

Analysis of Poly(3-Hydroxybutyrate) Granule-Associated Proteome in Recombinant *Escherichia coli*

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Abstract Poly(3-hydroxybutyrate) [P(3HB)] is a microbial polyester intracellularly accumulated as distinct granules in numerous microorganisms as an energy and carbon storage material. Recombinant *Escherichia coli* harboring the heterologous P(3HB) biosynthesis genes accumulates large amounts of P(3HB) granules, yet the granule-associated proteins have not been identified. Therefore, this study reports on an analysis of the P(3HB) granule-associated proteome in recombinant *E. coli*. Five proteins out of 7 spots identified were found to be involved in functions of translation, heat-stress responses, and P(3HB) biosynthesis. Two of the major granule-associated proteins, IbpA/B, which are already known to bind to recombinant proteins forming inclusion bodies in *E. coli*, were further analyzed. Immunoblotting and immunoelectron microscopic studies with IbpA/B antibodies clearly demonstrated the binding and localization of IbpA/B to P(3HB) granules. IbpA/B seemed to play an important role in recombinant *E. coli* producing P(3HB) by stabilizing the interface between the hydrophobic P(3HB) granules and the hydrophilic cytoplasm. Thus, IbpA/B were found to act like phasins in recombinant *E. coli*, as they are the major proteins bound to the P(3HB) granules, affect the morphology of the granules, and reduce the amount of cytosolic proteins bound to the P(3HB) granules.

Key words: *Escherichia coli*, IbpA/B, P(3HB) granule-associated proteome, small heat-shock proteins

Proteome analyses using two-dimensional gel electrophoresis (2-DE) combined with mass spectrometry (MS) have become

quite popular for the simultaneous systematic separation, identification, and quantification of cellular proteins. The development of advanced MS has also significantly increased the number of large-scale proteome studies. However, there are still serious limitations, as none of the currently available proteomic techniques can facilitate the analysis of an entire proteome in a single-step procedure. The recent development of advanced separation methodologies, such as subcellular fractionation, affinity purification, and the fractionation of proteins and peptides according to their physicochemical properties, provides powerful means of reducing the complexity of proteome profiles and detecting low abundant proteins. In particular, subcellular proteome profiling has been successfully employed to separate and identify a large number of organelle-specific proteins [16, 51] and inclusion body-associated proteins [12, 14, 15, 19] in a single experiment.

Polyhydroxyalkanoates (PHAs) are polyesters of various (R)-hydroxycarboxylic acids accumulated in the cytoplasm of microorganisms under unfavorable growth conditions in the presence of excess carbon [27, 30, 35, 45]. PHAs have attracted much research interest owing to their biodegradability, possibility to be produced from renewable resources, and similar mechanical properties to various petroleum-derived polymers. The genes, enzymes, and pathways involved in the biosynthesis of PHAs have already been examined extensively [21, 27, 30, 45, 46], and poly(3-hydroxybutyrate) [P(3HB)] is perhaps the best studied PHA, which is synthesized from acetyl-CoA in three sequential reaction steps catalyzed by β -ketothiolase (*phbA*), NADPH-dependent acetoacetyl-CoA reductase (*phbB*), and P(3HB) synthase (*phbC*) in *Wautersia eutropha* (formerly *Ralstonia eutropha*) and *Alcaligenes latus* [7, 27]. In natural PHA producers,

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PHAs exist in the cytoplasm as distinct granules coated with a layer of proteins and phospholipids [11]. These proteins are referred to as granule-associated proteins, and include PHA synthases, PHA depolymerases, small amphipathic proteins, and other proteins [47]. Phasins are small amphipathic proteins (12–28 kDa) that form a layer on the surface of PHA granules, and represent the most dominant protein in natural PHA producers. Several phasins have already been identified, such as the GA24 protein from *W. eutropha* [54], GA14 protein from *Rhodococcus ruber* [37, 38], GA13 protein from *Acinetobacter* spp. [43], GA 14 protein from *Chromatium vinosum* [29], GA16 protein from *Paracoccus denitrificans* [31], GA13 protein from *Aeromonas caviae* [10], and GA24 protein from *Pseudomonas* sp. 61-3 [32]. Phasins provide the interface between the hydrophilic cytoplasm and the much more hydrophobic core of PHA granules. Some of these PHA granule-associated proteins have been reported to regulate PHA biosynthesis [31, 34, 40, 41], although without any significant influence on the overall PHA content [17, 37]. Most notably, phasins have been found to influence the size and number of PHA granules; the overproduction of phasins resulted in the formation of many small PHA granules, and the removal of phasins caused the formation of a few large granules [31, 38, 54].

Recombinant *Escherichia coli* harboring heterologous PHA biosynthesis genes in a multicopy plasmid has been shown to be particularly efficient for the production of P(3HB) owing to fast growth, the accumulation of large amounts of P(3HB), and the availability of well-established high-cell-density culture techniques [7, 27]. For example, the production of P(3HB) by recombinant *E. coli* harboring the *A. latus* PHA biosynthesis operon (*phbCAB*) was up to 73% of the dry cell weight [7]. However, the existence of such large amounts of hydrophobic P(3HB) granules in the cytoplasm remains an issue, as *E. coli* does not possess the phasin gene (*phaP*).

Small heat-shock proteins (sHsps), which have a molecular mass of 12 to 43 kDa, are ubiquitous and diverse molecular chaperones that prevent protein aggregation under heat-shock conditions [6]. sHsps are also characterized by a conserved ~90 residue α -crystallin domain flanked by a variable hydrophobic N-terminal region and C-terminal extension. To exhibit chaperone activity, they must assemble into large oligomers with a molecular mass of 200 to 800 kDa [20, 28]. Many sHsps have dynamic and variable quaternary structures with subunits that can be freely and rapidly exchanged between oligomers depending on the substrates [3, 48, 49]. According to the present model, sHsps prevent the irreversible aggregation of denatured proteins, and facilitate their transfer to the DnaK-DnaJ-GrpE and/or GroELs chaperone systems for refolding [9, 26, 52].

Two sHsps from *E. coli*, IbpA/B, have been reported to bind to the inclusion bodies (IBs) of recombinant proteins [1, 25]. IbpA/B share a 50% amino acid sequence identity with each other, and *E. coli* strains overproducing IbpA/B have been found to accumulate less aggregated proteins [22, 23, 44, 52]. Recently, the current authors demonstrated that IbpA/B are essential for recombinant protein production in *E. coli* and play important roles in protecting recombinant proteins from degradation by proteases, resulting in the development of new methods for enhancing the yield of desired recombinant protein products [14]. Thus, IbpA/B seem to exhibit several different functions depending on the physiological conditions.

Accordingly, this study conducted a proteome analysis of the P(3HB) granule-associated proteins in recombinant *E. coli* to search for phasin-like proteins that function as a phase stabilizer at the interface of the hydrophilic cytoplasm and the much more hydrophobic P(3HB) granules. Seven major protein spots were detected that represented potential *in vivo* P(3HB) granule-associated proteins in *E. coli*, and 5 of these were identified using liquid chromatography-tandem mass spectrometers (LC-MS/MS). Among these proteins, the function and localization of the most abundant proteins, IbpA/B, were further examined during the biosynthesis of P(3HB).

MATERIALS AND METHODS

Bacterial Strains, Plasmids, and Growth Conditions

The *E. coli* strains used in this study were XL1-Blue (*supE44 hsdR17 recA1 gyrA96 thi relA1 lac F' [proAB⁺ lacI^q lacZ Δ M15 Tn10(Tet^r)]*) and XIB101 (XL1-Blue *ibpAB::Km*). The deletion of the *ibpAB* genes in *E. coli* XL1-Blue to construct XIB101 was carried out using the *red* operon of bacteriophage λ , as previously described [18]. The replacement of the *ibpAB* genes with a kanamycin-resistant gene was confirmed by a polymerase chain reaction (PCR) performed in a PCR Thermal Cycler MP (Takara Shuzo Co., Ltd., Shiga, Japan) using an ExpandTM High Fidelity PCR System (Roche Molecular Biochemicals, Mannheim, Germany). The DNA sequencing was carried out using a Bigdye terminator cycle sequencing kit (Perkin-Elmer Co., Boston, MA, U.S.A.), Taq polymerase, and ABI PrismTM 377 DNA sequencer (Perkin-Elmer Co.). All the DNA manipulations were carried out according to standard procedures [33, 35, 42].

Plasmid pJC4, which contains the *A. latus* PHA biosynthesis genes, has already been described [7], and the PHA biosynthesis genes are constitutively expressed in *E. coli* from their original promoter. Thus, for the co-expression of the *ibpAB* genes, plasmid pPSJ2 was constructed by the insertion of the PCR-amplified *ibpAB* genes containing their original promoter into pJC4 at the

EcoRI site. The primers used for the amplification of the *ibpAB* genes from the *E. coli* W3110 chromosome were 5'-GGAATTCAGCTGTGGATCACCGAAACTGAT and 5'-GGAATTCAGAACGTGCCGAAATATCTTA (the underlined sequences indicate the restriction sites).

To produce the P(3HB), the cells were cultured in 250-ml flasks containing 100 ml of a Luria-Bertani (LB) medium (10 g/l tryptone, 5 g/l yeast extract, 5 g/l sodium chloride) and 20 g/l of glucose in a shaking incubator at 30°C and 200 rpm. Then, to cultivate the recombinant *E. coli* strains, ampicillin was added at a concentration of 50 mg/l.

P(3HB) Analysis

The cell growth was monitored by measuring the absorbance at 600 nm (OD₆₀₀; DU Series 600 Spectrophotometer, Beckman, Fullerton, CA, U.S.A.). The P(3HB) concentration was then determined by measuring the concentration of 3-hydroxybutyric acid methyl ester, which was prepared by the methanolysis of P(3HB) using a gas chromatograph (6890N GC System, Agilent Technologies, Palo Alto, CA, U.S.A.) equipped with a fused silica capillary column (Supelco SPBTM-5, 30 m×0.32 mm ID 0.25 mm film, Bellefonte, PA, U.S.A.) with benzoic acid methyl ester as the internal standard [5]. The cell concentration, defined as the dry cell weight (DCW) per liter of culture broth, was determined as previously described [8], and the P(3HB) content (wt%) was defined as the percentage of the ratio of the P(3HB) concentration to the cell concentration.

Isolation of P(3HB) Granules

The P(3HB) granules were isolated using two different methods, as reported by Maehara *et al.* [31] and Wieczorek *et al.* [54]. In the first method [31], the cells were harvested by centrifugation for 15 min at 6,000 ×g and 4°C. The pellet was then washed and resuspended in 3 volumes of a TE buffer (10 mM Tris-HCl, 1 mM EDTA; pH 8.0), followed by sonication (for 1 min at 20% of the maximum output; High Intensity Ultrasonic Liquid Processor, Sonics & Material Inc., Newtown, CT, U.S.A.). After centrifuging the cell extract at 10,000 ×g and 4°C for 20 min, the supernatant and loosely packed material were then stored at -20°C as soluble proteins. Meanwhile, the pellet of P(3HB) granules was washed 3 times with the TE buffer, centrifuged again, and stored at -20°C as P(3HB)-associated proteins.

In the second method [54], the cells were washed and resuspended in 30 ml of a Tris-HCl buffer (100 mM, pH 7.5), and then passed through a French press twice (100×10⁶ Pa). Thereafter, 1 ml of the lysate was loaded on top of a linear glycerol gradient, obtained from a discontinuous gradient prepared with 1 ml of 88% and 4 ml of 44% glycerol in Tris-HCl (pH 7.5), and then frozen at -70°C and thawed at 4°C. After centrifuging for 30 min at

210,000 ×g and 4°C, a clear granule layer was obtained. The granules were then isolated from this layer and washed with 100 mM Tris-HCl (pH 7.5) by centrifugation for 10 min at 100,000 ×g and 4°C. Next, the granules were resuspended in 100 mM Tris-HCl (pH 7.5) and loaded on top of a linear sucrose gradient, prepared from 2 ml of 2.0, 1.66, 1.33, and 1.0 M sucrose, respectively, in 100 mM Tris-HCl (pH 7.5). After centrifuging for 2 h at 210,000 ×g and 4°C, the granules were sedimented, washed 3 times with 100 mM Tris-HCl (pH 7.5), and finally stored at -20°C.

Two-Dimensional Electrophoresis

The 2-DE experiments were carried out using a Protean II xi 2-D Cell (Bio-Rad, Hercules, CA, U.S.A.), as described previously [12]. The P(3HB)-associated proteins were solubilized from the granules by treatment with an IEF denaturation buffer (8 M urea, 0.5% CHAPS, 10 mM DTT, 0.2% (w/v) Bio-lyte, pH 3–10, 0.001% (w/v) Bromophenol Blue). The solubilized proteins were then separated from the granules by centrifugation at 68,000 ×g for 20 min. Meanwhile, the soluble proteins were dried down by vacuum centrifugation, suspended in the IEF denaturation buffer, and quantified by a Bradford assay using bovine serum albumin as the standard [4]. The protein samples were carefully loaded onto immobilized pH gradient (IPG) strips (18 cm, pH 3–10 NL; Amersham Biosciences, Uppsala, Sweden), which were then rehydrated for 12 h on a Protean IEF Cell (Bio-Rad) and focused at 20°C for 15 min at 250 V, followed by 8,000 V until reaching a total of 60,000 V·h. For the second dimension, the strips were incubated in equilibration buffer I (6 M urea, 0.375 M Tris-HCl (pH 8.8), 20% (w/v) glycerol, 2% (w/v) SDS, 130 mM DTT) for 15 min, followed by incubation in equilibration buffer II (6 M urea, 0.375 M Tris-HCl (pH 8.8), 20% (w/v) glycerol, 2% (w/v) SDS, 135 mM iodoacetamide) for 15 min. Thereafter, the strips were placed on a 12% (w/v) SDS-PAGE prepared by the standard protocol [24], and sealed with a 0.5% (w/v) low-melting-point agarose in a running buffer containing Bromophenol Blue. The gels were run in Protean II gel tanks (Bio-Rad) at 35 mA per gel until the dye front ran off the bottom of the gels. The protein spots were visualized using a silver staining kit (Amersham Biosciences), and the stained gels scanned using a GS710 Calibrated Imaging Densitometer (Bio-Rad). PDQuest 2-D Analysis Software (Bio-Rad) was used to automate the process of finding protein spots within the image and quantifying the density of the spots as a percentage of the volume basis (*i.e.*, the values were calculated based on integrating the spot optical intensity over the spot area). To check the reproducibility and estimate the standard deviation, protein samples were taken from duplicate cultures and analyzed using the 2-D gels in triplicate.

Protein Identification

The samples for the MS/MS analysis were prepared as described previously [13]. All the MS/MS experiments for peptide sequencing were performed using a nano-LC/MS system consisting of an Ultimate HPLC system (LC Packings, Amsterdam, The Netherlands) and Q-TOF MS (Micromass, Manchester, U.K.) equipped with a nano-ESI source. Ten μl of each sample was loaded using an autosampler (FAMOS; LC Packings) and sampling pump (LC-10AD; Shimadzu, Tokyo, Japan) onto a C18 precolumn (i.d. 300 μm , length 1 mm, particle size 5 μm ; LC Packings) for desalting and concentrating the sample. A flow rate of 30 ml/min was used, and the switching-valve position changed after 10 min. The peptides trapped on the precolumn were back-flushed and separated on a C18 nano column (i.d. 75 μm , length 150 mm, particle size 5 μm ; LC Packings). The gradient used was 0% (v/v) acetonitrile for 10 min, followed by 0% (v/v) to 50% (v/v) acetonitrile over 40 min, and then 50% (v/v) acetonitrile for 5 min at a flow rate of 200 nl/min. In the nano-electrospray ionization source, the end of the capillary tubing from the nano-LC column was connected to the emitter with picotip silica tubing (i.d. 5 μm ; New Objectives, Woburn, U.S.A.) using a stainless steel union, with a PEEK sleeve to couple the nanospray with the online nano-LC. The voltage applied to the union to produce the electrospray was 1.5–2 kV, and the cone voltage was 30 V. Argon was introduced as the collision gas at a pressure of 10 pounds per square inch. The MS/MS data were acquired in a data-dependent MS/MS mode, where the collision energy was increased in steps to 25, 30, and 35 eV. The Mascot search server (<http://www.matrixscience.com>) was used to identify the protein spots based on querying the sequence of the trypsin-digested peptide fragment data. The reference databases used to identify the target proteins were SWISS-PROT (<http://kr.expasy.org/>) and NCBI (<http://www.ncbi.nlm.nih.gov>).

SDS-PAGE and Western Immunoblotting

The protein samples were routinely analyzed using a 12% (w/v) SDS-PAGE, as described by Laemmli [24]. The gels were stained with Coomassie brilliant blue R250 (Bio-Rad). The Western blot analysis was performed according to standard protocols [42]. For the immunodetection of IbpA/B, polyclonal rabbit antisera against IbpA/B (kindly provided by Dr. E. Laskowska, Gdansk, Poland) and anti-rabbit immunoglobulin G-peroxidase conjugate (Sigma, St. Louis, MO, U.S.A.) were used. A light-emitting non-radioactive ECL kit (Amersham Biosciences) was used for the signal detection.

Electron Microscopic Studies

The cells were washed and suspended in a 50 mM potassium phosphate buffer (pH 6.8), prefixed in the presence of a mixture of 1% (w/v) paraformaldehyde and 1% (w/v) glutaraldehyde in a 0.1 M cacodylate buffer, and fixed with 1% (w/v) osmium tetroxide. After dehydration with a graded ethanol series, the cells were embedded in Lowicryl HM20. Ultrathin sections were then mounted on copper grids, stained with uranyl acetate and lead citrate, and examined under a Zeiss EM-109 electron microscope (Carl Zeiss, Jena, Germany) at an accelerating voltage of 80 kV. The postembedding immunogold labeling of the IbpA/B in the ultrathin sections of the recombinant *E. coli* cells was performed as described by Pieper-Fürst *et al.* [37] using IbpA/B antibodies and a goat-anti-rabbit IgG-gold (GARG) complex (Sigma). The specificity of the labeling was checked by a control experiment using just the GARG complex.

RESULTS

Proteome Analysis

The recombinant *E. coli* XL1-Blue (pJC4) was cultured in an LB medium containing 20 g/l of glucose. The cell

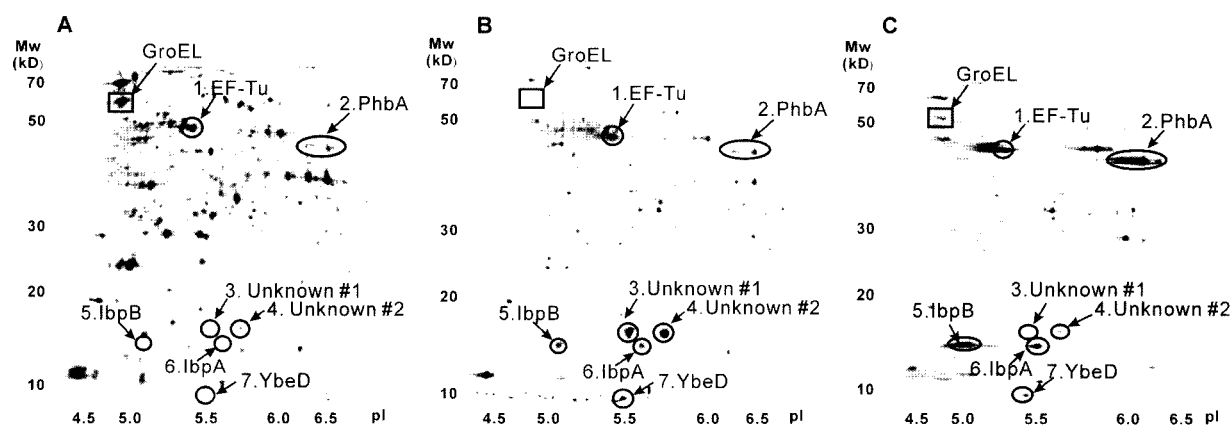


Fig. 1. Proteome profiles of recombinant *E. coli* XL1-Blue (pJC4) accumulating P(3HB).

A. Soluble protein fraction using Method 1; B. P(3HB) granule fraction using Method 1; C. P(3HB) granule fraction using Method 2. The circles indicate the notable P(3HB) granule-associated proteins, as listed in Table 1.

concentration and P(3HB) content in the sample taken for the proteome analysis were 3.1 g/l and 60% (w/w), respectively. The proteome profiles for the soluble fraction and granule-associated fraction of XL1-Blue (pJC4) are shown in Fig. 1. To avoid any cross-contamination of certain proteins during the granule fractionation, two different methods were used, including the general purification method of Maehara *et al.* [31] (Method 1) and glycerol gradient fractionation method of Wieczorek *et al.* [54] (Method 2). To identify the P(3HB) granule-associated proteins, the proteome profile for the soluble protein fraction was compared with that for each P(3HB) granule fraction. As a result, the amount of soluble proteins loaded on the gel was 200 µg, which was twice that of the granule-associated proteins (100 µg). The overall profiles of the cellular proteins within a pH range of 3–10 were quite reproducible and distinctive enough to be compared and matched. To ensure against the detection of soluble proteins accidentally bound to the granules during purification, only the proteins with at least a 3-fold higher intensity were selected as putative granule-associated proteins.

Among the several protein spots that appeared as putative P(3HB) granule-associated proteins (Figs. 1B and 1C), 5 spots were common between the 2 gels obtained using Methods 1 and 2, and identified as exact matches by LC-MS/MS, followed by a database search (Table 1). The identifications included EF-Tu, PhbA, IbpA, IbpB, and YbeD. Two spots, indicated as “Unknown,” remained unmatched after the database search. GroEL, indicated in

Fig. 1, is shown for comparison, as will be described later. Among the 5 granule-associated proteins, only PhbA had a direct relationship with P(3HB) biosynthesis. Most notably, two of the major granule-associated proteins were IbpA/B, which led to the examination of the roles of IbpA/B during P(3HB) production in *E. coli*.

In Vivo* Localization of IbpA/B During Production of P(3HB) in Recombinant *E. coli

To examine the localization of IbpA/B on the surface of the P(3HB) granules, Western immunoblotting and immunoelectron microscopic studies were performed. First, the soluble proteins and P(3HB) granule-associated proteins of XL1-Blue (pJC4) were analyzed by Western blot using polyclonal IbpA/B antibodies (Fig. 2A), and most of the IbpA/B were found to be associated with the P(3HB) granules. Next, the *in vivo* localization of IbpA/B in XL1-Blue (pJC4) was examined by transmission electron microscopy using GARG complexes and polyclonal IbpA/B antibodies (Fig. 2B). The postembedding immunogold labeling clearly revealed the localization of IbpA/B on the surface of the P(3HB) granules. In the control experiment using just the GARG complexes, no gold-conjugated IbpA/B was detected.

Effect of IbpA/B on P(3HB) Accumulation in Recombinant *E. coli*

To examine the effect of IbpA/B on the production of P(3HB), plasmid pJC4 was introduced to *E. coli* XIB101,

Table 1. Analysis of P(3HB) granule-associated proteins in recombinant *E. coli*.

Spot No.	Gene ^a	Accession no. ^b	pI/Mw (kDa)	Protein description	Ratio					
					Granule/Soluble [XL1-Blue (pJC4)] ^c		Granule/Soluble [XIB101 (pJC4)] ^d		XIB101 (pJC4)/XL1-Blue (pJC4) ^e	
					Method 1 ^f	Method 2 ^f	Method 1 ^f	Method 2 ^f	Method 1 ^f	Method 2 ^f
1	<i>tufB</i>	P02990	5.32/44.6	Elongation factor Tu (EF-Tu)	3.02±0.520	4.40±0.710	7.55±1.04	8.90±1.11	2.5	2.0
2	<i>phbA</i>	Q9ZG19	6.28/40.5 6.58/40.5	β-Ketothiolase	3.98±0.562	10.0±1.25	48.8±2.59	47±1.99	12	4.7
5	<i>ibpB</i>	P29210	5.19/16.1	16 kDa heat-shock protein B	∞ ^g	∞ ^g	0	0	0	0
6	<i>ibpA</i>	P29209	5.63/15.6	16 kDa heat-shock protein A	∞ ^g	∞ ^g	0	0	0	0
7	<i>ybeD</i>	P30977	5.50/9.83	Hypothetical protein YbeD	∞ ^g	∞ ^g	∞ ^g	∞ ^g	2.0 ^h	2.0 ^h

^aMascot search server (<http://www.matrixscience.com>) was used to identify proteins analyzed by LC-MS/MS.

^bAccession number is from SWISS-PROT.

^cRatio of protein levels in granule-associated fraction and soluble fraction in recombinant XL1-Blue (pJC4).

^dRatio of protein levels in granule-associated fraction and soluble fraction in recombinant XIB101 (pJC4).

^eRatio of two ratios obtained with XL1-Blue (pJC4) and XIB101 (pJC4).

^fGranule fractionation methods: general fractionation by Maehara *et al.* [31] (Method 1) and glycerol gradient fractionation by Wieczorek *et al.* [54] (Method 2).

^gOnly present in granule-associated fraction.

^hRatio of granule-associated fractions in XIB101 (pJC4) and XL1-Blue (pJC4).

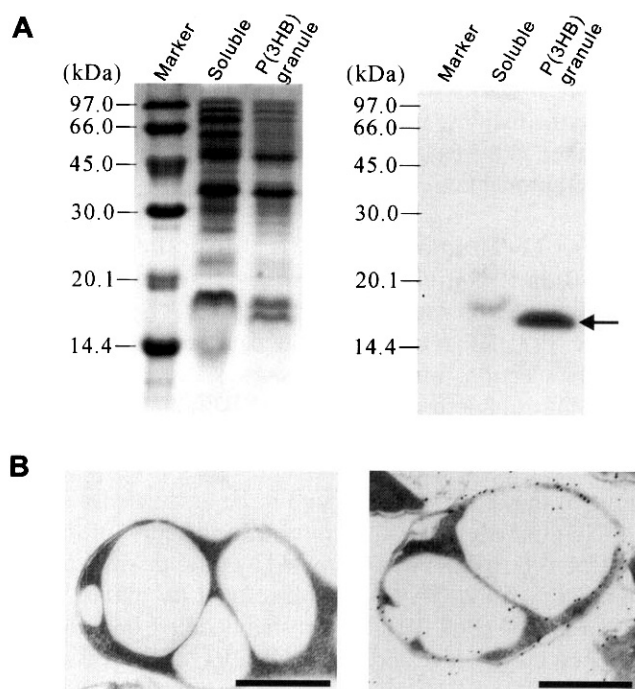


Fig. 2. Localization of IbpA/B during production of P(3HB) in recombinant *E. coli*.

A. SDS-PAGE (left panel) and immunoblotting analysis (right panel) of recombinant *E. coli* XL1-Blue (pJC4) accumulating P(3HB). Molecular mass standard proteins, with masses indicated on the left (from top to bottom, phosphorylase b of rabbit muscle, 97 kDa; albumin of bovine serum, 66 kDa; ovalbumin of chicken egg white, 45 kDa; carbonic anhydrase of bovine erythrocyte, 30 kDa; trypsin inhibitor of soybeans, 20.1 kDa; α -lactalbumin of bovine milk, 14.4 kDa, respectively). The Western blotting was performed using polyclonal rabbit antisera against IbpA/B. The arrow indicates the position of the IbpA/B proteins. **B.** Immunoelectron microscopic localization of the IbpA/B in the recombinant *E. coli* based on postembedding immunogold labeling. The right panel shows a section of an *E. coli* XL1-Blue (pJC4) cell labeled with antibodies against the IbpA/B and goat anti-rabbit immunoglobulin G-gold (GARG) complexes. The left panel shows a section of a control *E. coli* XL1-Blue (pJC4) cell labeled with just the GARG complexes. The bar represents 0.5 μ m.

which is an *ibpAB* gene-deficient derivative of *E. coli* XL1-Blue. Flask cultures of XL1-Blue (pJC4) and XIB101 (pJC4) were carried out in an LB medium with 20 g/l of glucose at 30°C. The final cell concentration and P(3HB) content were 3.6 g/l and 65%, respectively, for XL1-Blue (pJC4) and 2.7 g/l and 52%, respectively, for XIB101 (pJC4), indicating that IbpA/B had a somewhat positive effect on the P(3HB) accumulation in the recombinant *E. coli*. More notably, IbpA/B affected the morphology of the P(3HB) granules in the *E. coli*. Most of the P(3HB) granules accumulated in *E. coli* XL1-Blue (pJC4) were smooth and round (Fig. 3A). Interestingly, the P(3HB) granules in *E. coli* XIB101 (pJC4) were significantly distorted and shrunken with wrinkles (Fig. 3B). To examine the effect of complementing IbpA/B in *E. coli* XIB101, plasmid pSJ2 was constructed, as described in

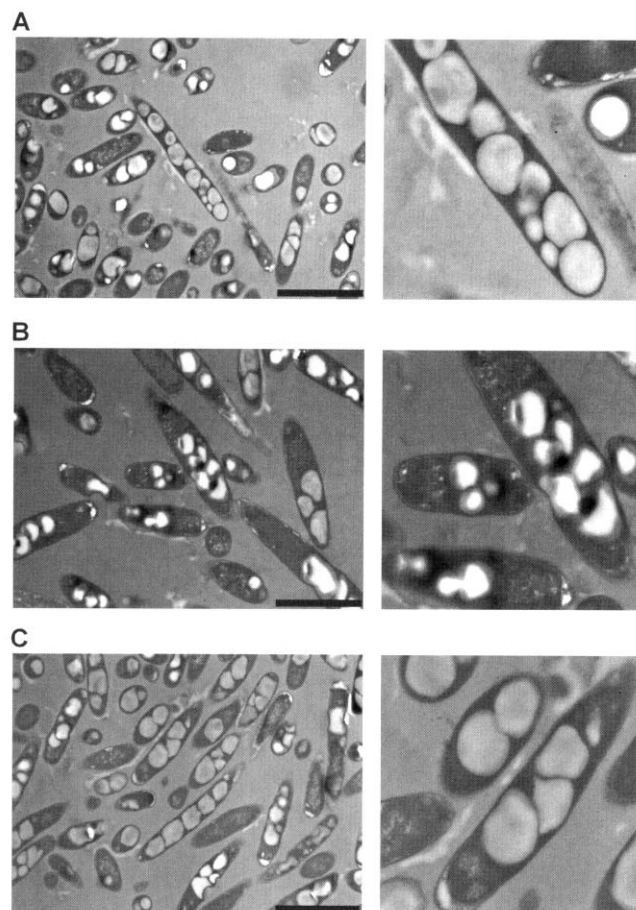


Fig. 3. Transmission electron microscopic observation of granule morphology in recombinant *E. coli*. The right panels show enlarged images.

A. *E. coli* XL1-Blue (pJC4). **B.** *E. coli* XIB101 (pJC4). **C.** *E. coli* XIB101 (pSJ2). The bar represents 1 μ m.

Materials and Methods. As a result, the morphology of the P(3HB) granules in *E. coli* XIB101 (pSJ2) was restored to that observed in the wild-type strain (Fig. 3C). Thus, from the distorted morphology of the P(3HB) granules in the *ibpAB* mutant, it would appear that IbpA/B stabilized the interface between the hydrophobic P(3HB) granules and the hydrophilic cytoplasm. It should also be mentioned that the XL1-Blue (pJC4), XIB101 (pJC4), and XIB101 (pSJ2) strains showed similar cell concentrations and granule contents.

Proteome Analysis of *ibpAB* Mutant Strain Accumulating P(3HB)

It was investigated whether IbpA/B play an important role in preventing cytosolic proteins from aggregating on the hydrophobic surface of the P(3HB) granules. For the two strains with and without IbpA/B, the protein concentrations in the soluble and granule-associated fractions were measured using a Bradford assay; and were

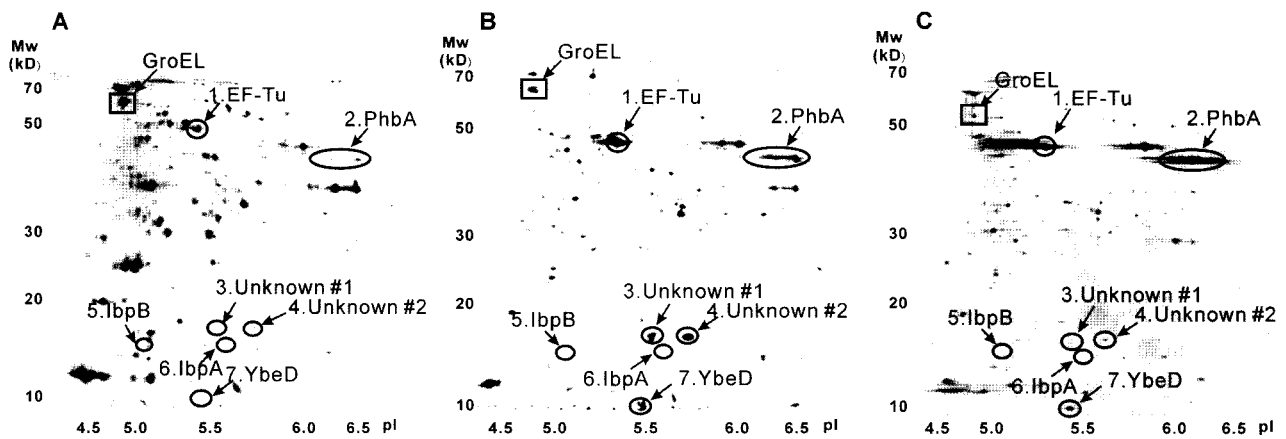


Fig. 4. Proteome profiles of *ibpAB*-negative recombinant *E. coli* XIB101 (pJC4) accumulating P(3HB).

A. Soluble protein fraction using Method 1. B. P(3HB) granule fraction using Method 1. C. P(3HB) granule fraction using Method 2. The circles indicate the notable P(3HB) granule-associated proteins, as listed in Table 1.

1.00 and 0.17 mg/ml, respectively, for XL1-Blue (pJC4) and 1.02 and 0.29 mg/ml, respectively, for XIB101 (pJC4) with Method 1, whereas they were 0.92 and 0.15 mg/ml, respectively, for XL1-Blue (pJC4) and 0.88 and 0.21 mg/ml, respectively, for XIB101 (pJC4) with Method 2. The protein percentage bound to the P(3HB) granules in XL1-Blue (pJC4) and XIB101 (pJC4) was 14.5% and 22.1% of the total proteins, respectively, with Method 1, and 14.0% and 19.3% of the total proteins, respectively, with Method 2. As such, these results clearly showed that more cytosolic proteins were bound to the P(3HB) granules when IbpA/B were absent. To examine which proteins were prominently bound to the P(3HB) granules, the proteome profiles of the soluble and granule-associated protein fractions from *E. coli* XIB101 (pJC4) were analyzed (Fig. 4). The cell concentration and P(3HB) content in the sample taken for the proteome analysis were 2.7 g/l and 52%, respectively. The proteome profiles for XIB101 (pJC4) were compared with those obtained for the parent strain (Figs. 1 and 4). The ratios of the 5 prominent proteins identified in common by the two methods were compared and summarized in Table 1. Considerably more EF-Tu, PhbA, and YbeD were bound to the P(3HB) granules in the *ibpAB* mutant strain compared with the parent strain. When Method 1 was used, only a small amount of GroEL was found to be associated with the P(3HB) granules in the parent strain XL1-Blue. Conversely, the amount of GroEL bound to the P(3HB) granules was increased 38-fold in the *ibpAB* mutant strain. When Method 2 was used, the amount of GroEL bound to the P(3HB) granules was also increased 2.5-fold in the *ibpAB* mutant strain. Therefore, these results suggest that IbpA/B may play an important role by reducing the amount of cytosolic proteins that bind to the P(3HB) granules, thereby protecting them from possible denaturation on the surface of a hydrophobic biopolymer.

DISCUSSION

E. coli has been shown to be one of the most promising candidate strains for the efficient production of P(3HB). Various metabolic engineering strategies, including the amplification of the heterologous P(3HB) biosynthesis genes and/or manipulation of other metabolic pathways, have resulted in the successful accumulation of large amounts of the polymer inside the cells [7, 53]. However, during the accumulation of P(3HB), heat-shock-like responses with increased levels of GroEL, GroES, and DnaK have been observed [13], which is not surprising, as P(3HB) is not one of the normal metabolic products of *E. coli* and is accumulated as relatively large granules taking up the actual cytosolic space, causing stress to the cells. Furthermore, since there are no known phasins in *E. coli*, unlike natural PHA producers, the hydrophobic P(3HB) granules are likely to be directly exposed to the cytoplasm, potentially causing more problems related to cytosolic proteins binding to the granules. Therefore, it was postulated that *E. coli* may contain some proteins that play similar roles to the phasins in natural PHA producers. Large differences in the amount of GroEL bound to the P(3HB) granules were observed when two different methods were employed for granule isolation. This was because the two methods widely used for granule isolation are inherently different in their procedures. Clearly, Method 2 allows isolation of much more pure P(3HB) granules and their associated proteins, which in other words means that loosely bound proteins were removed when Method 2 was used. Nonetheless, it is important to note that GroEL was still bound to the P(3HB) granules in the recombinant *ibpAB* mutant strains.

In the present study, it was found that the *E. coli* sHsps, IbpA/B, were the major granule-associated proteins in P(3HB) granules, and localized on the surface of the

P(3HB) granules. As far as is known, this is the first *in vivo* electron microscopic observation of the localization of IbpA/B in *E. coli*. sHsps have been shown to be very efficient at binding to denatured proteins, and current models propose that ATP-independent sHsps prevent the irreversible aggregation of denatured proteins by binding and stabilizing them [9, 22, 26, 52]. It has also been shown that temperature-induced structural rearrangements promote the exposure of normally hidden hydrophobic regions that allow IbpA/B to bind and stabilize partially folded proteins as they denature [25, 44]. Similarly, the present study found that IbpA/B were induced by the accumulation of P(3HB) and bound to the hydrophobic surface of the P(3HB) granules in recombinant *E. coli*, possibly to stabilize both the P(3HB) granules and the denatured cytosolic proteins. These denatured proteins were refolded by ATP-dependent chaperone systems, such as DnaK-DnaJ-GrpE and/or GroELS.

One of the notable findings in the *ibpAB* mutant strain was a significant change in the P(3HB) granule morphology, which became distorted and wrinkled when IbpA/B were absent. This finding indicates that IbpA/B stabilize the surface of P(3HB) in the cytoplasm. In the *ibpAB* mutant strain, the P(3HB) granules may have come into direct contact with intracellular biomolecules, including DNA, RNA, and proteins, without an interface, thereby binding with more cytosolic proteins compared with the parent strain. This agrees with the previous finding that deleting the *ibpAB* genes increases protein aggregation under stressful conditions [22, 23]. Basha *et al.* [2] reported that sHsps are important for the protection of various cellular proteins and a wide variety of cellular activities, including translation, transcription, cell signaling, and secondary metabolism. Previously, the current authors reported a significant reduction in the level of EF-Tu in the soluble fraction of *E. coli* cells accumulating a large amount of P(3HB) [13]. The present study then found that EF-Tu was bound on the surface of the hydrophobic P(3HB) granules, suggesting that cell growth after P(3HB) accumulation may be negatively affected by the loss of functional EF-Tu, an important component of the protein biosynthetic machinery. In addition, one enzyme of the P(3HB) biosynthesis operon, β -ketothiolase (*phbA*), was detected in the P(3HB) granule fraction, suggesting a reduction in the active β -ketothiolase with a negative affect on P(3HB) biosynthesis. Although it was unclear whether or not this was the main reason, the P(3HB) accumulation was somewhat reduced in the *ibpAB* mutant strain of *E. coli*.

Moreover, a higher level of GroEL (38-fold in Method 1 and 2.5-fold in Method 2) was associated with the P(3HB) granules in the *ibpA/B* mutant strain when compared with the parent strain. Pötter *et al.* [39] reported on a proteome analysis of *W. eutropha* using Coomassie blue-stained 2D gels and showed that PhaP1, PhaP3, and PhaP4 were

bound to the P(3HB) granules. As observed in the *ibpAB* mutant, the amounts of PhaP3, GroEL, and PhaA bound to the P(3HB) granules increased in the phasin-negative mutant strain. Therefore, IbpA/B appeared to act like phasins during the PHA accumulation in the recombinant *E. coli*.

Analysis of the granule-associated proteome enabled the identification of new proteins specifically bound to the P(3HB) granules in the recombinant *E. coli*. In this study, two granule fractionation methods were used to exclude cross-contamination during the protein extraction process. Both methods provided similar 2D-gel results, except for spot numbers 3 and 4. There was no contamination with membrane proteins in the granule fraction, as no membrane proteins were found in the 2D gels. Despite using the same method of glycerol gradient centrifugation (Method 2), many more cellular proteins were seen compared with the results of Pötter *et al.* [39], owing to the use of silver-stained 2D gels, which are at least 100 times more sensitive than Coomassie blue-stained 2D gels [50]. However, account was taken of the potential of cross-contamination of some proteins during the preparation of the two fractions. Furthermore, several methods verified that IbpA/B were the major proteins bound to the P(3HB) granules in the recombinant *E. coli*.

In conclusion, the proteome analysis of the P(3HB) granule-associated proteins in a recombinant *E. coli* producing a large amount of P(3HB) revealed several granule-associated proteins in the recombinant *E. coli*. Among these proteins, it was found that IbpA/B acted like phasins found in natural PHA-producing bacteria. Consequently, IbpA/B seem to play an important role in recombinant *E. coli* producing P(3HB) by stabilizing the interface between the hydrophobic P(3HB) granules and the hydrophilic cytoplasm, and reducing the amount of cytosolic proteins that bind to the P(3HB) granules.

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