacearum* GMI1000. All of these bacteria had PHB synthase genes. Because these species belong to the genus *Wautersia* or *Ralstonia*, *W. eutropha* H16 may have a similar putative intracellular PHB depolymerase.

Among these ORFs, ZP_00166687 of *W. eutropha* JMP134, ZP_00276315 of *W. metallidurans* CH34, and NP_520270 of *R. solanacearum* GMI1000 showed high levels of identity and similarity. Therefore, the DNA sequences of these putative intracellular PHB depolymerase genes were used to design the degenerate primers for PCR. With these primers and a template of *W. eutropha* H16 genomic DNA, a product of the predicted size (about 500 bp) was amplified by PCR. The DNA sequence of the amplified fragment closely resembled that of

the putative intracellular PHB depolymerase genes of the genus *Wautersia* (data not shown). The PCR product was used as a probe for Southern hybridization.

Cloning and sequencing of *phaZd* in *W. eutropha* H16. Using Southern hybridization and colony PCR, two positive clones, designated CharomidZdR108 and CharomidZdR112, were obtained. The different restriction maps of the two charomids indicated that these fragments were different.

One of two positive clones, CharomidZdR108, was subcloned and 2 kbp of the PstI DNA fragment containing *phaZd* was sequenced. The fragment included an ORF (*phaZd*) of 1,089 bp and the putative gene product deduced from the ORF was composed of 362 amino acids (accession number AB206256). A typical catalytic center, a lipase box containing a putative essential serine residue (Ser¹⁹⁰), was located in the middle of the amino acid sequence of PhaZd. The positions of the catalytic triad (Asp²⁶⁶ and His³³⁰) and putative oxyanion hole (His¹⁰⁸) accorded with those in type I catalytic domain of the extracellular PHB depolymerase of *R. pickettii* T1 and some other extracellular PHB depolymerases such as PhaZ1 in *Paucimonas lemoignei* (13) (Fig. 1). No linker domain or substrate binding domain was found in the deduced amino acid sequences of the *phaZd* product. In addition, no signal peptide was identified by the SignalP 3.0 server (28). The other positive clone, CharomidZdR112, is still under investigation.

Expression and purification of recombinant *phaZd*. *E. coli* harboring pE3ReZd1 (*phaZd*) showed strong activity to degrade artificial PHB granules, though the crude cell supernatant fraction from *E. coli* harboring pET23b (control) showed no activity. The proteins of the crude cell supernatant and the insoluble fraction (inclusion bodies) were analyzed by SDS-PAGE and immunological staining. Stained bands with the same molecular mass were detected in both fractions (Fig. 2B, lanes 2 and 3). The chemically determined N-terminal amino acid sequence of PhaZd_{Weu} from inclusion bodies corresponded to the deduced amino acid sequence from *phaZd*, having methionine at the first position (MTKSFAADWH). The immunostained band of PhaZd_{Weu} in the total cell extract of *W. eutropha* H16 was the same size as that of PhaZd_{Weu} from *E. coli* harboring pE3ReZd1 (Fig. 2B, lane 1, 2, and 3). These results indicate that *phaZd* in *W. eutropha* H16 is expressed intact, as deduced from its nucleotide sequence.

To purify PhaZd_{Weu}, histidine-tagged forms of the protein were constructed. The purified N-terminally histidine-tagged PhaZd_{Weu} showed a single band with an apparent molecular mass of 41 kDa on SDS-PAGE (Fig. 2A and B, lane 4). The size corresponded to the molecular mass (41,349 Da) deduced from the nucleotide sequence. The purified N-terminally histidine-tagged PhaZd_{Weu} was used for the characterization of PhaZd_{Weu}.

Properties of PhaZd_{Weu}. Table 2 shows the substrate specificity of the purified N-terminally histidine-tagged PhaZd_{Weu}. Although purified PhaZd_{Weu} degraded both artificial amorphous PHB granules and native PHB inclusion bodies, the depolymerase activity was much stronger for the former. The specific PHB-degrading activity of PhaZd_{Weu} was 40- to 150-fold that of PhaZa1_{Weu}, PhaZb_{Weu}, or PhaZc_{Weu}. The enzyme like other intracellular PHB depolymerases did not degrade semicrystalline PHB. Poly(3-hydroxyoctanoate), medium-chain-length poly(3-hydroxyalkanoate), was not degraded. Al-

FIG. 1. Alignment of amino acid sequence for PhaZd in *W. eutropha* H16 and other putative PhaZs and the catalytic domain (type I) of extracellular PHB depolymerase. PhaZdWe, PhaZd of *W. eutropha* H16; PhaZdJMP, putative PhaZd of *W. eutropha* JMP134; PhaZdWm, putative PhaZd of *W. metallidurans* CH34; PhaZ1Ple, catalytic domain of extracellular PHB depolymerase PhaZ1 in *Paucimonas lemoignei*; eDepRpT1, catalytic domain of extracellular PHB depolymerase in *R. pickettii* T1. The alignment was performed with ClustalW 1.8.3. The lipase box and catalytic triad are indicated by an underline and reversed characters, respectively. Boxed H and C show the histidine of the oxyanion hole and cysteines. *, conserved in all proteins; ;, substitution of the amino acid residue with one of high similarity; ., substitution of the amino acid residue with one of slightly lower similarity.

though its 3HB oligomer-hydrolyzing activity was relatively weak compared with that of PhaZb_{Weu} or PhaZc_{Weu}, PhaZd_{Weu} degraded the 3HB dimer and 3HB trimer. The products of the degradation of amorphous PHB by PhaZd_{Weu} were mainly the 3HB dimer and 3HB trimer as detected by high-pressure liquid chromatography (date not shown). The

monomer was rarely detected in the hydrolyzed products of PHB and 3HB oligomers.

To estimate the kinds of 3HB oligomers among the hydrolytic products of amorphous PHB generated by PhaZd_{Weu}, a 3HB oligomer hydrolase of *Acidovorax* sp. strain SA1 (PhaZc_{Asp}), which hydrolyzes mainly the 3HB dimer and 3HB trimer (41), and PhaZb_{Weu}, which hydrolyzes 3HB oligomers from the 3HB dimer to 3HB pentamer at a similar rate (20), were used. Judging from the difference in the amount of 3HB produced by the two hydrolases, nearly 50% of the products of the degradation by PhaZd_{Weu} were 3HB tetramers and higher 3HB oligomers, 45% were 3HB dimers and trimers, and 5% were monomers. The hydrolyzing activity of PhaZd_{Weu}, PhaZa1_{Weu}, PhaZb_{Weu}, and PhaZc_{Weu} for *p*-nitrophenyl esters or olive oil was examined (Table 2). PhaZd_{Weu} hydrolyzed *p*-nitrophenyl acetate or *p*-nitrophenyl butyrate faster than other depolymerases and 3HB oligomer hydrolases. *p*-Nitrophenyl palmitate was not hydrolyzed. Only PhaZb_{Weu} slightly hydrolyzed olive oil.

The effect of various reagents on the activity of PhaZd_{Weu} was determined (Table 3). The PHB-degrading activity of PhaZd_{Weu} was completely inhibited by 100 μM of diisopropyl-fluorophosphate, which is a serine hydrolase inhibitor (1), but not by 1 mM of phenylmethylsulfonyl fluoride. 1,4-Dithiothreitol (DTT) (1 mM) inhibited the depolymerase activity. The activity of PhaZd_{Weu} inhibited by DTT was restored completely by oxidation with hydrogen peroxide (2 mM) (Fig. 3). SDS (0.1% [wt/vol]) inhibited the activity completely, but Triton X-100 (1% [wt/vol]) did not fully inhibit it. MgCl₂ and CaCl₂ partially inhibited the activity. Chelating by adding

FIG. 2. SDS-PAGE of total protein of *W. eutropha* H16 and PhaZds expressed in *E. coli*. Coomassie brilliant blue stain (A) and immunostain (B). Lane 1, total cell extract of *W. eutropha* H16 cultured in PHB-accumulating conditions for 48 h (A, 5.0 μg; B, 25 μg); lane 2, crude cell supernatant of *E. coli* harboring pE3ReZd1 (A, 10 μg; B, 0.14 μg); lane 3, inclusion bodies of *E. coli* harboring pE3ReZd1 (A, 2.0 μg; B, 0.040 μg); lane 4, purified N-terminally histidine-tagged PhaZd (A, 0.22 μg; B, 3.0 ng).

TABLE 2. Substrate specificity of PhaZd and other intracellular PHB depolymerases in *W. eutropha* H16

Substrate ^a	Activity ($\mu\text{mol}/\text{min}/\text{mg}$) ^b			
	PhaZd	PhaZa1	PhaZb	PhaZc
Amorphous PHB granules (P)	110	0.71	3	3
Native PHB granules (P)	0.43	0	0	0
Semicrystalline PHB granules (P)	0	0	0	0
PHO suspension (T)	0	n.d.	n.d.	n.d.
3HB oligomers (P)				
Dimer	1.2	0	58	31
Trimer	2.6	0	59	200
Tetramer	0	1.1	54	240
Pentamer	n.d.	1.2	50	310
<i>p</i> -Nitrophenyl acetate (R)	12	0.093	0	0.022
<i>p</i> -Nitrophenyl butyrate (R)	10	0.074	1.1	0.24
<i>p</i> -Nitrophenyl palmitate (R)	0	0	0	0
Olive oil (D)	0	0	1.8	0

^a Assay methods were: P, photometric (3HB released); T, photometric (turbidity); R, photometric (*p*-nitrophenol released); D, titration (pH stat, acid released).

^b n.d., not determined. The final concentration of substrates was 0.5 mg/ml for amorphous PHB granules, native PHB granules, semicrystalline PHB granules, and the PHO suspension; 10 mM for 3HB oligomers; 40 mM for *p*-nitrophenyl acetate and *p*-nitrophenyl butyrate; 5 mM for *p*-nitrophenyl palmitate; and 12.5% (vol/vol) for olive oil. Activities were assayed at pH 8.5 except for *p*-nitrophenyl esters and olive oil. The hydrolytic activity for *p*-nitrophenyl esters and olive oil was measured at pH 8.0.

EDTA in an equimolar amount to these metals restored the activity completely.

The optimal pH of PhaZd_{Wcu} was 8.5 to 9.0 in Tris-HCl buffer. The optimal temperature of the enzyme was 20 to 30°C. PhaZd_{Wcu} was stable at 30°C for 10 min, but after 10 min at 40°C, more than 80% of the activity was lost. At 50°C or above, PhaZd_{Wcu} lost all activity. On long-term storage, at least 3 months, PhaZd_{Wcu} was stable in 20 mM Tris-HCl buffer (pH 8.0) containing 50% glycerol at -20°C.

FIG. 3. Effect of DTT and hydrogen peroxide on PHB-degrading activity of PhaZd. The reaction mixture (50 μl) contained purified PhaZd (0.18 μg), 100 mM Tris-HCl (pH 8.5), and 2 mM DTT. The mixture was incubated at room temperature and then assayed for remaining activity. After 20 min, 2 mM hydrogen peroxide was added to the solution; 100% activity was equal to 110 $\mu\text{mol}/\text{min}/\text{mg}$.

The existence of a thermostable activator (7, 27) for PhaZd_{Wcu} was investigated using the boiled cytosolic fraction of *W. eutropha* H16. No activation of PhaZd_{Wcu} was found when native PHB inclusions or artificial PHB granules was used as a substrate. Additionally, the activator of *R. rubrum* (7) did not activate the PHB-degrading activity of PhaZd_{Wcu} (data not shown).

Subcellular localization of PhaZd. Since PhaZd_{Wcu} was not secreted out of the cell (data not shown), the subcellular localization of PhaZd_{Wcu} in *W. eutropha* H16 was examined by sucrose density gradient centrifugation (Fig. 4). PhaZa1_{Wcu} is known to localize solely in PHB inclusion bodies (20, 31) and was used as a marker of the protein in PHB inclusion bodies. 3HB dehydrogenase (3HBDH) was used as a marker of the protein in the cytosol of the cell (20). As judged from the intensity of the immunostained bands, PhaZd_{Wcu} seemed to be distributed nearly equally between the PHB inclusion bodies and the cytosolic fraction. We also separated the periplasmic fraction from the cells by lysozyme-EDTA treatment (2, 3). An immunostained band was detected in the PHB inclusion bodies and the cytosolic fraction, but not in the periplasmic fraction (data not shown).

Accumulation of PHB in the *phaZd* deletion mutant. Since PhaZd_{Wcu} efficiently degrades amorphous PHB, it probably participates in the mobilization of PHB. We constructed a *phaZd* deletion (ΔphaZd) mutant, strain H16DZd1, and compared it with the wild type in terms of the accumulation of PHB. When cultured in nutrient-rich medium (Fig. 5A), a temporary accumulation of PHB in the logarithmic phase with a rapid mobilization of PHB in the wild-type and ΔphaZd mutant strains was observed as reported earlier (20, 31). Although the amount of PHB reached a larger maximum and a

TABLE 3. Effect of agents on PHB depolymerase activity of PhaZd

Agent ^a	Concn	Relative activity ^b (%)
DFP (μM)	10	47
	100	0
PMSF (mM)	1	100
	10	110
DTT (mM)	0.1	14
	1	0
PCMB (mM)	0.1	100
	1	92
Triton X-100 (% wt/vol)	0.001	120
	0.1	43
	1	36
SDS (% wt/vol)	0.01	72
	0.1	0
EDTA (mM)	1	130
	10	140
MgCl ₂ (mM)	1	91
	10	43
CaCl ₂ (mM)	1	100
	10	61
MgCl ₂ (mM) + EDTA (equimolar)	1	120
	10	120
CaCl ₂ (mM) + EDTA (equimolar)	1	120
	10	110

^a DFP, diisopropylfluorophosphate; PMSF, phenylmethylsulfonyl fluoride; DTT, 1,4-dithiothreitol; PCMB, *p*-(chloromercuri)benzoic acid.

^b Artificial amorphous PHB granules were used as a substrate; 0.05 μg of the purified enzyme was used, and 100% activity was equal to 110 $\mu\text{mol}/\text{min}/\text{mg}$.

FIG. 4. Subcellular localization of PhaZd determined by sucrose density gradient centrifugation. Ten microliters of each fraction (1.1 ml) was analyzed by Western blotting and immunostaining with antiserum against PhaZd_{Wcu}, PhaZa1_{Wcu}, or 3HB dehydrogenase (3HBDH). Columns and circles show the amount of PHB and protein, respectively.

rapid PHB accumulation was observed in the $\Delta phaZd$ mutant than the wild-type strain, it decreased at a similar rate in both forms (Fig. 5A).

Under PHB-accumulating conditions, the $\Delta phaZd$ mutant accumulated about 10% (wt/dry cell wt) more PHB than the wild-type strain in the stationary phase (Fig. 5C, full line). When the cells were transferred to PHB-degrading conditions, a rapid decrease of PHB was found in both the wild-type and $\Delta phaZd$ mutant cells (Fig. 5C, dotted line).

PHB accumulation-dependent expression of *phaZd* in *W. eutropha* H16. The total cell extract of *W. eutropha* H16 grown under several sets of conditions was analyzed by Western blotting and immunostaining using antiserum against PhaZd_{Wcu} (Fig. 5B, D). In nutrient-rich medium, immunostaining analysis showed a temporary expression of *phaZd* in the logarithmic phase and immunostained bands of PhaZd_{Wcu} were only found until the 19th h. Under PHB-accumulating conditions, immunostained bands were found in all samples. The amount of PhaZd_{Wcu} reached a maximum after 48 h. From the intensity of the immunostained bands, the molecular ratio of PhaZa1_{Wcu}, PhaZb_{Wcu}, PhaZc_{Wcu}, and PhaZd_{Wcu} in the cell at 48 h was estimated to be 7.2:0.4:20:1.0. When the cells were transferred to PHB-degrading conditions, a very faint immunostained band was found at 72 h (12 h after the cells were transferred). No band was found after 84 h.

DISCUSSION

PhaZd_{Wcu} showed unique properties different from those of known intracellular PHB depolymerases or 3HB oligomer hydrolases PhaZa1_{Wcu}, PhaZb_{Wcu}, and PhaZc_{Wcu}. Table 4 presents the properties of PhaZd_{Wcu} and other PhaZs in *W. eutropha* H16. Although the properties of each enzyme are

different, PhaZb_{Wcu} and PhaZc_{Wcu} have an overall similarity in substrate specificity. The strong PHB-hydrolyzing activity of PhaZd_{Wcu} is a remarkable feature that is not found in other intracellular PHB depolymerases except for PhaZ1_{Rru} in *R. rubrum* (8). Although PhaZd_{Wcu} showed relatively intense esterase activity toward *p*-nitrophenyl esters (Table 2), the ratio of PHB-hydrolyzing activity to esterase activity is about 10:1. Since no lipase activity was detected, PhaZd_{Wcu} seems to be a specific enzyme for the mobilization of PHB. The degradation product was mainly 3HB oligomers when artificial amorphous PHB granules were used as a substrate. Since only a small amount of 3HB monomer was detected in the degradation product, PhaZd_{Wcu} probably works as an endo-type depolymerase.

PhaZd_{Wcu} is the second PHB depolymerase to show similarity to the extracellular PHB depolymerases. Bacterial extracellular PHB depolymerases are known to have a composite domain structure. Most of these depolymerases consist of a signal peptide, a catalytic domain, a linker domain, and a substrate binding domain from the N terminus to C terminus (14). Three types of linker domain and two types of substrate binding domain are known. In regard to the order of the active amino acids in the catalytic domain, two types of catalytic domain of extracellular PHB depolymerase are known (14). In type I (*R. pickettii* T1, *Paucimonas lemoignei*, etc.), the sequential order of these active amino acids is histidine (oxyanion hole)-serine-aspartate-histidine from the N terminus to the C terminus. Therefore, the active center (lipase box) serine is located in the middle of the catalytic domain. In type II (*Acidovorax* sp. strain TP4, *Comamonas* sp., etc.), the order is serine-aspartate-histidine-histidine (oxyanion hole). The active center is located at the N-terminal side. There is no significant similarity in amino acid sequence between type I and type II catalytic domains.

PhaZd_{Wcu} is similar to the type I catalytic domain of the extracellular PHB depolymerase of *R. pickettii* T1 in the positions of its catalytic triad and putative oxyanion hole (Fig. 1). On the other hand, PhaZ1_{Rru} is similar to the extracellular PHB depolymerase having a type II catalytic domain. Another structural difference between PhaZd_{Wcu} and PhaZ1_{Rru} is that PhaZ1_{Rru} has a signal peptide, but PhaZd_{Wcu} does not. In *R. rubrum*, PhaZ1_{Rru} localizes to the periplasm in a form lacking the signal peptide (8). PhaZd_{Wcu} from *W. eutropha* H16 is distributed nearly equally between the PHB inclusion bodies and the cytosolic fraction (Fig. 4), and was not found in the periplasmic fraction. In Fig. 2, the size of an immunologically stained band of PhaZd_{Wcu} in *W. eutropha* H16 was same to the size of PhaZd_{Wcu} from *E. coli* whose N-terminal portion was not cleaved.

Recently, amorphous PHB-specific extracellular PHB depolymerase was found from *Paucimonas lemoignei* (PhaZ7_{ple}) (9). Although PhaZ7_{ple} show a high PHB-hydrolyzing specific activity, the amino acid sequence was not similar to either the type I or type II catalytic domain. These structural differences are summarized in Fig. 6. Furthermore, PhaZ1_{Rru} has been reported to be activated by a thermostable activator within the cell (7, 27). However, there seems to be no thermostable activator for PhaZd_{Wcu} in the cytosolic fraction of *W. eutropha* H16. PhaZd_{Wcu} was strongly inhibited by diisopropylfluorophosphate, suggesting that the active center of the enzyme is a

FIG. 5. Growth, PHB accumulation, and expression of PhaZd. A and C: dry cell weight (upper) and accumulation of PHB (lower). The wild-type and $\Delta phaZd$ mutant cells were cultured in nutrient-rich medium (A) or PHB-accumulating (C, solid line) or PHB-degrading (C, dotted line) conditions. PHB accumulation: cells were grown in minimal salt medium containing 2% (wt/vol) fructose and 0.1% (wt/vol) ammonium sulfate. PHB degradation; 60-h-cultured cells that had accumulated PHB were transferred to medium containing 0.5% (wt/vol) ammonium sulfate (arrows). Solid circle, wild type; open circle, H16DZd1 ($\Delta phaZd$ mutant). Results are the means for three independent measurements. The range of error bars was within $\pm 5\%$ of each value in the wild-type and $\pm 10\%$ in H16DZd1. B and D: time-dependent immunostaining analysis using the antiserum against PhaZd_{Weu}. Asterisks show the time after the transfer. Each lane contained 50 μ g of protein.

serine residue in the lipase box (Ser¹⁹⁰). Furthermore, DTT inhibited the activity of PhaZd_{Weu}. These effects were similar in PhaZd_{Weu} and PhaZ1_{Rru}. However, PhaZ1_{Rru} is inhibited by EDTA and slightly activated by monovalent cations such as Na⁺ or K⁺, but these effects were not found in PhaZd_{Weu}. The temperature optimum is 50°C in PhaZ1_{Rru} and 20 to 30°C in PhaZd_{Weu}. Although PhaZ1_{Rru} has strong hydrolyzing activity for both PHB and 3HB oligomers, the 3HB oligomer-hydro-

lyzing activity of PhaZd_{Weu} was relatively weak. As a whole, PhaZd_{Weu} seems to be a novel type of intracellular PHB depolymerase which is apparently different from PhaZ1_{Rru} in *R. rubrum*.

Although PhaZd_{Weu} was located in the cell, it was inhibited completely by DTT. Only two cysteines (Cys¹¹⁰ and Cys¹⁴⁸) exist in the amino acid sequence of PhaZd_{Weu}. These two cysteines probably form an essential disulfide bond. DTT in-

TABLE 4. Properties of PhaZd and other intracellular PHB depolymerases in *W. eutropha* H16

Property	PhaZd	PhaZa1	PhaZb	PhaZc
Substrate ^a				
PHB	+++	+	+	+
3HB oligomers	+	+	++	+++
Type of hydrolysis	Endo	Endo	Exo and endo	Exo
Localization	PHB and cytosol	PHB	Cytosol and PHB	Cytosol
Molecular mass* (Da)	39,168	47,316	78,461	31,542
Similarity	Extracellular PHB depolymerase (type I catalytic domain)	PHB synthase	Extracellular 3HB oligomer hydrolase (<i>R. pickettii</i> T1)	Intracellular 3HB oligomer hydrolase (<i>Acidovorax</i> sp. stain SA1)
Reference(s)	This study	21, 31	20, 32	19

^a +, <10 μ mol/min/mg; ++, >10 μ mol/min/mg; +++, >100 μ mol/min/mg; *, deduced from the nucleotide sequence.

FIG. 6. Comparison of structures of PhaZd_{Weu}, PhaZ1_{Rru}, and extracellular PHB depolymerases. Reversed characters (S, D, H) show amino acid residues in the catalytic triad. An asterisk indicates the histidine of the supposed oxyanion hole. SP, signal peptide; SBD, substrate binding domain.

hibits all extracellular PHB depolymerases as far as we know (12). From the results of experiments with PhaZd_{Weu}, we may specify an essential disulfide bond in the extracellular PHB depolymerase of *R. pickettii* T1 which has 4 cysteine residues in the catalytic domain (Fig. 1). Cytoplasmic proteins do not generally contain structural disulfide bonds, although certain cytoplasmic enzymes form such bonds as part of their catalysis (4, 40). The activation or inactivation of PhaZd_{Weu} may be regulated by oxidation-reduction in vivo. Alternatively, the disulfide bond of PhaZd_{Weu} may be required for the association of PhaZd_{Weu} with highly hydrophobic PHB inclusion bodies to maintain the structure of PhaZd_{Weu}, or the structure of the type I catalytic domain of the extracellular PHB depolymerase may be simply conserved.

Immunostaining showed that there was less PhaZd_{Weu} in the cell than other intracellular PHB depolymerases or 3HB oligomer hydrolase except for PhaZb_{Weu} in *W. eutropha* H16. Although PhaZd_{Weu} distributed nearly equally between PHB inclusion bodies and the cytosolic fraction, the enzyme in the cytosol may not participate in the mobilization of PHB considering its relatively weak 3HB oligomer hydrolase activity. More detailed study is necessary to elucidate why the enzyme is also found in the cytosol. A comparison of PHB content in the cell revealed a clear difference between the wild-type and Δ phaZd mutant in the PHB-accumulating period. In nutrient-rich medium, the difference was also observed during the temporary accumulation of PHB. Since the rate of decrease after the accumulation of PHB is almost the same in both the wild-type and Δ phaZd mutant, PhaZd_{Weu} seems not to participate in the rapid degradation of PHB (Fig. 5A). As a rapid decrease in the amount of PHB was also found in both the wild-type and Δ phaZd mutant cells under PHB-degrading conditions (Fig. 5C, dotted line), again the enzyme seems not to participate in the rapid degradation induced by the addition of ammonium sulfate.

The difference in PHB content between the wild-type and Δ phaZd mutant well matched the time-dependent analysis of PhaZd_{Weu} (Fig. 5B), in which PhaZd_{Weu} was not detected in the degradation phase. Both the accumulation and degradation of PHB have been reported to occur at the same time under certain conditions in vivo (16). Expression of PhaZd_{Weu}

was observed in PHB-accumulating conditions. These results may indicate that PhaZd_{Weu} controls the amount of PHB in the cell.

PhaZa1_{Weu}, PhaZb_{Weu}, PhaZc_{Weu}, and PhaZd_{Weu} differed in substrate specificity and subcellular localization (Table 4). PhaZa1_{Weu} and PhaZd_{Weu} were found in the PHB inclusion bodies. They may participate in the degradation of PHB in the cell. PhaZb_{Weu} probably participates also in the degradation of PHB, because a synergistic effect for PhaZa1_{Weu} has been reported (20). Although PhaZc_{Weu} also has weak PHB-hydrolyzing activity, most PhaZc_{Weu} occurs in the cytosolic fraction (19). Therefore, PhaZc_{Weu} probably mainly hydrolyzes 3HB oligomers produced by other depolymerases during the mobilization of PHB together with PhaZb_{Weu}. Since PhaZd_{Weu} had high specific activity for PHB and hardly hydrolyzed 3HB oligomers at all, the enzyme may specifically degrade the PHB within the cell. PHB depolymerase activity in the cytosolic fraction in *W. eutropha* H16, which is different from PhaZb_{Weu} and PhaZc_{Weu}, has been suggested (35). PhaZd_{Weu} probably contributes to the PHB depolymerase activity in the cytosolic fraction in *W. eutropha* H16.

A BLAST search indicated the existence of another phaZd-like hydrolase (PhaZd homolog) in *W. eutropha* JMP 134 and in *W. metallidurans* CH34. In this study, two positive fragments from *W. eutropha* H16 were obtained by Southern hybridization. Clarification of the other PhaZd_{Weu} is required. Analysis of the other positive clone obtained by Southern hybridization is in progress.

ACKNOWLEDGMENTS

This study was supported in part by a grant-in-aid for the High-Tech Research Center Project (2002) from the Ministry of Education, Culture, Sports, Science and Technology of Japan.

We thank M. Shiraki and M. Takanashi for their advice and A. Sugiyama for 3HB oligomers.

REFERENCES

- Berg, J., J. Tymoczko, and L. Stryer. 2002. Biochemistry, 5th ed., p. 190–225. W. H. Freeman and Company, New York, N.Y.
- Bernhard, M., B. Benelli, A. Hochkoeppler, D. Zannoni, and B. Friedrich. 1997. Functional and structural role of the cytochrome b subunit of the membrane-bound hydrogenase complex of *Alcaligenes eutrophus* H16. *Eur. J. Biochem.* **248**:179–186.
- Bernhard, M., B. Friedrich, and R. A. Siddiqui. 2000. *Ralstonia eutropha* TF93 is blocked in tat-mediated protein export. *J. Bacteriol.* **182**:581–588.
- Cumming, R. C., N. L. Andon, P. A. Haynes, M. Park, W. H. Fischer, and D. Schubert. 2004. Protein disulfide bond formation in the cytoplasm during oxidative stress. *J. Biol. Chem.* **279**:21749–21758.
- Daws, E. A., and D. W. Ribbons. 1964. Some aspects of the endogenous metabolism of bacteria. *Bacteriol. Rev.* **28**:126–149.
- Gao, D., A. Maehara, T. Yamane, and S. Ueda. 2001. Identification of the intracellular polyhydroxyalkanoate depolymerase gene of *Paracoccus denitrificans* and some properties of the gene product. *FEMS Microbiol. Lett.* **196**:159–164.
- Handrick, R., S. Reinhardt, D. Schultheiss, T. Reichart, D. Schuler, V. Jendrossek, and D. Jendrossek. 2004. Unraveling the function of the *Rhodospirillum rubrum* activator of polyhydroxybutyrate (PHB) degradation: the activator is a PHB granule-bound protein (phasin). *J. Bacteriol.* **186**:2466–2475.
- Handrick, R., S. Reinhardt, P. Kimmig, and D. Jendrossek. 2004. The “intracellular” poly(3-hydroxybutyrate) (PHB) depolymerase of *Rhodospirillum rubrum* is a periplasm-located protein with specificity for native PHB and with structural similarity to extracellular PHB depolymerases. *J. Bacteriol.* **186**:7243–7253.
- Handrick, R., S. Reinhardt, M. L. Focarete, M. Scandola, G. Adamus, M. Kowalczyk, and D. Jendrossek. 2001. A new type of thermoalkalophilic hydrolase of *Paucimonas lemoignei* with high specificity for amorphous polyesters of short chain-length hydroxyalkanoic acids. *J. Biol. Chem.* **276**:36215–36224.

10. Handrick, R., S. Simone, and D. Jendrossek. 2000. Mobilization of poly(3-hydroxybutyrate) in *Ralstonia eutropha*. J. Bacteriol. **182**:5916–5918.
11. Horowitz, D. M., and J. K. M. Sanders. 1994. Amorphous, biomimetic granules of polyhydroxybutyrate: preparation, characterization, and biological implications. J. Am. Chem. Soc. **116**:2695–2702.
12. Jendrossek, D. 2002. Extracellular polyhydroxyalkanoate depolymerases: the key enzymes of PHA degradation, p. 41–83. In Y. Doi, and A. Steinbüchel (ed.), Biopolymers, vol. 3b: polyesters II. Wiley-VCH, Weinheim, Germany.
13. Jendrossek, D., B. Müller, and H. G. Schlegel. 1993. Cloning and characterization of the poly(hydroxyalkanoic acid)-depolymerase gene locus, phaZ1, of *Pseudomonas lemoignei* and its gene product. Eur. J. Biochem. **170**:1–710.
14. Jendrossek, D., and R. Handrick. 2002. Microbial degradation of polyalkanoates. Annu. Rev. Microbiol. **56**:403–443.
15. Karr, D. B., J. K. Waters, and D. W. Emerich. 1983. Analysis of poly- β -hydroxybutyrate in *Rhizobium japonicum* bacteroids by ion-exclusion high-pressure liquid chromatography UV detection. Appl. Environ. Microbiol. **46**:1339–1344.
16. Kawaguchi, Y., and Y. Doi. 1992. Kinetics and mechanism of synthesis and degradation of poly(3-hydroxybutyrate) in *Alcaligenes eutrophus*. Macromolecules **25**:2324–2329.
17. Kobayashi, T., A. Sugiyama, Y. Kawase, T. Saito, J. Mergaert, and J. Swings. 1999. Biochemical and genetic characterization of an extracellular poly(3-hydroxybutyrate) depolymerase from *Acidovorax* sp. strain TP4. J. Environ. Polym. Degrad. **7**:9–17.
18. Kobayashi, T., K. Nishikori, and T. Saito. 2004. Properties of an intracellular poly(3-hydroxybutyrate) depolymerase (PhaZ1) from *Rhodobacter sphaeroides*. Curr. Microbiol. **49**:199–202.
19. Kobayashi, T., K. Uchino, T. Abe, Y. Yamazaki, and T. Saito. 2005. Novel intracellular 3-hydroxybutyrate oligomer hydrolase in *Wautersia eutropha* H16. J. Bacteriol. **187**:5129–5135.
20. Kobayashi, T., M. Shiraki, T. Abe, A. Sugiyama, and T. Saito. 2003. Purification and properties of an intracellular 3-hydroxybutyrate oligomer hydrolase (PhaZ2) in *Ralstonia eutropha* H16 and its identification as a novel intracellular poly(3-hydroxybutyrate) depolymerase. J. Bacteriol. **185**:3485–3490.
21. Kobayashi, T., and T. Saito. 2003. Catalytic triad of intracellular poly(3-hydroxybutyrate) depolymerase (PhaZ1) in *Ralstonia eutropha* H16. J. Biosci. Bioeng. **96**:487–492.
22. Laemmli, U. K. 1970. Cleavage of structural proteins during the assembly of the head of bacteriophage T4. Nature **227**:680–685.
23. Lenz, O., and B. Friedrich. 1998. A novel multicomponent regulatory system mediates H₂ sensing in *Alcaligenes eutrophus*. Proc. Natl. Acad. Sci. USA **95**:12474–12479.
24. Lenz, O., E. Schwartz, J. Dernerode, M. Eitinger, and B. Friedrich. 1994. The *Alcaligenes eutrophus* H16 *hoxX* gene participates in hydrogenase regulation. J. Bacteriol. **176**:4385–4393.
25. Lowry, O. H., N. J. Rosebrough, A. L. Farr, and R. J. Randall. 1951. Protein measurement with the Folin phenol reagent. J. Biol. Chem. **193**:265–275.
26. Merrick, J. M. 2002. Microbial water-insoluble aliphatic polyesters (PHA), p. 105–122. In Y. Doi and A. Steinbüchel (ed.), Biopolymers: polyesters I. Wiley-VCH, Weinheim, Germany.
27. Merrick, J. M., and M. Doudoroff. 1964. Depolymerization of poly- β -hydroxybutyrate by an intracellular enzyme system. J. Bacteriol. **88**:60–71.
28. Nielsen, H., J. Engelbrecht, S. Brunak, and G. von Heijne. 1997. A neural network method for identification of prokaryotic and eukaryotic signal peptides and prediction of their cleavage sites. Int. J. Neurol. Syst. **8**:581–599.
29. Potter, M., H. Müller, F. Reinecke, R. Wiczorek, F. Fricke, B. Bowien, B. Friedrich, and A. Steinbüchel. 2004. The complex structure of polyhydroxybutyrate (PHB) granules: four orthologous and paralogous phasins occur in *Ralstonia eutropha*. Microbiology **150**:2301–2311.
30. Ramsay, B. A., I. Saracovan, J. A. Ramsay, and H. Marchessault. 1994. A method for isolation of microorganisms producing extracellular long-side-chain poly(β -hydroxybutyrate) depolymerase. J. Environ. Polym. Degrad. **2**:1–7.
31. Saegusa, H., M. Shiraki, C. Kanai, and T. Saito. 2001. Cloning of an intracellular poly[D(-)-3-hydroxybutyrate] depolymerase gene from *Ralstonia eutropha* H16 and characterization of the gene product. J. Bacteriol. **183**:94–100.
32. Saegusa, H., M. Shiraki, and T. Saito. 2002. Cloning of an intracellular D(-)-hydroxybutyrate oligomer hydrolase gene from *Ralstonia eutropha* H16 and identification of the active site serine residue by site-directed mutagenesis. J. Biosci. Bioeng. **94**:106–112.
33. Saito, I., and G. R. Stark. 1986. Charomids: cosmid vectors for efficient cloning and mapping of large or small restriction fragment. Proc. Natl. Acad. Sci. USA **83**:8664–8668.
34. Saito, T., K. Suzuki, J. Yamamoto, T. Fukui, K. Miwa, K. Tomita, S. Nakanishi, S. Odani, J. Suzuki, and K. Ishikawa. 1989. Cloning, nucleotide sequence, and expression in *Escherichia coli* of the gene for poly(3-hydroxybutyrate) depolymerase from *Alcaligenes faecalis*. J. Bacteriol. **171**:184–189.
35. Saito, T., K. Takizawa, and H. Saegusa. 1995. Intracellular poly(3-hydroxybutyrate) depolymerase in *Alcaligenes eutrophus*. Can. J. Microbiol. **41**(Suppl. 1):187–191.
36. Saito, T., and T. Kobayashi. 2002. Intracellular degradation of PHAs, p. 23–39. In Y. Doi and A. Steinbüchel (ed.), Biopolymers, vol. 3b: polyesters II. Wiley-VCH, Weinheim, Germany.
37. Sambrook, J., E. F. Fritsch, and T. Maniatis. 1989. Molecular cloning: a laboratory manual, 2nd ed. Cold Spring Harbor Laboratory Press, Cold Spring Harbor, N.Y.
38. Simon, R., U. Priefer, and A. Pühler. 1983. A broad host range mobilization system for in vivo genetic engineering: transposon mutagenesis in gram negative bacteria. Bio/Technology **1**:784–791.
39. Smibert, R. M., and N. R. Krieg. 1994. Phenotypic characterization, p. 650. In P. Gerhardt, R. G. E. Murry, W. A. Wood, and N. R. Krieg (ed.), Methods for general and molecular bacteriology. American Society for Microbiology, Washington, D.C.
40. Stewart, E. J., F. Åslund, and J. Beckwith. 1998. Disulfide bond formation in the *Escherichia coli* cytoplasm: an in vivo role reversal for the thioredoxins. EMBO. J. **17**:5543–5550.
41. Sugiyama, A., T. Kobayashi, M. Shiraki, and T. Saito. 2004. Roles of poly(3-hydroxybutyrate) depolymerase and 3HB oligomer hydrolase in bacterial metabolism. Curr. Microbiol. **48**:424–427.
42. Tanaka, Y., T. Saito, T. Fukui, T. Tanio, and K. Tomita. 1981. Purification and properties of D(-)-3-hydroxybutyrate dimer hydrolase from *Zoogloea ramigera* I-16-M. Eur. J. Biochem. **118**:177–182.
43. Vanechoutte, M., P. Kampfer, T. De Baere, E. Falsen, and G. Verschraegen. 2004. *Wautersia* gen. nov., a novel genus accommodating the phylogenetic lineage including *Ralstonia eutropha* and related species, and proposal of *Ralstonia* [*Pseudomonas*] *syzygii* (Roberts et al. 1990) comb. nov. Int. J. Syst. Evol. Microbiol. **54**:317–327.
44. York, G. M., J. Lupberger, J. Tian, A. G. Lawrence, J. A. Stubbe, and A. J. Sinskey. 2003. *Ralstonia eutropha* H16 encodes two and possibly three intracellular poly[D(-)-3-hydroxybutyrate] depolymerase genes. J. Bacteriol. **185**:3788–3794.
45. Zhang, K., M. Shiraki, and T. Saito. 1997. Purification of an extracellular D(-)-3-hydroxybutyrate oligomer hydrolase from *Pseudomonas* sp. strain A1 and cloning and sequencing of its gene. J. Bacteriol. **179**:72–77.