Cloning of an Intracellular Poly[D(–)-3-Hydroxybutyrate] Depolymerase Gene from *Ralstonia eutropha* H16 and Characterization of the Gene Product

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An intracellular poly[D(–)-3-hydroxybutyrate] (PHB) depolymerase gene (*phaZ*) has been cloned from *Ralstonia eutropha* H16 by the shotgun method, sequenced, and characterized. Nucleotide sequence analysis of a 2.3-kbp DNA fragment revealed an open reading frame of 1,260 bp, encoding a protein of 419 amino acids with a predicted molecular mass of 47,316 Da. The crude extract of *Escherichia coli* containing the PHB depolymerase gene digested artificial amorphous PHB granules and released mainly oligomeric D(–)-3-hydroxybutyrate, with some monomer. The gene product did not hydrolyze crystalline PHB or freeze-dried artificial amorphous PHB granules. The deduced amino acid sequence lacked sequence corresponding to a classical lipase box, Gly-X-Ser-X-Gly. The gene product was expressed in *R. eutropha* cells concomitant with the synthesis of PHB and localized in PHB granules. Although a mutant of *R. eutropha* whose *phaZ* gene was disrupted showed a higher PHB content compared to the wild type in a nutrient-rich medium, it accumulated PHB as much as the wild type did in a nitrogen-free, carbon-rich medium. These results indicate that the cloned *phaZ* gene encodes an intracellular PHB depolymerase in *R. eutropha*.

Poly[D(–)-3-hydroxybutyrate] (PHB), a homopolymer of D(–)-3-hydroxybutyrate (3HB), is a storage material produced by some bacteria in response to environmental stress. The PHB biosynthesis genes from such bacteria have been cloned in *Escherichia coli* and studied in detail (13, 24, 26). However, only a few studies on the intracellular degradation of PHB have been published. In an in vitro system consisting of native PHB granules from *Bacillus megaterium* and the soluble fraction from PHB-depleted cells of *Rhodospirillum rubrum*, the existence of a thermostable activator and a thermolabile depolymerase has been reported (16). Various chemical and physical treatments inactivate the native PHB granules. In intracellular degradation of PHB in *Ralstonia eutropha*, weak hydrolysis activity against [¹⁴C]PHB granules independent of the harvest time of the cells was reported (5). We have detected intracellular PHB depolymerase activity in *Zoogloea ramigera* I-16-M (18) and *R. eutropha* H16 (21), using protease-treated native PHB granules as a substrate.

A few intracellular poly-3-hydroxyoctanoate (PHO) depolymerase genes have been cloned. Huisman et al. have cloned an intracellular PHO depolymerase gene from *Pseudomonas oleovorans* using a PHO degradation mutant that cannot degrade PHO (8). Timm and Steinbüchel have cloned a PHO depolymerase gene from *Pseudomonas aeruginosa* PAO1 by hybridization using information on the DNA sequence of *P. oleovorans* (29). In both cases, the gene products have yet to be characterized. Although many extracellular PHB depolymer-

ase genes have been cloned (11), no intracellular PHB depolymerase gene has been cloned to date. We tried unsuccessfully to clone the intracellular PHB depolymerase gene (*phaZ*) in *R. eutropha* by Southern hybridization using an extracellular PHB depolymerase gene as a probe. Therefore, we performed shotgun gene cloning by assaying enzyme activity of clones expressed in *E. coli*. It is not easy to measure activity of the intracellular PHB depolymerase, because the enzyme can digest only amorphous PHB (16). Protease-treated native PHB granules show activity against cell extract from *Z. ramigera* I-16-M and *R. eutropha* H16, but they still have some autolytic activity (18, 21). Therefore, they may not be suitable for measuring low-level activity. Recently, artificial granules made from purified PHB and detergents have been reported (7). In these granules, PHB assumes an amorphous morphology similar to that of the native PHB granules. By assaying intracellular PHB depolymerase activity with the artificial granules, we have succeeded in cloning a *phaZ* gene from *R. eutropha*. In this report, we describe the cloning of a *phaZ* gene, characterization of its product, and properties of a *phaZ* null *R. eutropha* mutant.

MATERIALS AND METHODS

Bacterial strains, plasmids, and culture. Bacterial strains and plasmids used in this study are listed in Table 1. All *R. eutropha* strains were grown in nutrient-rich medium containing 1% (wt/vol) yeast extract, 1% (wt/vol) Polypeptone, 0.5% (wt/vol) beef extract, and 0.5% (wt/vol) (NH₄)₂SO₄ at 30°C with appropriate antibiotics. To produce PHB, cells grown on a nutrient-rich medium were transferred to a nitrogen-free medium containing 0.27% (wt/vol) KH₂PO₄, 0.99% (wt/vol) K₂HPO₄, 0.02% (wt/vol) MgSO₄ · 7H₂O, 0.1% (wt/vol) mineral solution, and 2% (wt/vol) fructose and were cultured at 30°C as described previously (21, 23). *E. coli* strains were grown in Luria-Bertani medium (LB) at 37°C with or without antibiotics (ampicillin [50 µg/ml], tetracycline [10 µg/ml], chloramphenicol [34 µg/ml], kanamycin [50 µg/ml], streptomycin [50 µg/ml], and gentamicin [10 µg/ml]).

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TABLE 1. Strains and plasmids used in this study

Strain or plasmid	Relevant features	Source or reference
<i>E. coli</i>		
DH5	<i>supE44 hsdR17 recA1 endA1 gyrA96 thi-1 relA1</i>	Takara
JM109	<i>recA1 endA1 gyrA96 thi hsdR17 supE44 relA1 Δ(lac-proAB)/F' [traD36 proAB⁺ lacI^a lacZΔM15]</i>	Takara
S17-1/λpir	<i>Tra⁺ recA pro thi hsdR</i> chr::RP4-2	25
XLI-BlueMR/pDPT51	<i>Δ(mcrA)183 Δ(mcrCB-hsdSMR-mrr) 173 endA1 supE44 thi-1 recA1 gyrA96 relA1 lac(Con) pDPT51(Amp^r)</i>	28
BLR(DE3)pLysS	<i>F⁻ ompT hsdS_B(r_B⁻ m_B⁻) gal dcm Δ(srl-recA)306::Tn10(Tc^r)DE3pLysS(Cm^r)</i>	Novagen
<i>R. eutropha</i>		
H16	Wild type	ATCC 17699
D1	<i>phaZ</i> ::pJpPH171, Km ^r	This study
Plasmids		
pJDR215	Wide-host-range cosmid, cloning vector; Km ^r Sm ^r	3
pMC1871	Fusion vector; Tc ^r	Amersham
pJP5603	R6K-based suicide vector; Km ^r	17
pUC18	Cloning vector; Amp ^r	Takara
pET23b	Expression vector; Amp ^r	Novagen
Charomid 9-36	Cosmid; Amp ^r	Nippon Gene
pAE17	pUC18 with <i>R. eutropha phaZ</i>	This study
pET171	pET23b carrying <i>XbaI/Bpu1102I</i> fragment of pAE171	This study
pET171H	pET23b carrying <i>NdeI/XhoI</i> fragment (1,263 bp) containing His-tagged <i>phaZ</i>	This study
pJPPH171	pJP5603 carrying <i>PstI/HincII</i> fragment of pAE171	This study
pJDR215T	pJDR215 carrying <i>EcoT14/HindIII</i> fragment of pMC1871 containing Tc ^r gene	This study
pJDR171T	pJDR215T carrying <i>SnaBI/EcoRV</i> fragment of pAE171 containing <i>phaZ</i>	This study

DNA manipulation. Preparation of chromosomal DNA and plasmid DNA, isolation and purification of DNA fragments, gel electrophoresis, Southern hybridization, and nucleotide sequencing were carried out according to standard techniques (22). The *Sau3A1*-digested chromosomal DNA fragments (10 to 15 kbp) from *R. eutropha* H16 were ligated to a cosmid vector, charomid 9-36 (19). The ligation mixture was packaged by using a LAMBDA INN in vitro packaging kit (Nippon Gene, Toyama, Japan), and the packaged charomid was used to transfect *E. coli* DH5. The bacteria were inoculated onto LB-ampicillin plates, and the resulting colonies were used as a genomic library.

Construction of *phaZ* null *R. eutropha* mutant (strain D1). A part of *phaZ* (268 bp, *PstI-HincII* fragment) was inserted into a suicide vector, pJP5603 (Km^r), which can replicate in *E. coli* but not in *R. eutropha*. The resultant plasmid (pJPPH171) was introduced into *E. coli* S-17 by transformation and was mobilized into *R. eutropha* via conjugation. Transconjugants were selected on kanamycin (50 µg/ml) and ampicillin (25 µg/ml). The selected strain (D1) was confirmed based on Southern blots and antibiotic susceptibility to carry pJPPH171 in the *phaZ* locus. No expression of *phaZ* in D1 grown in the conditions under which PHB was accumulated was detected by immunoblot analysis.

Preparation of cell extract for enzyme assay. Cells harvested from an overnight culture in LB were suspended in 50 mM Tris-HCl (pH 7.5) (5 ml/g [wet weight] of cells). The cell suspension was disrupted by sonication (20-kHz tip, 30 W for 5 min). The sonicated cells were centrifuged at 10,000 × *g* for 10 min, and the supernatant fraction was used as the crude extract.

Preparation of PHB granules. Artificial amorphous PHB granules were prepared by the method described by Horowitz and Sanders (7) as follows. Purified PHB was dissolved in chloroform, and then 0.05% (wt/vol) sodium oleate was added. The mixture was sonicated (20-kHz tip, 200 W for 10 min); then the emulsion was heated at 75°C for 90 min with stirring to remove chloroform and dialyzed for 24 h against 0.01% (wt/vol) oleate at room temperature. Crystalline PHB granules were prepared from *R. eutropha* by a hypochlorite procedure described by Smibert and Krieg (27).

Enzyme assay. PHB depolymerase activity was assayed as follows. The reaction mixture (0.5 ml) contained Tris-HCl (40 µmol, pH 9.0) and artificial PHB granules (0.3 mg as PHB) and enzyme. After addition of enzyme, the mixture was incubated at 30°C for 15 min, and the reaction was stopped by heating at 100°C for 5 min. To hydrolyze the resulting 3HB oligomers, 0.05 U of 3HB oligomer hydrolase (31) was added, and the mixture was incubated at 30°C for 15 min and centrifuged. The 3HB in the supernatant fraction was measured enzymatically with 3HB dehydrogenase as described previously (31). One unit of the enzyme catalyzes the formation of 1 µmol of 3HB per min under the assay conditions used.

Purification of His-tagged PHB depolymerase from *E. coli*. The *phaZ* gene was amplified by PCR with primers Nde (AGGCAGAAACATATGCTCTAC) and Xho (CGTTCTCGAGCCTGGTGCCGA). primer Nde introduces an *NdeI*

site at the translation start codon; primer Xho introduces an *XhoI* site at the stop codon. The amplified DNA was cloned into the protein fusion vector pET23b as an *NdeI-XhoI* fragment. The fusion contained decahistidine at the carboxy-terminal end of the protein, allowing the purification of the modified protein (His-PHB depolymerase) from the inclusion body on a metal chelation column under denaturing conditions (6). Expression in *E. coli* BLR(DE3)pLysS and column purification of the protein were performed as recommended by the supplier (Novagen). After metal chelating purification, the protein was finally purified by a continuous elution sodium dodecyl sulfate (SDS)-polyacrylamide gel electrophoresis (Prep cell model 491; Bio-Rad). The antibody against purified His-PHB depolymerase was prepared in rabbits as described previously (31).

Purification of PHB granules. *R. eutropha* cells containing PHB were suspended in 20 mM Tris-HCl (pH 7.5) (5 ml/g [wet weight] of cells) and disrupted by sonication (20-kHz tip, 40 W for 15 min). The resulting suspension (5 ml) was loaded onto a discontinuous glycerol gradient, which was prepared from 3 ml each of 88 and 44% glycerol. After centrifugation for 30 min at 210,000 × *g* and 4°C, the granules were collected between 88 and 44% glycerol. They were washed with 20 mM Tris-HCl (pH 7.5) and loaded onto another discontinuous sucrose gradient, which was prepared from 4 ml each of 1.66 and 1.5 M sucrose. After centrifugation for 2 h at 210,000 × *g* and 4°C, the granules were collected between 1.66 and 1.5 M sucrose. The purified granules were withdrawn, washed, and suspended in 20 mM Tris-HCl (pH 7.5).

Other analytical methods. Wide-angle X-ray scattering (WAXS) was performed using a MAC Science MXP-18 diffractometer and Cu Kα radiation (λ = 1.5405 Å). Protein was measured by the method of Lowry et al. (14). SDS-polyacrylamide gel electrophoresis was done by the procedure of Laemmli (12). Electrophoretic transfer of proteins was done using nitrocellulose membranes (Schleicher & Schuell, Dassel, Germany) according to the method of Towbin et al. (30). PHB content was determined by gas chromatography as described by Braunneg et al. (2).

Nucleotide sequence accession number. The nucleotide sequence data reported in this paper have been deposited in the DDBJ, EMBL, and GenBank nucleotide sequence databases under accession number AB017612.

RESULTS

Identification and cloning of a genomic fragment relevant to PHB depolymerase. A library of chromosomal DNA from *R. eutropha* H16 was introduced by transfection into *E. coli* DH5. Approximately 2,000 colonies were picked for enzymatic assay. To facilitate screening, 5-ml cultures of each clone were grown in LB overnight, and five cultures were pooled in one group for

FIG. 1. Restriction endonuclease sites of the cloned DNA (13 kbp) in a charomid DNA and the ability of recombinant plasmids to express PHB depolymerase activity in *E. coli* DH5. B, *Bam*HI; P, *Pst*I; Sa, *Sac*I; S, *Sma*I.

assay. The mixed cultures were centrifuged, and cells were disrupted by sonication. The resulting crude extracts were assayed for PHB depolymerase activity. One of about 400 pools showed PHB depolymerase activity (about 0.087 U/ml). One clone in this pool harbored a genomic DNA fragment of 13 kbp. Judging from a restriction map of this DNA fragment, this region was not in or near the polyhydroxyalkanoate (PHA) synthase gene locus reported by Schubert et al. (24).

After subcloning of the 13-kbp fragment, a 1.7-kbp *Pst*I-*Pst*I fragment that was responsible for the PHB depolymerase activity (Fig. 1) expressed an ~48-kDa protein in an in vitro transcription-translation system (data not shown). Southern hybridization using the 1.7-kbp *Pst*I fragment as a probe showed that the cloned DNA fragment was derived from a single chromosomal site in DNA of *R. eutropha* (data not shown).

Nucleotide sequence analysis of the 2.3-kbp fragment. The nucleotide sequence of the 2.3-kbp *Sac*I-*Pst*I fragment (Fig. 1) was determined. In this region, there were two open reading frames (ORFs): ORF1, which probably corresponds to a portion of the C-terminal region of some protein, and ORF2. ORF2, assigned for *phaZ*, has 1,257 nucleotides and encodes a protein of 419 amino acid residues with a calculated molecular mass of 47,316 Da. The nucleotide sequence of ORF2 revealed relatively high similarity with several proteins in recent databases: a hypothetical protein, RP681 (1,269 bp) of *Rickettsia prowazekii*; ORF1 (1,341 bp) of the *Paracoccus denitrificans* PHA synthase gene locus; and ORF1 (843 bp) of the *Rhodobacter sphaeroides* PHA synthase gene locus (identities in nucleotides and in amino acids of 47.0, 49.0, and 58.9% and 44.4, 37.9, and 41.1%, respectively) (Fig. 2) (1, 9, 15). Analysis

of the amino acid sequence deduced from ORF2 did not reveal any similarity with intracellular PHO depolymerase sequences of *P. oleovorans* or *P. aeruginosa* (8, 29) or with extracellular PHB depolymerases of *Ralstonia pickettii* (formerly *Alcaligenes faecalis*) T1 and *P. lemoignei* (10, 20).

Properties of the PHB depolymerase expressed in *E. coli*.

The properties of the PHB depolymerase expressed in *E. coli* were examined with crude extracts of recombinant *E. coli*. The ORF2 product (PhaZ) expressed in *E. coli* JM109 showed an alkaline pH optimum (pH 8.5 to 10). The ability of the enzyme to degrade PHB granules of different morphology was examined (Fig. 3). Analysis by WAXS of the PHB preparations used (Fig. 3B) revealed that the depolymerase hydrolyzed only amorphous PHB; it did not degrade purified, crystalline PHB granules or freeze-dried artificial amorphous PHB granules (Fig. 3A). Even the slight crystallization observed in the freeze-dried artificial PHB granules hindered PHB degradation under the experimental conditions. To examine the water-soluble products of the PHB depolymerase reaction, we determined the amounts of 3HB monomer in the reaction mixture with or without treatment with the extracellular 3HB oligomer hydrolase (31) after the PHB depolymerizing reaction. About eight times more 3HB monomer was formed with than without treatment with the 3HB oligomer hydrolase. Since 3HB dehydrogenase does not use 3HB oligomers as a substrate, these results show that the PHB depolymerase expressed in *E. coli* degraded amorphous PHB granules to 3HB oligomers as major products.

The enzyme activity in the crude extract was inhibited 50% by 3 mM diisopropylfluorophosphate. The nucleotide se-

<i>R. eutropha</i>	1:	MLY-Q-IHE-FORSILHPLTAWAQATAKTFTNPL-SPL-----SLVPGAP-RFAAGYELLY	51
<i>Rickettsia</i>	1:	MMHCD-IETNYTYIILEWLR--AQIAPMHLGIKILKELYEHEPLEDNGFARTIHAYLTLC	57
<i>Paracoccus</i>	1:	MTTYKGLRGIISYDAMETIRNTNEWMGASARALASYFAFA---LLPNPLFKLMSAWGRVT	57
<i>Rhodobacter</i>	1:	MAS-----YPLFA---LSMNPMLALTAAWGEVT	25
<i>R. eutropha</i>	52:	RLGKEYE-KPAFDIKSVRSNGRDIPIVEQTVL-EKPFCKLVRFKRYADDETIKLLKDE	108
<i>Rickettsia</i>	58:	ERMTRKYT-KPEFNILETIINDKTYNINEQVIF-KKPFCELRHFQK-----IGFKKEL	108
<i>Paracoccus</i>	58:	ERSFARMVIKPDWEIPPVVSEDGQDHVVYVEPVIERFFGDLVHFRVARRAPLAR-----	111
<i>Rhodobacter</i>	26:	ERSFARMVAKPDWGRSIVGDGGQDHLVDVTVVEKPFGNLQFVYRRREPMAR-----	79
<i>R. eutropha</i>	109:	PVVLVAAPLSGHHATLLRDTVRLTLLQDHKVVYVTDWIDARMVPEECAFHLSDYIYYIQEF	168
<i>Rickettsia</i>	109:	PKLLIVAPMAGHHATLLRSTVHALLEPYTDIYITDWTENYVPLEACHFMDDDYIDYLIIEF	168
<i>Paracoccus</i>	112:	KVLLVAPMSGHYATLLRRTVTSLLPDCVYVTDWHDNARDIPVSCCKFDIDDYTCYLVDF	170
<i>Rhodobacter</i>	80:	KVLLVAPMSGHYATLLRSTVLSLLPDCDVYVTDWHDNARDIPVSACKFDVEDYTYLVVDF	138
<i>R. eutropha</i>	169:	IRHIGAENLHVISVCOPTVPVLAATISLMASAGEKTPR-TMTMMGGPIDARKSPITAVNSLA	227
<i>Rickettsia</i>	169:	INFMGQNI-HTMAVCOPTVPVLAATISLMSENNSPNVSSMILIGGPIDARKNPTVNEFA	227
<i>Paracoccus</i>	171:	IRHLGPDT-NVIAVCOPAPLALAAATALLAEEDPKAOPRSLILIGGPIDPDAAATVETDFG	229
<i>Rhodobacter</i>	139:	MRALGPET-NVIAVCOPAPVLAATAYLAGEDPAACPTTLTILIGGPIDPDAAATVETDFG	197
<i>R. eutropha</i>	228:	TNKSFEWFENNVIYTVFANYPCHGRVYPGFLQHAGFVAMNPDRLSSHDFYLSLVEGD	287
<i>Rickettsia</i>	228:	LSKSLEWFCEMLTMOVPPNYPCYGRKVPYGFLOLTGFIISNLLRHIDSHLELWQSLNSD	287
<i>Paracoccus</i>	230:	NRITMGELDYLMTQOVGFKYRGAGRVYPGLAOLSSFIAAMNAETAKAFLDKIFAEARQ	289
<i>Rhodobacter</i>	198:	RRITMGQLEQLALQRVGFKYMGAGRLVYPGLLOLQSFISMMMERHSAKAFQEQIVRVARQ	257
<i>R. eutropha</i>	288:	ADDAEAHVRFYDEYNVAVLDMAAEYVLDITIREVFOEFRLANGTWAIDGNVVRPQDIKSTAL	347
<i>Rickettsia</i>	288:	YQKADYIIFKYDEYLSGMDMPAEFYLOTIDEVFOQFSLARGKFISEKRPIDLKHKTKCAL	347
<i>Paracoccus</i>	290:	GSEGDKNHTFYDEYLAVMDMTAEFYLSSTVERIFKEREIAQNRFTVNGRPVDLGKITDVAV	349
<i>Rhodobacter</i>	258:	ASDHDAAHNRFYDEYLAVMDMTAEF-----	281
<i>R. eutropha</i>	348:	MTVEGELDDISGAGOTAAAHDLCAIGIPKIRKOH-LNAACHGHYGFISGRRWREETYPQLR	406
<i>Rickettsia</i>	348:	LGIEGELDDIAAVGOTKAALKLCVNISESMKRYHONGV-GHYGVFSGSKFKQFIVPLIK	406
<i>Paracoccus</i>	350:	MTVEGANDDISAPGOCVAALALCTGVPANRKTQHLEPGA-GHYGFPAGKSWRLNIRPLVL	408
<i>Rhodobacter</i>	282:	-----	282
<i>R. eutropha</i>	407:	DFIRKYHQASAT-----R-----	419
<i>Rickettsia</i>	407:	EFIYDFDKTNVQ-----K---L-----KLK-	423
<i>Paracoccus</i>	409:	DFIDEQVQAPAGQRKRRRGGPVVDCCGLANLSEHDSKIAV	447
<i>Rhodobacter</i>	282:	-----	282

FIG. 2. Comparison of deduced amino acid sequences that showed relatively high similarity with the intracellular PHB depolymerase of *R. eutropha*. Amino acids identical among more than three proteins are marked in black. *R. eutropha*, *R. eutropha* intracellular PHB depolymerase; *Rickettsia*, hypothetical protein RP681 of *R. prowazekii*; *Paracoccus*, ORF1 product of *P. denitrificans*; *Rhodobacter*, ORF1 product of *R. sphaeroides*.

quence of the cloned intracellular PHB depolymerase does not have a lipase box sequence (Gly-X-Ser-X-Gly).

Detection of PhaZ in *R. eutropha*. With polyclonal antibody against the purified His-PHB depolymerase, the gene product was examined in *R. eutropha* cells (Fig. 4). No immunostained band was detected in the whole-cell lysate of *R. eutropha* cells grown in a nutrient-rich medium for 2 days. When the cells were transferred to the nitrogen-starved, carbon-rich medium for PHB production, an ~50-kDa protein band was detected in the PHB granule fraction and in whole-cell lysate; then the intensity of the band increased with time after the onset of PHB synthesis. No immunostained band was observed in the supernatant fraction of PHB-rich cells. These results indicate that the cloned gene encodes the *R. eutropha* intracellular PHB depolymerase.

PHB accumulation in the *phaZ* null mutant. The *phaZ* null *R. eutropha* mutant strain D1 was constructed using a suicide vector. Although PhaZ was lost in PHB granules of D1 as judged by immunoblot analysis, PHB depolymerase activity in the supernatant fraction of cell extracts (21) was still present in D1 (wild type, 2.3 U/ml; D1, 1.8 U/ml). The effect of PhaZ on PHB accumulation was examined. In a nutrient-rich medium, *R. eutropha* maximized PHB content at 12 h (log phase of cell growth) to about 40% (wt/wt [dry] of cell), which decreased

quickly to below 5% (wt/wt [dry] of cell) after 43 h (Fig. 5A and B). On the other hand, D1 showed a smaller peak of accumulation of PHB, and the decrease of PHB content at 80 h was not great (36% of the maximum value). In the mutant D1 harboring *phaZ*, the PHB content of the cells decreased quickly after 12 h in a fashion similar to that for wild-type *R. eutropha* (Fig. 5B). Under PHB-producing conditions in a nitrogen-free, carbon-rich medium, however, the wild type and D1 did not differ significantly in the accumulation of PHB (Fig. 5C).

DISCUSSION

The nucleotide sequence of the cloned DNA showed relatively high similarity to the DNA sequence corresponding to the hypothetical protein RP681 of *R. prowazekii*, ORF1 of the *P. denitrificans* PHB synthase gene locus, and ORF1 of the *R. sphaeroides* PHA synthase gene locus (1, 9, 15) (Fig. 2). These three bacteria have a PHB synthase gene, and the latter two ORFs containing a PHB depolymerase-like sequence were located very close to this gene. Although the roles of these genes are not known, they may encode intracellular PHB depolymerases. Since the amino acid sequence of the intracellular PHB depolymerase of *R. eutropha* has no lipase box, whereas

FIG. 3. (A) Degradation of crystalline PHB (□), artificial amorphous PHB granules (○), and freeze-dried artificial amorphous PHB granules (△) by intracellular PHB depolymerase. The reaction mixture contained crude extract from *E. coli* JM109 with pUC18 carrying a 1.7-kbp *PstI*-*PstI* fragment (100 μg of protein) and various PHB granules (0.3 mg). The 3HB formed in the supernatant was measured enzymatically after treatment with 3HB oligomer hydrolase. (B) WAXS patterns for crystalline PHB granules (a), artificial amorphous PHB granules (b), and freeze-dried artificial amorphous PHB granules (c).

sequences of all known intracellular PHO depolymerases and extracellular PHB depolymerases do, the PHB depolymerase of *R. eutropha* seems to differ from related enzymes in the structure of its active center. Alignment of the deduced amino acid sequences shows several common regions containing histidine or aspartate residues known to be key amino acids of the charge relay system of the catalytic triad in lipase, but there is no common sequence containing serine residues (Fig. 2). Therefore, the intracellular PHB depolymerase may have in its active center an amino acid other than serine.

The enzyme expressed in *E. coli* showed high activity at alkaline pH (8.5 to 10). The soluble intracellular PHB depolymerase from *R. rubrum* is active at pH 8.0 toward the native PHB granules from *B. megaterium* (16). The intracellular PHB depolymerase in soluble fractions from *Z. ramigera* I-16-M (18) and *R. eutropha* (21) also has a high optimum pH. PHB depolymerase characterized in this study was found only as a PHB granule-bound form. Therefore, the local pH of PHB granules is important for enzyme action, but we do not know the exact pH of or near the surface of the inclusion bodies.

Our results confirm that the intracellular PHB depolymerase

degrades only amorphous PHB as first suggested by Merrick and Doudoroff (16) (Fig. 3). It is surprising that the enzyme cannot hydrolyze freeze-dried amorphous PHB, which was only slightly crystallized as judged by WAXS. It is possible that the crystallization occurred at the surface of PHB granules in such preparations. The water-soluble products of the enzymatic reaction seemed to be mainly 3HB oligomers. This means that *R. eutropha* probably has an intracellular 3HB oligomer hydrolase or another type of intracellular PHB depolymerase for intracellular PHB metabolism. As described above, PHB depolymerase activity has been found in the supernatant of *R. eutropha* cells (21). The product of the cloned gene was found only in the PHB granule fraction of *R. eutropha* (Fig. 4) by immunoblot analysis. It is possible that the concentration of PHB depolymerase in the soluble fraction was not as high as the concentration that binds to PHB granules, and the enzymes in the supernatant and PHB granules are derived from the same protein molecule. However, *phaZ* null mutant D1 still has intracellular PHB depolymerase activity in the supernatant fraction of cell extracts. Therefore, we concluded that there are at least two intracellular PHB depolymerases in *R. eutropha* and that PhaZ localizes to PHB granules. Charac-

FIG. 4. Detection of PhaZ in *R. eutropha* H16. SDS-polyacrylamide gel electrophoresis of whole cells and PHB granules from *R. eutropha* was carried out. The gels were stained with Coomassie brilliant blue R-250 (A) or immunostained (Western blot) (B). Lanes: a-0, whole cells grown for 2 days in nitrogen-rich medium (91 μg of protein); a-1, whole cells after 1 day of PHB synthesis (86 μg of protein, 21 μg of PHB); a-3, whole cells after 3 days of PHB synthesis (88 μg of protein, 73 μg of PHB); b-1, PHB granules after 1 day of PHB synthesis (19 μg of protein, 160 μg of PHB); b-3, PHB granules after 3 days of PHB synthesis (20 μg of protein, 200 μg of PHB); c-1, supernatant fraction of cells after 1 day of PHB synthesis (91 μg of protein); c-3, supernatant fraction of cells after 3 days of PHB synthesis (57 μg of protein); Ni, purified His-tagged depolymerase (0.1 μg of protein); M, molecular mass markers. Sizes are indicated in kilodaltons.

FIG. 5. Growth and accumulation of PHB in various *R. eutropha* derivative strains and *phaZ* null mutant D1. (A) Absorbance at 600 nm in nitrogen-rich medium; (B) accumulation of PHB in nitrogen-rich medium; (C) accumulation of PHB in nitrogen-free medium with 2% (wt/vol) fructose. Cells grown on nutrient-rich medium were transferred at time zero to nitrogen-free medium containing 2% (wt/vol) fructose. ○, *R. eutropha*; ●, D1; △, *R. eutropha* harboring pJDR215T; □, *R. eutropha* harboring pJDR171T; ▲, D1 harboring pJDR215T; ■, D1 harboring pJDR171T. Results are the means for three independent measurements.

terization of the depolymerase in the supernatant fraction of cell extracts in *R. eutropha* should be performed.

The *phaZ* null mutant had a higher PHB content than the wild type in nutrient-rich conditions after 40 to 80 h of cultivation (Fig. 5). It is interesting that PhaZ seems to function well only in *R. eutropha* cells grown in a nutrient-rich medium (Fig. 5B). The *phaZ* null mutant accumulated an amount of PHB similar to that of the wild type in PHB accumulation conditions (Fig. 5C). These results indicate that the cloned PhaZ is probably inhibited during active synthesis of PHB without growth. Although PHB metabolism in *R. eutropha* has been reported to be cyclic in nature (4), it seems uneconomical for bacteria to synthesize and degrade PHB in granules at the same time. Some unknown regulation of PHB degradation in the granules may occur. Some factor like the heat-stable activator found by Merrick and Doudoroff (16) in *R. rubrum* may also work in the PHB depolymerizing system in *R. eutropha*. However, it is also possible that some physical factors, for example, morphological changes of PHB granules during PHB synthesis, are involved in the regulation of PHB degradation.

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