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NOTE

Poly(3-hydroxybutyrate) Extrusion by Cells of Recombinant *Escherichia coli*

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Poly(3-hydroxybutyrate) (PHB) was synthesized and accumulated intracellularly to a high concentration (7 g/l) by cultivating recombinant *Escherichia coli* XL1-Blue (pSYL105) in a complex medium containing 20 g/l glucose. The morphology of PHB granules was examined by transmission electron microscopy. The PHB granules synthesized in recombinant *E. coli* were much larger than reported values for wild type microorganisms, and were often irregularly shaped. Some cells were apparently extruding PHB into the medium, which suggests that PHB granules maintain some fluidity and cells become fragile due to PHB accumulation.

Poly(3-hydroxybutyric acid) (PHB) is a bacterial storage polymer produced by various microorganisms in response to nutrient limitation (1). It is a member of the family of polyhydroxyalkanoic acids (PHAs) which hold promise as a biodegradable alternative to existing commodity plastics such as polyethylene and polypropylene. PHB could be efficiently produced by recombinant *Escherichia coli* harboring a stable high-copy-number plasmid containing *Alcaligenes eutrophus* polyhydroxyalkanotate biosynthesis genes (6, 8). More than 80 g/l of PHB with a productivity greater than 2 g/l-h could be obtained by the pH-stat fed-batch culture of recombinant *E. coli* (7-9). Recombinant *E. coli* accumulated PHB to a content as high as 86% of dry cell weight (10). It was observed that cells of recombinant *E. coli* accumulating a large amount of PHB underwent considerable morphological changes. Cells were heavily distorted by protruding PHB granules, and some cells were elongated by filamentation (5, 10). Size analysis of PHB granules synthesized in recombinant *E. coli* by photosedimentation suggested that the average diameter was in the range of 1.13-1.25 μm (11). Granules synthesized in *A. eutrophus* or *Bacillus megaterium* are typically spherical with a diameter of 0.1-0.8 μm (4). This study examines the morphology of PHB granules synthesized in recombinant *E. coli* by transmission electron microscopy (TEM). The possible reasons for the apparent extrusion of PHB by cells are discussed.

Bacterial Strain and Culture Condition

The recombinant *E. coli* strain used was XL1-Blue harboring pSYL105 which contains the *A. eutrophus* PHA biosynthesis genes (10). The medium used was Luria-Bertani (LB) medium supplemented with 20 g/l glucose. Cells were cultivated in a 250 ml flask containing 50 ml medium for 48 h at 37°C and 300 rpm. Cell mass (gram dry cell weight per liter of culture broth) and PHB concentration were determined as previously described (9, 10). The PHB content (%) was defined as the percentage of the ratio of PHB concentration to cell mass.

Transmission Electron Microscopy

Cells were fixed in 2% (vol/vol) glutaraldehyde containing 2% (vol/vol) paraformaldehyde in 0.1 M phosphate buffer (pH 7.2) and were postfixed in 1% (wt/vol) osmium tetroxide. Cells were dehydrated in an ascending series of ethanol (10% steps from 30 to 100%, 10 min for each step) and were embedded in Epon 812 (Agar Co., Cambridge, UK). Thin sections were prepared with the LKB 2088 Ultrotome V (Surrley, UK), stained with 2% (wt/vol) uranyl acetate and 2% (wt/vol) lead citrate, and were examined with a Philips CM20 transmission electron microscope (Eindhoven, Netherlands) at an accelerating voltage of 80 kV.

PHB Synthesis by Recombinant *E. coli*

The recombinant *E. coli* strain XL1-Blue (pSYL105) was cultured for 48 h in LB+20 g/l glucose, which supported the highest PHB accumulation among the various media tested (10). The time profiles of cell mass, PHB concentration, and residual cell mass are shown in Fig. 1. The final cell mass and PHB concentration were as high as 8.5 and 7.0 g/l, respectively, resulting in a PHB content of 82%. Cells were virtually full of PHB granules,

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often swollen and distorted, and elongated by filamentation as previously reported (10).

PHB Granules and PHB Extrusion

Due to the difficulty of resolving the individual PHB granules by phase contrast microscopy, transmission electron microscopy was used to examine granule morphology. The TEM pictures of XL1-Blue (pSYL105) cultured for 22 h are shown in Fig. 2A. At this time, the PHB concentration and the PHB content were 2.5 g/l and 53%, respectively. It can be seen that the size and shape of the individual granules are highly heterogeneous. This is due to the non-synchronous nature of PHB synthesis by recombinant *E. coli*. Different cells would not possess the same amount of PHB because new daughter cells had just started to synthesize PHB whereas old cells had already accumulated a large amount of PHB. By contrast, PHB synthesis by wild type microorganisms such as *A. eutrophus* occurs rather synchronously since cells start to synthesize PHB upon the depletion of an essential nutrient (e.g. nitrogen or phosphate) without further growth (1). Recombinant *E. coli* accumulates PHB during growth without the requirement for nutrient limitation (9, 10). The diameter of PHB granules shown in Fig. 2A is in the range of 0.5-2.0 μm, which is in good agreement with the results obtained by photosedimentation measurements (11). The most interesting observation was the apparent extrusion of PHB from some of the cells (see arrows in Fig. 2A). More detailed pictures of cells extruding PHB are shown in Fig. 2B and 2C. PHB was extruded from large holes in the cell wall (arrows in Fig. 2). Polymer released had an amorphous globular appearance. Since cells leaking PHB have never been observed under phase contrast microscopic examinations, it seems that the PHB extrusion is an artifact generated during the preparation of the cells for the TEM. The PHB extrusion phenomenon has never been observed in *A. eutrophus* and most other wild type microorganisms. Recently, Page *et al.* (13) also observed PHB extrusion in the TEMs of pleomorphic cells of *Azotobacter vinelandii* UWD cultured in fish peptone medium. It has been previously reported that *A. vinelandii* became extremely fragile when grown in fish peptone medium (12). Page *et al.* (13) suggested that the ex-

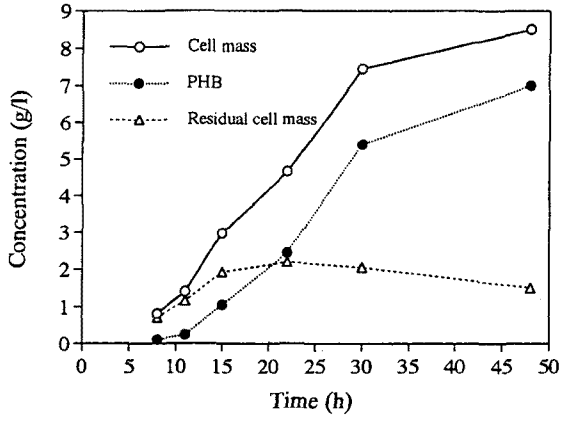

Fig. 1. Time profiles of cell mass, PHB concentration, and residual cell mass.
XL1-Blue (pSYL105) was cultivated in LB medium supplemented with 20 g/l glucose for 48 h at 37°C and 300 rpm.

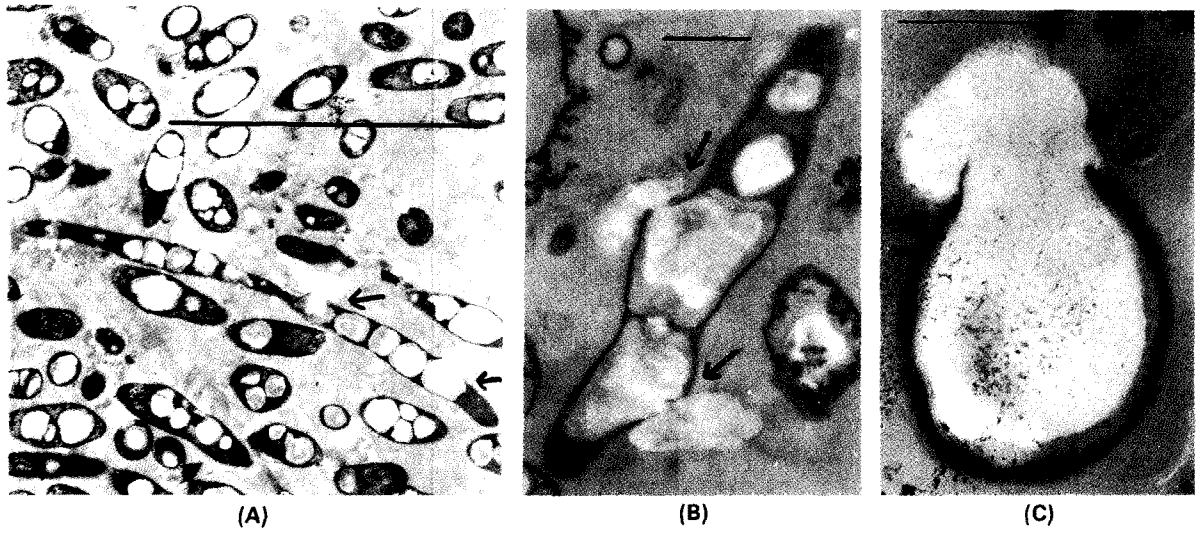

Fig. 2. Transmission electron micrographs of XL1-Blue (pSYL105).
Cells were cultivated in LB+20 g/l glucose for (A) 22 h, (B) and (C) 48 h. PHB extrusion is indicated by arrows. Bar represents 10 μm in (A) and 1 μm in (B) and (C).

trusion of PHB is due to polymer expansion caused by the dehydration of the specimen for the TEM. No matter what the exact reason may be, PHB extrusion is observed only with cells that become extremely fragile. This is supported by the finding that *Haloferax mediterranei*, an extremely halophilic bacterium, also showed PHB extrusion phenomenon in TEMs (14). This halobacterium requires a high salt concentration for cell viability, and is extremely fragile and lysed without salts. Therefore, I believe that PHB extrusion from recombinant *E. coli* is also caused by the weakness of cells accumulating a large amount of polymer to tolerate sample treatment for TEM. The fragility of recombinant *E. coli* cells accumulating PHB will prove beneficial for the recovery of this polymer.

The *in vivo* state of PHB granules accumulated in wild type microorganisms has been investigated by several groups, and was found to be amorphous rather than crystalline (2). The PHB extrusion observed in this study suggests that PHB granules synthesized in recombinant *E. coli* are also in the amorphous state to a large extent. Our group previously reported that PHB might be more crystalline in recombinant *E. coli* compared with *A. eutrophus*. The crystallinity of the PHB in the lyophilized cell powder of recombinant *E. coli* and *A. eutrophus* were 60 and 16%, respectively (3). It is well known that the crystallization of PHB can be induced by several treatments including lyophilization (2). It is possible that lyophilization affected the morphology of PHB to a different extent in recombinant *E. coli* and *A. eutrophus* due to their different cell structure, the former being more fragile with a large accumulation of PHB. In conclusion, the TEM observations made in this study strongly suggest that recombinant *E. coli* becomes fragile upon accumulating large amounts of PHB and that PHB granules in recombinant *E. coli* are to a large extent in the mobile amorphous state.

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