Identification and Characterization of the *Bacillus thuringiensis* phaZ Gene, Encoding New Intracellular Poly-3-Hydroxybutyrate Depolymerase

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A gene that codes for a novel intracellular poly-3-hydroxybutyrate (PHB) depolymerase has now been identified in the genome of *Bacillus thuringiensis* subsp. *israelensis* ATCC 35646. This gene, previously annotated as a hypothetical 3-oxoadipate enol-lactonase (PcaD) gene and now designated *phaZ*, encodes a protein that shows no significant similarity with any known PHB depolymerase. Purified His-tagged PhaZ could efficiently degrade trypsin-activated native PHB granules as well as artificial amorphous PHB granules and release 3-hydroxybutyrate monomer as a hydrolytic product, but it could not hydrolyze denatured semicrystalline PHB.

In contrast, purified His-tagged PcaD of *Pseudomonas putida* was unable to degrade trypsin-activated native PHB granules and artificial amorphous PHB granules. The *B. thuringiensis* PhaZ was inactive against p-nitrophenylpalmitate, tributyrin, and triolein. Sonication supernatants of the wild-type *B. thuringiensis* cells exhibited a PHB-hydrolyzing activity in vitro, whereas those prepared from a *phaZ* mutant lost this activity. The *phaZ* mutant showed a higher PHB content than the wild type at late stationary phase of growth in a nutrient-rich medium, indicating that this PhaZ can function as a PHB depolymerase in vivo. PhaZ contains a lipase box-like sequence (G-W-S<sub>10</sub>-M-G) but lacks a signal peptide. A purified His-tagged S102A variant had lost the PHB-hydrolyzing activity. Taken together, these results indicate that *B. thuringiensis* harbors a new type of intracellular PHB depolymerase.

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Poly-3-hydroxybutyrate (PHB) is a storage material produced by a variety of bacteria in response to nutritional stress (17, 32). Intracellular PHB, which accumulates along with some PHB binding proteins in bacteria as native PHB (nPHB) granules, is in an amorphous state. When PHB-producing cells die, PHB is released into the environment, where it is transformed into a denatured semicrystalline state. While the extracellular degradation of denatured semicrystalline PHB (dPHB) has been clarified in many bacteria (16, 17, 37), not much is known about intracellular mobilization of PHB. So far, only a few novel intracellular PHB depolymerase (PhaZ) genes have been identified and characterized (1, 33). *phaZa1* (formerly *phaZ1*) of *Ralstonia eutropha* H16, which was the first cloned intracellular PHB depolymerase gene (33), encodes a protein with no classical lipase box (G-X-S-X-G) (15). PhaZa1 was found to exist only as a PHB granule-bound form in cells, and its main hydrolytic products in enzymatic degradation of amorphous PHB (aPHB) are 3-hydroxybutyrate (3HB) oligomers. PhaZa1 exhibits high similarity with a great number of proteins in databases, some of which were later demonstrated to be intracellular PHB depolymerases (8, 18, 19). These PhaZa1 homologs also lack the typical lipase box motif. Recently, a novel intracellular PHB depolymerase gene (*phaZd*) was cloned from *Ralstonia eutropha* H16 (1), *phaZd* encodes a protein which shows similarity with the type I catalytic domain of extracellular PHB depolymerases from bacteria such as *Ralstonia pickettii* T1 and *Pseudomonas lemoignei* (17). Its hydrolytic products in enzymatic degradation of amorphous PHB are various 3HB oligomers. 3HB monomer was rarely detected as a hydrolytic product.

Although genes involved in biosynthesis of PHB granules from *Bacillus megaterium* have been cloned and characterized (22, 24, 25), little is known about genes involved in mobilization of PHB in *B. megaterium* or any other PHB producer belonging to the genus *Bacillus*. *B. thuringiensis* is known to a PHB producer (4, 13, 38). Its genome sequence is available now (GenBank accession no. NC_005957 for the genome sequence of the human-pathogenic isolate *B. thuringiensis* serovar konkukian strain 97-27; GenBank accession no. NZ_AAJM01000001-NZ_AAJM0100866 for the incomplete genome sequence of *Bacillus thuringiensis* subsp. *israelensis* ATCC 35646). However, BLAST searches revealed that no open reading frame in the genome of *B. thuringiensis* codes for a protein that shows significant similarity with any known intracellular or extracellular PHB depolymerase. The same is the case with genomes of other sequenced *Bacillus* species. In this study, we report the identification of an intracellular PHB depolymerase gene of *B. thuringiensis* subsp. *israelensis* ATCC 35646, whose counterpart is also present in the genome of *B. thuringiensis* serovar konkukian strain 97-27. This PhaZ was previously annotated as a hypothetical 3-oxoadipate enol-lactonase (PcaD) gene and has no significant similarity with any known intracellular or extracellular PHB depolymerase (30). It appears to be a new type of intracellular PHB depolymerase.

**MATERIALS AND METHODS**

**Bacterial strains and growth conditions.** The bacterial strains and plasmids used in this study are listed in Table 1. *Escherichia coli*, *B. megaterium*, and *Bacillus thuringiensis* cells were grown in Luria-Bertani (LB) medium (34). An-
A two-step PCR method (14). All DNA sequences were confirmed by DNA-directed mutagenesis that was used to introduce S102A mutation was carried out by HindIII and BamHI sites of the thermosensitive replicative plasmid pRN5101 (7).

HindIII and BamHI sites was amplified by PCR and cloned between HindIII and pLC4 carrying the promoter region and N-terminal coding region of phaZ

To construct plasmid pGS1243 that overproduces His-tagged D-3-hydroxybutyrate dehydrogenase and 3-oxoadipate enol-lactonase, E. coli

B. thuringiensis

Plasmids

pLC4

Promoter probe vector with xylE as the reporter gene; Ap’, Cm’

pQE30

Expression vector for producing His-tagged proteins; Ap’, Em’

pRN5101

Thermosensitive replicative plasmid used for gene disruption; Ap’, Em’

pGS1185

pQEs0 carrying the wild-type phaZ gene

pGS1209

pQEs0 carrying the mutated phaZ gene (S102A substitution)

pGS1243

pQEs0 carrying the ω-3-hydroxybutyrate dehydrogenase gene

pGS1266

pLC4 carrying the promoter region and N-terminal coding region of phaZ

pGS1289

pRN5101 derivative; phaZ disruptive plasmid

pGS1544

pQEs0 carrying the 3-oxoacyclic enol-lactonase gene of P. putida KT2440

**TABLE 1. Bacterial strains and plasmids**

<table>
<thead>
<tr>
<th>Strain or plasmid</th>
<th>Description*</th>
<th>Reference or source†</th>
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<td><strong>Strains</strong></td>
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<tr>
<td>E. coli JM109</td>
<td>recA1 supE44 endA1 hsdR17 gyrA96 relA1 thi-1 (lac-proAB) [F’ traD36 proAB- lacIq lacZM15]</td>
<td>Takara</td>
</tr>
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<td>B. thuringiensis subsp. israelensis ATCC 35646 BM863</td>
<td>Wild-type strain ATCC 35646 derivative; phaZ:pGS1289</td>
<td>ATCC</td>
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<tr>
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<td>This study</td>
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<tr>
<td><strong>Plasmids</strong></td>
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<td>pLC4</td>
<td>Promoter probe vector with xylE as the reporter gene; Ap’, Cm’</td>
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<td>pQE30</td>
<td>Expression vector for producing His-tagged proteins; Ap’, Em’</td>
<td>QIAGEN</td>
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<td>pRN5101</td>
<td>Thermosensitive replicative plasmid used for gene disruption; Ap’, Em’</td>
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<td>pQEs0 carrying the wild-type phaZ gene</td>
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<td>pGS1544</td>
<td>pQEs0 carrying the 3-oxoacyclic enol-lactonase gene of P. putida KT2440</td>
<td>This study</td>
</tr>
</tbody>
</table>

* Ap’, ampicillin resistant; Cm’, chloramphenicol resistant; Em’, erythromycin resistant; Tc’, tetracycline resistant.

† ATCC, American Type Culture Collection.

tibiotics were used at the following concentrations (μg/ml): ampicillin, 100 (for E. coli); chloramphenicol, 8; erythromycin, 2 (for B. thuringiensis).

**Construction of plasmids.** To construct the phaZ disruptive plasmid pGS1208, an 0.3-kb DNA fragment containing the internal region of phaZ and flanked by HindIII and BamHI sites was amplified by PCR and cloned between HindIII and BamHI sites of the thermosensitive replicative plasmid PRN5101 (7).

To construct a plasmid that overproduces His-tagged PhaZ or its variant S102A, an 0.93-kb DNA fragment carrying the wild-type phaZ gene or the mutated phaZ gene and flanked by BamHI and HindIII sites was amplified by PCR and cloned between the BamHI and HindIII sites of pQE30 (QIAGEN Inc.). This resulted in plasmids pGS1185 and pGS1209, respectively. Site-directed mutagenesis that was used to introduce S102A mutation was carried out by two-step PCR method (14). All DNA sequences were confirmed by DNA sequencing.

To construct plasmid pGS1243 that overproduces His-tagged ω-3-hydroxybutyrate dehydrogenase of B. thuringiensis, an 0.95-kb DNA fragment containing this gene and flanked by BamHI and HindIII sites was amplified by PCR and cloned between the BamHI and HindIII sites of pQE30.

To construct plasmid pGS1266, an 0.2-kb DNA fragment that contains the promoter region and N-terminal coding region of phaZ and is flanked by EcoRI and HindIII sites was amplified by PCR and cloned between the EcoRI and HindIII sites of pLC4 (31).

To construct plasmid pGS1544 that overproduces the His-tagged 3-oxoacyclic enol-lactonase gene of Pseudomonas putida KT2440, an 0.83-kb DNA fragment containing this gene and flanked by BamHI and HindIII sites was amplified by PCR and cloned between the BamHI and HindIII sites of pGS1266.

**Disruption of the chromosomal phaZ gene.** Disruption of the chromosomal phaZ gene by integration of plasmid pRN5101-derived pGS1208 through a Campbell-like single-crossover recombination was performed as previously described (7). Plasmid pGS1208 was introduced into B. thuringiensis cells by electroporation. Transformants were first grown at the permissive temperature 30°C and then transferred to the nonpermissive temperature 37°C. Finally, integrants were selected on LB agar plates at 39°C for resistance to erythromycin. The correctness of integrants was verified by both PCR and Southern blot analysis.

**Overproduction and purification of His-tagged proteins.** For overproduction and purification of His-tagged PhaZ and its variant S102A as well as His-tagged ω-3HB dehydrogenase and 3-oxoacyclic enol-lactonase, E. coli JM109 cells bearing plasmid pGS1185, pGS1209, pGS1243, or pGS1544 were grown in LB medium to an absorbance at 600 nm of 0.5 and were then treated with 0.5 mM IPTG (isopropyl-β-D-thiogalactopyranoside) for 2 h. After cells were collected by centrifugation and disrupted by sonication, the disrupted cells were centrifuged at 15,000 x g for 10 min. Purification of His-tagged proteins from the supernatants by affinity chromatography on a Ni-nitrioltriacetic acid (NTA) agarose column was performed according to the instructions of the matrix manufacturer (QIAGEN Inc.).

**Preparation of native PHB granules, artificial amorphous PHB granules, and denatured semicrystalline PHB.** PHB granules were isolated from French press-disrupted B. megaterium or B. thuringiensis cells by sucrose density gradient centrifugation as previously described (26). dPHB was isolated from B. megaterium or B. thuringiensis cells by a procedure involving sodium hypochlorite digestion and subsequent solvent extraction with acetone-ether (2:1, vol/vol) as previously described (6). Artificial aPHB granules were prepared from dPHB of B. megaterium according to a previous report (1) by using sodium deoxycholate as surfactant.

**Turbidimetric determination of PHB depolymerase activity.** The decrease in turbidity of a suspension made from nPHB granules, aPHB granules, or dPHB due to hydrolysis by PHB depolymerase was monitored as previously described (11) in order to determine PHB depolymerase activity. The turbidity of reaction mixtures was assayed spectrophotometrically at 650 nm by using a microplate reader, SpectraMax 190 (Molecular Devices Corp.). Each reaction mixture (200 μl) contained 100 mM Tris-HCl (pH 8.0); 0.7 mg of nPHB granules, aPHB granules, or dPHB; and 3 μg of purified protein or 200 μg of cell-free sonication supernatant. The term “trypsin-activated nPHB granules” designates nPHB granules that were preincubated with 4 μg of trypsin at 37°C for 10 min prior to the addition of a purified enzyme. One unit of activity is defined as the decrease of one unit of optical density at 650 nm in 1 min.

**Assay for hydrolysis of ω-nitrophenyl esters by PHB depolymerase and lipase activity.** The release of yellow ω-nitrophenol due to hydrolysis of ω-nitrophenyl esters by PHB depolymerase was measured spectrophotometrically at 405 nm. A 200-μl reaction mixture that contains 0.25 mM ω-nitrophenyl ester (dissolved in ethanol), 50 mM Tris-HCl (pH 8.0), and 1 mM ω-nitrophenol was incubated at 37°C. Since autohydrolysis of substrates produced low but significant background values at 405 nm, the absorbance in each assay was measured against a substrate-buffer mixture. A series of known amounts of ω-nitrophenol were used to construct a standard curve. The apparent molar absorptivity (ε) for ω-nitrophenol at 405 nm is 1.66 x 10^4 M^-1 cm^-1. The lipase activity was measured at 37°C with a pH meter in a 20-ml emulsified solution (pH 8.0) containing 250 μl of tributyrin or triolein, 4 mM CaCl2, and 10 mM NaCl. One hundred fifty micrograms of purified PHB depolymerase or 50 μg of lipase from Chromobacterium viscosum (Sigma) was added to the emulsified solution.

**Analysis of PHB content of B. thuringiensis cells or nPHB granules by gas chromatography.** Analysis of PHB content of B. thuringiensis cells or nPHB granules by gas chromatography. Analysis of PHB content of B. thuringiensis cells or nPHB granules by gas chromatography was performed as previously described (3). Briefly, approximately 4 mg of lyophilized B. thuringiensis cells or nPHB granules...
was reacted in a screw-cap glass tube with a solution containing 1 ml of chloroform, 0.85 ml of methanol, and 0.15 ml of sulfuric acid at 100°C for 140 min to convert PHB to 3-hydroxybutyrate methyl ester. After extraction with 0.5 ml of distilled water, the methyl ester was assayed by an Agilent 6890N gas chromatograph equipped with a capillary column, HP-5MS (30 m by 0.25 mm).

**Enzymatic quantitation of D-3-hydroxybutyrate.** The amount of 3HB monomer released from hydrolysis of nPHB granules by PHB depolymerase was quantified by the enzymatic method using NAD⁺-dependent 3HB dehydrogenase as previously described (39). The reaction mixture (200 μl) contained 15 mM Tris-HCl (pH 8.5), 5 mM NAD⁺, 0.3 M hydrazine hydrate (pH 8.5), 30 μg of 3HB dehydrogenase, and the sample containing the substrate 3HB or a series of 3HB standards solutions. The reaction was started by the addition of 3HB dehydrogenase at 25°C. Readings were taken spectrophotometrically at 5-min intervals until the absorbance at 340 nm was constant. A standard curve made from readings of a series of 3HB standard solutions was used to estimate the amount of 3HB monomer in the sample.

**RNA extraction and primer extension analysis.** Total RNA was extracted from *B. thuringiensis* cells carrying plasmid pGS1266 and grown to an absorbance at 600 nm of 0.5 as previously described (5), and the phaZ transcriptional start site was determined by the previously described method of primer extension (5) using synthetic oligonucleotides 5'-CGTTTCTCCGTTCGATAGTG-3'.

**Protein sequence analyses.** The BLAST network server of the National Center for Biotechnology Information was used for database searches. Overall amino acid identities and similarities between proteins were calculated with the Clustal W program (36). Signal peptide sequence was analyzed by the SignalP 3.0 server (29).

**Other methods.** Transformation of *B. thuringiensis* cells by electroporation was carried out as previously described (2). Southern blot analysis was performed using standard methods (34).

**RESULTS**

**Identification of a putative PHB depolymerase by a BLAST search using PhaC as a probe.** The *B. megaterium* phaC gene, which encodes a subunit of class IV PHB synthase, was previously cloned along with other PHB metabolism-related genes, including phaB, phaP, phaQ, and phaR (24). An open reading frame that encodes the *B. thuringiensis* homolog of the *B. megaterium* PhaC is present in genomes of both *B. thuringiensis* subsp. *israelensis* ATCC 35646 (GenBank accession number ZP_00742249) and *B. thuringiensis* serovar konkukian strain 97-27 (YP_035543). *B. thuringiensis* subsp. *israelensis* ATCC 35646 was used throughout this study. The overall amino acid sequence identity and similarity between the *B. megaterium* PhaC and the *B. thuringiensis* PhaC are 70.2% and 84.8%, respectively. The open reading frames that code for the *B. thuringiensis* counterparts of the *B. megaterium* PhaB, PhaP, PhaQ, and PhaR are also present in the vicinity of the *B. thuringiensis* phaC gene. Blast searches revealed that no open reading frame in the genome of *B. thuringiensis* codes for a protein showing significant similarity with any currently known intracellular or extracellular PHB depolymerase, such as the intracellular PHB depolymerase PhaZa1 (33, 40), HB oligomer hydrolyase PhaZb (20), HB oligomer hydrolyase PhaZc (21), and PHB depolymerase PhaZd (1) of *Ralstonia eutropha* H16, a periplasm-located PHB depolymerase of *Rhodospirillum rubrum* (10), and the amorphous PHB-specific extracellular PHB depolymerase PhaZ of *Paucimonas lemoignei* (9). Assuming that the intracellular PHB synthase of *B. thuringiensis* might possibly have a low level of similarity with its intracellular PHB depolymerase, we used the *B. thuringiensis* PHB synthase (PhaC) as a probe to search the genome of *B. thuringiensis*. This BLAST search yielded some matches including a protein of 300 amino acids (see below) that was previously annotated as a putative 3-oxoadipate enol-lactonase (now identified as an intracellular PHB depolymerase in this study and renamed PhaZ; see below). This putative phaZ gene is also present in the genome of *B. thuringiensis* serovar konkukian strain 97-27. The overall amino acid sequence identity and similarity between the putative PhaZ and PhaC of *B. thuringiensis* are 18.7% and 35.6%, respectively. This putative PhaZ contains a pentapeptide sequence (G-W-S102-M-G) that is similar to the lipase box motif (G-X-S-X-G) (15).

**Overproduction and purification of His-tagged PhaZ.** In an attempt to investigate whether this putative PhaZ had PHB depolymerase activity, we first constructed plasmid pGS1185, which could overproduce His-tagged PhaZ in *E. coli*. This His-tagged PhaZ was then purified in a single step by affinity chromatography on a Ni-NTA agarose column. The purified His-tagged PhaZ migrated as a single protein band with an apparent molecular mass of approximately 33 kDa on a sodium dodecyl sulfate (SDS)-13% PAGE and stained with Coomassie blue. Molecular mass standards. (A) Lanes: 1 and 2, the control plasmid pQE30 without (lane 1) or with (lane 2) the addition of IPTG; 3 and 4, the phaZ-expressing plasmid pGS1185 without (lane 3) or with (lane 4) the addition of IPTG; 5, purified His-tagged PhaZ; 6 and 7, the pcaD-expressing plasmid pGS1544 without (lane 6) or with (lane 7) the addition of IPTG; 8, purified His-tagged PcaD. (B) Lanes: 1 and 2, the S102A variant-producing plasmid pGS1209 without (lane 1) or with (lane 2) the addition of IPTG; 3, purified His-tagged S102A variant.

**PHB depolymerase activity and other enzymatic activities of the *B. thuringiensis* PhaZ.** To determine whether this putative PhaZ could degrade nPHB granules or dPHB, we prepared these substrates from *B. megaterium* and *B. thuringiensis* cells as described in Materials and Methods. A turbidimetric method that monitored the decrease in turbidity of a suspension made from nPHB granules or dPHB was used to examine
degradation of PHB by PhaZ. It was previously reported that in vitro degradation of nPHB granules by a PHB depolymerase of *Rhodospirillum rubrum* requires pretreatment of granules with trypsin or a heat-stable activator (11, 27). Therefore, we also tested the effect of trypsin on in vitro degradation of nPHB granules by this putative PhaZ. Figure 2A shows enzymatic hydrolysis of nPHB granules isolated from *B. megaterium* cells. Treatment of nPHB granules with trypsin alone did not lead to PHB degradation under the assay condition. Besides, nPHB granules without activation by trypsin could not be efficiently degraded by PhaZ. Only trypsin-activated nPHB granules could be rapidly degraded by PhaZ (Fig. 2A). The specific PHB-degrading activity of purified His-tagged PhaZ against trypsin-activated nPHB granules is approximately 83.3 units/mg of protein. This PhaZ could also efficiently degrade trypsin-activated nPHB granules prepared from *B. thuringiensis* cells (Fig. 2B). However, it could not hydrolyze dPHB prepared from *B. megaterium* or *B. thuringiensis* cells (Fig. 2B).

Analysis of the hydrolytic products of nPHB granules by using NADH-dependent 3HB dehydrogenase as described in Materials and Methods revealed that 3HB monomer had been released. The amount of released 3HB monomer corresponded to approximately 42% and 34% of total 3HB equivalents present in the nPHB granules of *B. megaterium* and *B. thuringiensis*, respectively. We also prepared artificial amorphous PHB granules from dPHB of *B. megaterium* according to a previous report (1) by using sodium deoxycholate as surfactant. As shown in Fig. 2D, the *B. thuringiensis* PhaZ could also efficiently degrade aPHB granules. The specific PHB-degrading activity of purified His-tagged PhaZ against aPHB is approximately 86.5 units/mg of protein.

The hydrolyzing activities of this PhaZ for *p*-nitrophenyl esters and triglycerides were also examined. As shown in Table 2, PhaZ was relatively more active towards *p*-nitrophenylbutyrate but was inactive against *p*-nitrophenylpalmitate. This property is similar to that exhibited by the PhaZa1 of *R. eutropha* H16 (1). The *B. thuringiensis* PhaZ was also inactive against tributyrin and triolein, which are typical substrates for esterase and lipase, respectively (Table 2). In contrast, the lipase from *Chromobacterium viscosum* (Sigma) as a positive control could degrade these two substrates (data not shown).

**PHB-hydrolyzing ability of PhaZ variant S102A of *B. thuringiensis* and PcaD of *Pseudomonas putida*.** In most lipases and other serine hydrolases, the active site serine is within the highly conserved lipase box (15). Since the *B. thuringiensis* PhaZ contains a lipase box-like sequence (G-W-S102-M-G), we next investigated whether S102 would be important for the PHB-hydrolyzing activity of PhaZ. Construction of the S102A variant by site-directed mutagenesis as well as overproduction and purification of His-tagged S102A variant was carried out as described in Materials and Methods. The mutant protein showed the same binding property as the wild-type PhaZ during degradation.

![FIG. 2. Turbidimetric determination of PHB depolymerase activity. OD₆₅₀, optical density at 650 nm. Purified His-tagged proteins were used in assays. (A) nPHB granules were isolated from *B. megaterium* cells. Solid circles, trypsin-activated nPHB granules plus PhaZ; open circles, nPHB granules plus PhaZ; triangles, trypsin-activated nPHB granules alone. (B) Solid circles, trypsin-activated nPHB granules of *B. thuringiensis* plus PhaZ; open circles, dPHB of *B. megaterium* plus PhaZ; triangles, dPHB of *B. thuringiensis* plus PhaZ. (C) nPHB granules were isolated from *B. megaterium* cells. Solid circles, trypsin-activated nPHB granules plus PhaZ as a positive control; open circles, trypsin-activated nPHB granules plus S102A variant; triangles, trypsin-activated nPHB granules plus PcaD. (D) Solid circles, aPHB granules plus PhaZ; open circles, aPHB granules plus PcaD.](http://jb.asm.org/)

**TABLE 2. Substrate specificity of *B. thuringiensis* PhaZ**

<table>
<thead>
<tr>
<th>Substrate</th>
<th>Activity (μmol/min/mg)</th>
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<tr>
<td><em>p</em>-Nitrophenylbutyrate (C₄)</td>
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<tr>
<td><em>p</em>-Nitrophenyldecanoate (C₁₀)</td>
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<tr>
<td><em>p</em>-Nitrophenyllaurate (C₁₃)</td>
<td>0.027</td>
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<tr>
<td><em>p</em>-Nitrophenylpalmitate (C₁₆)</td>
<td>0.0</td>
</tr>
<tr>
<td>Tributyrin</td>
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<tr>
<td>Triolein</td>
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ing the purification procedure. Degrees of expression and relative mobilities of wild-type and mutant proteins on SDS-polyacrylamide gels were also unchanged (Fig. 1). However, when the PHB-hydrolyzing activities were measured, large differences were found. Turbidimetric analysis showed that S102A variants were inactive toward trypsin-activated nPHB granules (Fig. 2C), suggesting that S102 is important for the PHB-hydrolyzing activity of PhaZ.

The lack of commercially available 3-oxoadipate enol-lactone as substrate and the difficulty in synthesizing this substrate did not allow us to analyze whether the B. thuringiensis PhaZ could display 3-oxoadipate enol-lactonase activity. Instead, we attempted to investigate whether the authentic 3-oxoadipate enol-lactonase of Pseudomonas putida KT2440 (23, 28) could exhibit PHB-hydrolyzing activity. Overproduction and purification of His-tagged PcaD were carried out as described in Materials and Methods, and SDS-polyacrylamide gel electrophoresis (PAGE) analysis of purified His-tagged PcaD is shown in Fig. 1A. Turbidimetric analysis showed that PcaD was inactive towards both trypsin-activated nPHB granules (Fig. 2C) and aPHB granules (Fig. 2D) even after prolonged incubation (up to 90 min) (data not shown). However, due to the lack of a suitable substrate as a positive control for PcaD activity, we are not sure that the water-soluble His-tagged PcaD purified with the Ni-NTA agarose column is indeed in an active state.

Effect of phaZ disruption on PHB-hydrolyzing activity and PHB content. To assess the effect of disruption of the B. thuringiensis phaZ gene on PHB-hydrolyzing activity, we constructed a phaZ disruptive mutant by using a thermosensitive replicative plasmid as described in Materials and Methods. The results of Southern blot analysis of phaZ disruption are shown in Fig. 3A. Sonication supernatants were prepared from the wild-type B. thuringiensis cells and the phaZ mutant BM863. nPHB granules were isolated from B. megaterium cells. Turbidimetric analysis showed that sonication supernatant from the wild type displayed PHB depolymerase activity, whereas sonication supernatant from the phaZ mutant lost this activity (Fig. 3B).

We then investigated the effect of phaZ disruption on PHB accumulation in vivo. As shown in Fig. 3C, PHB contents of the wild-type B. thuringiensis and the phaZ mutant grown in LB medium were not significantly different after 12-, 24-, and 48-h cultivations. However, PHB contents of the wild type were considerably lower than those of the phaZ mutant after 72- and 96-h cultivations. This result indicates that the B. thuringiensis PhaZ can function as a PHB depolymerase in vivo. Similar results were also observed for R. eutropha H16 (33). It was reported that a higher PHB content in the phaZ (now renamed phaZa1) mutant of R. eutropha than in the wild type was observed only in a nutrient-rich medium after 40 to 80 h of cultivation. PhaZa1 seemed to function as a PHB depolymerase only in R. eutropha cells grown in a nutrient-rich medium (33).

We also tested if there was PHB depolymerase activity in the cell-free culture supernatant of the wild-type B. thuringiensis cells. The result revealed that no significant PHB depolymerase activity was detected in the culture supernatant even after 15-fold concentration of the culture supernatants (data not shown). In addition, analysis of the B. thuringiensis PhaZ by the SignalP 3.0 server failed to detect any signal peptide sequence (29). Taken together, these results suggest that the B. thuringiensis phaZ gene encodes an intracellular PHB depolymerase.

Identification of the transcriptional initiation site of phaZ. The phaZ gene of B. thuringiensis serovar konkukian strain 97-27 was predicted to encode a protein of 300 amino acids (GenBank accession number YP_037407). However, the phaZ gene of B. thuringiensis subsp. israelensis ATCC 35646

![FIG. 3. Effects of phaZ disruption on PHB-hydrolyzing activity and PHB content. (A) Southern blot analysis of phaZ disruption. HindIII-digested genomic DNAs isolated from the wild-type B. thuringiensis (lane 1) and the phaZ mutant BM863 (lane 2) were run on a 1% agarose gel. A 32P-labeled internal fragment of phaZ was used as the probe. The probe hybridized to a 2.8-kb DNA fragment from the wild type and to 2.1- and 8.8-kb DNA fragments from the phaZ mutant. (B) In vitro hydrolysis of nPHB granules by sonication supernatants of wild-type B. thuringiensis cells and the phaZ mutant. nPHB granules were isolated from B. megaterium cells. Sonication supernatants were prepared from wild-type B. thuringiensis cells and the phaZ mutant BM863 that were grown to an absorbance at 600 nm of 1.4. Equal amounts of total proteins in sonication supernatants of wild-type B. thuringiensis cells and the phaZ mutant were used in assays. Circles, sonication supernatants from the wild type plus trypsin-activated nPHB granules (solid) or plus nPHB granules (open); triangles, sonication supernatants from the phaZ mutant plus trypsin-activated nPHB granules (solid) or plus nPHB granules (open). (C) PHB contents and growth curves of the wild-type B. thuringiensis and the phaZ mutant that were grown in LB medium for various periods of time. Solid symbols, PHB contents of the wild type (circles) and the phaZ mutant (triangles). Each value represents the mean of at least three determinations. Each error bar indicates the standard error of the mean. Open symbols, growth curves of the wild type (open circles) and the phaZ mutant (open triangles).]
PHB synthesis and degradation help bacteria to survive in times of stress. PHB-producing bacteria should contain intracellular PHB depolymerase(s) for catalyzing the first step of intracellular PHB degradation pathway. In this report, we have identified an intracellular PHB depolymerase of *B. thuringiensis*, which can function at the late stationary phase of growth in a nutrient-rich medium. It is not unprecedented that bacteria when grown in a nutrient-rich medium can make and degrade PHB (33). Multiple factors are probably involved in regulation of PHB metabolism in *Bacillus* species. The *B. megaterium* PhaC, one of the two subunits of the *B. megaterium* PHB synthase, is considered to be produced constitutively. It has been shown that two forms of PHB synthase exist in *B. megaterium* cells: an active form in PHB-accumulating cells and an inactive form in nonaccumulating cells (25). It is still unknown whether this is the case with the PHB synthase of *B. thuringiensis*. The *B. thuringiensis* PhaZ can hydrolyze artificial amorphous PHB granules in vitro without the need for trypsin pretreatment. However, when nPHB granules are used as substrate in vitro, protein removal from the surface layer by trypsin digestion is required in order for the *B. thuringiensis* PhaZ to gain access to PHB. Further studies of identification and characterization of a trypsin-like “activator” for mobilization of PHB by the *B. thuringiensis* PhaZ in vivo are under way.

The *B. thuringiensis* PhaZ bears no significant similarity with any known intracellular or extracellular PHB depolymerase (Table 3). It is inactive against *p*-nitrophenylpalmitate, dPHB, tributyrin, and triolein. The esterase activity of the *B. thuringiensis* PhaZ toward *p*-nitrophenylbutyrate is comparable to that shown by PhaZd of *R. eutropha* but is much weaker than that shown by PhaZd of *R. eutropha* (1). Since neither esterase activity toward tributyrin nor lipase activity toward triolein was

**DISCUSSION**

*Table 3. Percentages of identities and similarities between the *B. thuringiensis* PhaZ and other bacterial proteins**

<table>
<thead>
<tr>
<th>Bacterial protein</th>
<th>Protein length (amino acids)</th>
<th>% Identity/similarity</th>
</tr>
</thead>
<tbody>
<tr>
<td><em>B. thuringiensis</em> PhaZ</td>
<td>300</td>
<td></td>
</tr>
<tr>
<td><em>B. thuringiensis</em> PhaC</td>
<td>361</td>
<td>18.7/35.6</td>
</tr>
<tr>
<td><em>P. putida</em> PcaD</td>
<td>263</td>
<td>20.6/31.0</td>
</tr>
<tr>
<td><em>R. eutropha</em> PhaZa1</td>
<td>419</td>
<td>15.0/24.1</td>
</tr>
<tr>
<td><em>R. eutropha</em> PhaZb</td>
<td>718</td>
<td>3.4/7.0</td>
</tr>
<tr>
<td><em>R. eutropha</em> PhaZc</td>
<td>293</td>
<td>22.3/35.3</td>
</tr>
<tr>
<td><em>R. eutropha</em> PhaZd</td>
<td>362</td>
<td>11.5/21.8</td>
</tr>
<tr>
<td><em>R. rubrum</em> PhaZ</td>
<td>362</td>
<td>4.6/7.0</td>
</tr>
<tr>
<td><em>P. lemoignei</em> PhaZ7</td>
<td>380</td>
<td>4.2/8.3</td>
</tr>
<tr>
<td>Hypothetical protein of <em>Bacillus anthracis</em></td>
<td>300</td>
<td>95.0/98.0</td>
</tr>
<tr>
<td>Hypothetical protein of <em>Bacillus cereus</em></td>
<td>301</td>
<td>98.7/99.3</td>
</tr>
</tbody>
</table>

* Hypothetical protein of *B. anthracis* strain Ames (GenBank accession number AAP27115); hypothetical protein of *B. cereus* ATCC 14579 (AAP10252).
detected for the <i>B. thuringiensis</i> PhaZ, it seems that the <i>B. thuringiensis</i> PhaZ is a specific enzyme for PHB mobilization.

In contrast to other known intracellular PHB depolymerases, the <i>B. thuringiensis</i> PhaZ generates much more 3HB monomers as hydrolytic product. This PhaZ appears to be a new type of intracellular PHB depolymerase. As far as is known, the intracellular PHB depolymerase PhaZa1 of <i>B. thuringiensis</i> is a specific enzyme for PHB mobilization. The intracellular PhaZd of <i>B. thuringiensis</i> detected for the intracellular 3-hydroxybutyrate-oligomer hydrolase in <i>Ralstonia eutropha</i> H16 shows similarity with the type I catalytic domain of extracellular PHB depolymerases from bacteria such as <i>R. pickettii</i> T1 and <i>Pseudomonas lumeoni</i> (17) and produces various 3HB oligomers from amorphous PHB as hydrolytic products. 3HB monomer was rarely detected as a hydrolytic product (1). The periplasm-located PHB depolymerase of <i>R. rubrum</i> (10) shows similarity with the type II catalytic domain of extracellular PHB depolymerases from bacteria such as <i>Acidovorax</i> sp. strain TP4 (17). The amount of 3HB monomer released from hydrolysis of nPHB granules by the amorphous PHB-specific extracellular PHB depolymerase PhaZ7 of <i>Pseudomonas lumeoni</i> corresponds to only 0.5 to 2.5% of total 3HB equivalents present in the nPHB granules (9). In this report we found that the <i>B. thuringiensis</i> PhaZ has a strong amorphous PHB-hydrolyzing activity and the amount of 3HB monomer released from hydrolysis of nPHB granules corresponds to approximately 42% and 34% of total 3HB equivalents present in the nPHB granules of <i>B. megaterium</i> and <i>B. thuringiensis</i>, respectively.

3-Carboxy-cis,cis-muconate cycloisomerase (PcaB), 4-carboxymuconolactone decarboxylase (PcaC), and 3-oxoapate enol-lactonase (PcaD), which participate in the conversion of protocatechuate to succinate and acetyl coenzyme A, are encoded by the pcaB, pcaC, and pcaD genes, respectively (23). These three genes are usually organized in a cluster in bacteria (12, 23, 28). However, no gene that encodes a homolog of PcaB or PcaC is present in the vicinity of the <i>B. thuringiensis</i> phaZ gene, which was previously annotated as a hypothetical pcd gene. In its upstream flanking gene encodes a putative transcriptional regulator of the MarR family and its downstream flanking gene codes for a putative protein of 82 amino acids with unknown function. Blast searches also revealed that the <i>B. thuringiensis</i> genome contains no gene that codes for a homolog of PcaB or PcaC. These observations imply that the <i>B. thuringiensis</i> PhaZ is unlikely to be an authentic 3-oxoadipate enol-lactonase involved in the 3-oxoapate pathway. Moreover, the overall amino acid sequence identities between PcaD from <i>Acinetobacter</i> sp. strain ADP1, <i>Bradyrhizobium japonicum</i> USDA 110, or <i>Pseudomonas putida</i> KT2440 range from 36 to 43%, whereas the <i>B. thuringiensis</i> PhaZ has only 20 to 24% overall amino acid sequence identities with PcaD from these bacteria. Due to the lack of commercially available substrate 3-oxoapate enol-lactone, we were unable to analyze whether the <i>B. thuringiensis</i> PhaZ could display 3-oxoapate enol-lactonase activity. Taken together, these observations favor the notion that the <i>B. thuringiensis</i> PhaZ is likely not to be a 3-oxoapate enol-lactonase.

**ACKNOWLEDGMENTS**

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3. Brandl, H., R. A. Gross, R. W. Lenz, and R. C. Fuller. 1988. <i>Pseudomonas</i> sp. strain T1 and <i>Pseudomonas lumeoni</i> (17) and produces various 3HB oligomers from amorphous PHB as hydrolytic products. 3HB monomer was rarely detected as a hydrolytic product (1). The periplasm-located PHB depolymerase of <i>R. rubrum</i> (10) shows similarity with the type II catalytic domain of extracellular PHB depolymerases from bacteria such as <i>Acidovorax</i> sp. strain TP4 (17). The amount of 3HB monomer released from hydrolysis of nPHB granules by the amorphous PHB-specific extracellular PHB depolymerase PhaZ7 of <i>Pseudomonas lumeoni</i> corresponds to only 0.5 to 2.5% of total 3HB equivalents present in the nPHB granules (9). In this report we found that the <i>B. thuringiensis</i> PhaZ has a strong amorphous PHB-hydrolyzing activity and the amount of 3HB monomer released from hydrolysis of nPHB granules corresponds to approximately 42% and 34% of total 3HB equivalents present in the nPHB granules of <i>B. megaterium</i> and <i>B. thuringiensis</i>, respectively.

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ERRATUM

Global Phylogeny of *Mycobacterium tuberculosis* Based on Single Nucleotide Polymorphism (SNP) Analysis: Insights into Tuberculosis Evolution, Phylogenetic Accuracy of Other DNA Fingerprinting Systems, and Recommendations for a Minimal Standard SNP Set

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Volume 188, no. 2, p. 759–772, 2006. Page 762: Figure 3 should appear as shown on the following page.