Journal of Bioscience and Bioengineering
VOL. 127 No. 6, 686–689, 2019

www.elsevier.com/locate/jbiosc

# Physiological characterization of poly-β-hydroxybutyrate accumulation in the moderately thermophilic hydrogen-oxidizing bacterium *Hydrogenophilus thermoluteolus* TH-1

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Received 21 June 2018; accepted 21 November 2018
Available online 19 December 2018

***Hydrogenophilus thermoluteolus*** **strain TH-1 is a thermophilic hydrogen-oxidizing microorganism that has the highest growth rate among autotrophs. Genomic analysis revealed that this strain comprises the complete gene set for poly-β-hydroxybutyrate (PHB) synthesis, i.e., three copies of acetyl-CoA acetyltransferase and polyhydroxyalkanoate synthase and one copy of acetoacetyl-CoA reductase and 3-hydroxyacyl-CoA dehydrogenase/3-hydroxybutyryl-CoA epimerase. An investigation on PHB accumulation in strain TH-1 demonstrated that PHB accumulation was induced by nitrogen limitation under autotrophic as well as heterotrophic conditions. This strain accumulated up to 430.4 ± 14.3 mg $L^{-1}$ PHB during a 3-h incubation under nitrogen-limited heterotrophic conditions. The highest PHB accumulation rates under autotrophic and heterotrophic conditions were 38.6% (w/w) of the dry cells after a 6-h induction and 53.8% after 3 h, respectively. Although PHB granules started to accumulate after 15 min of nitrogen limitation under heterotrophic conditions, a drastic decrease of PHB was observed after 9 h of induction.**



[**Key words:** *Hydrogenophilus thermoluteolus*; Thermophile; Hydrogen bacterium; Poly-β-hydroxybutyrate; Transmission electron microscopy]

In the field of materials science during the 20th century, there was a strong trend of inventing and developing new synthetic plastics (1). However, these plastics are mostly nonbiodegradable and their increasing consumption is becoming a major source of environmental pollution (2). Thus, there is an urgent need to develop a new synthetic material that can be biologically degraded. Poly-β-hydroxybutyrate (PHB) is a biocompatible and biodegradable plastic belonging to the group of endocellular polyhydroxyalkanoates, with potential applications in agriculture, industry, medicine, and other fields (3,4). PHB is produced by various microorganisms as they enter the stationary phase of growth under pH stress, nitrogen limitation, or phosphate limitation with an excess carbon source (5,6). To date, most reported studies about PHB production have been conducted under heterotrophic conditions. In contrast, there are only few reports of PHB production by autotrophs, mainly due to their slow growth (7–15).

*Hydrogenophilus thermoluteolus* strain TH-1$^T$ (DSM 6765) is a gram-negative, non-spore-forming, thermophilic hydrogen-oxidizing microorganism. It was isolated from a hot spring in Izu District, Japan. The optimal temperature and pH for its growth are 50°C and pH 7.0 (16). The maximum specific growth rate ($\mu_{max}$) under optimal autotrophic conditions was determined to be 0.68 $h^{-1}$, which is the highest among autotrophs.

In this study, we demonstrate that strain TH-1 has multiple gene sets for PHB synthesis. In addition, we biochemically investigated PHB accumulation under autotrophic and heterotrophic conditions using a two-phase culture system.

## MATERIALS AND METHODS

**Bacterial strains and growth conditions** *H. thermoluteolus* strain TH-1 (DSM 6765) was utilized throughout this study. The DDBJ accession numbers of the sequences of a chromosome and a plasmid of strain TH-1 are AP018558 and AP018559, respectively.

The mineral salt medium used for the growth comprised 2.0 g $L^{-1}$ $(NH_4)_2SO_4$, 1.0 g $L^{-1}$ $KH_2PO_4$, 2.0 g $L^{-1}$ $K_2HPO_4$, 0.5 g $L^{-1}$ NaCl, 1.1 mg $L^{-1}$ $FeSO_4 \cdot 7H_2O$, 30.0 mg $L^{-1}$ $CaCl_2$, 0.5 g$L^{-1}$ $MgSO_4 \cdot 7H_2O$, and a trace element solution of pH 7.0 (17).

Autotrophic cultivation was performed using the mineral salt medium with gas phase comprising $H_2$:$O_2$:$CO_2$ (75:10:15). Heterotrophic cultivation was performed in an air atmosphere using the mineral salt medium with the addition of 60 mM organic acid (acetate, pyruvate, lactate, butyrate, or malate) as the sole carbon source. Strain TH-1 was cultivated using a reciprocating shaker at 50°C, with a culture volume of 10 mL in 100-mL vials. The preculture for all experiments was autotrophically performed. The growth was monitored by measuring the optical density at 540 nm ($OD_{540}$).

**PHB formation** After 10 h of autotrophic cultivation or 7 h of heterotrophic cultivation with malate, the $OD_{540}$ reached approximately 2.0. Cultivated cells were harvested by centrifugation at 40,000 × *g*, at 4°C for 2 min, and a cell suspension was made to provide 10 mL of the corresponding medium without $(NH_4)_2SO_4$ per gram

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1389-1723/$ – see front matter 
https://doi.org/10.1016/j.jbiosc.2018.11.011

of wet cells. Herein, the medium without $(NH_4)_2SO_4$ is referred as induction medium. Subsequently, the cell suspension autotrophically prepared was incubated with $H_2$:$O_2$:$CO_2$ (75:10:15) using a reciprocating shaker at 50°C, whereas the cell suspension heterotrophically prepared was incubated in air using a reciprocating shaker at 50°C.

**High performance liquid chromatography analysis for PHB** Total biomass was determined by dry weight measurement (drying conditions, 100°C overnight). The PHB content was determined by crotonic acid quantification through high performance liquid chromatography (HPLC) analysis. The appropriate dried cells were digested with 1 mL of 96% $H_2SO_4$ at 100°C for 60 min to form crotonic acid from PHB. This reaction mixture was cooled to room temperature, the sample was then diluted with nine volumes of distilled water, and crotonic acid was determined using an HPLC system (Waters 2695 Separation with a TSKgel OApak-A ion-exclusion column, 7.8 mm × 30 cm; Tosoh, Tokyo, Japan). For the eluent, 0.75 mM $H_2SO_4$ was used at a flow rate of 0.6 mL $min^{-1}$. Detection was performed by monitoring absorption at 210 nm. Crotonic acid content was calculated using a calibration curve drawn using commercial PHB (18).

**Transmission electron microscope** After appropriate cultivation as described above, the cells were suspended in phosphate-buffered saline (PBS), prechemically fixed in PBS comprising 40.2% (w/v) paraformaldehyde and 20.3% (v/v) glutardialdehyde overnight at 4°C, and washed in PBS. The samples were washed and postfixed with 2% osmium oxide (w/v) at 4°C for 3 h. After being washed in PBS, the samples were dehydrated in a graded series of ethanol and propylene oxide solutions and then embedded in Spurr Lowicry K4M resin (Spurr Low Viscosity Embedding Kit; Polysciences, Warrington, PA, USA). Ultrathin resin sections of approximately 80-nm thickness were prepared using a diamond knife. The sections were stained with 4% uranyl acetate (v/w) for 30 min and washed with distilled water. The sections were then stained with lead citrate for 3 min, washed with distilled water, and dried. Samples were observed under a JEM-2000EX transmission electron microscope (JEOL, Tokyo, Japan), and the obtained images were analyzed using ImageJ v.1.48 software (National Institutes of Health, Bethesda, ME, USA, http://imagej.nih.gov/ij/).

**Phylogenetic analysis** Three PHA synthase genes of strain TH-1 were aligned with 90 sequences of PHA synthase from NCBI (http://www.ncbi.nlm.nih.gov/) using the ClustalW Multiple Sequence Alignment program. A phylogenetic tree was constructed and drawn using the neighbor-joining method and MEGA 7 program (http://www.megasoftware.net/mega.php).

## RESULTS

**Genomic analyses focusing on PHB synthesis** Genomic analyses focusing on PHB synthetic metabolism revealed that strain TH-1 comprises three copies of the acetyl-coenzyme A (CoA) acetyltransferase gene *atoB* (HPTL0267, HPTL0484, and HPTL0992), one copy of the acetoacetyl-CoA reductase gene *phbB* (HPTL1375), one copy of the 3-hydroxyacyl-CoA dehydrogenase/3-hydroxybutyryl-CoA epimerase gene *fadJ* (HPTL0993), and three copies of the polyhydroxyalkanoate synthase gene *phbC* (HPTL0263, HPTL0635, and HPTL1376). Among these synthetic genes, polyhydroxyalkanoate synthase (*phbC*) is the key enzyme for the polymerization (19). Also, to the best of our knowledge, this is the first time that the existence of three copies for polyhydroxyalkanoate synthase has been reported. Therefore, detailed analyses of *phbC* genes were performed. When alignment with the 90 polyhydroxyalkanoate synthases was performed, the three polyhydroxyalkanoate synthases from strain TH-1 were found to belong to class I of the polyhydroxyalkanoate synthases (Fig. S1). Class I polyhydroxyalkanoate synthase has been reported to preferentially make short-chain-length PHAs (19).

Five amino acids within the synthases are required for enzymatic activity; they are cysteine 319, aspartate 480, histidine 508, serine 260, and serine 546 in PhaC from *Ralstonia eutropha* (5,19,20). These amino acid residues within PHA synthases from strain TH-1 were also conserved (Fig. 1).

**Growth profile of strain TH-1** TH-1 had a specific growth rate of 0.68 $h^{-1}$ at 50°C under autotrophic conditions, whereas its growth on acetate, pyruvate, lactate, butyrate, and malate was analyzed under heterotrophic conditions (data not shown). The highest growth rate was recorded when strain TH-1 was grown on malate (0.88 $h^{-1}$). Therefore, malate was chosen as a substrate for heterotrophic growth in the PHB accumulation experiment.

**PHB production via a two-step batch cultivation** To investigate PHB accumulation and the effect of carbon source, strain TH-1 was cultivated by two-stage incubation. At the first stage, strain TH-1 was grown under autotrophic conditions with a gas component of $H_2$:$O_2$:$CO_2$ (75:10:15) or under heterotrophic conditions with malate until the optical density reached 2.0. However, PHB accumulation was not observed during this stage. Subsequently, the cells were transferred into a corresponding induction medium and incubated at 50°C to induce PHB production.

Table 1 summarizes the time course during the PHB accumulation experiment. PHB granules appeared after induction for 3 h under autotrophic induction conditions (Fig. 2C). Under heterotrophic conditions, PHB granules started to appear after induction for 15 min (Fig. 2F). Under autotrophic conditions, PHB amount reached 270.1 ± 14.6 mg $L^{-1}$, corresponding to 38.61% ± 4.06% (w/w) of the dry cells, after induction for 6 h. In addition, under heterotrophic conditions after a 3-h induction, PHB amount reached


FIG. 1. Multiple alignment of the partially deduced amino acid sequences of *phaC*. Three amino acid sequences for *phaC* from *H. thermoluteolus* TH-1 were aligned with their corresponding amino acid sequences from *R. sphaeroides* 2.4.1 (GenBank accession no. YP353458.1) and *R. eutropha* H16 (GenBank accession no. P23608.1).

**TABLE 1.** Result of two-phase culture of strain TH-1 for PHB production under autotrophic and heterotrophic condition.

| | Induced phase | | | |
|---|---|---|---|---|
| | Induction time | Dry cell weight (mg $L^{-1}$) | PHB concentration (mg $L^{-1}$) | PHB content within cells (%) |
| Autotrophic condition $H_2$:$O_2$:$CO_2$ (75:10:15) | 15 min | 667.8 ± 36.7 | ND | ND |
| | 3 h | 671.1 ± 25.5 | 107.3 ± 4.0 | 16.01 ± 1.27 |
| | 6 h | 702.2 ± 38.6 | 270.1 ± 14.6 | 38.61 ± 4.06 |
| | 9 h | 771.1 ± 56.7 | 167.4 ± 9.2 | 21.85 ± 2.82 |
| Heterotrophic condition (malate) | 15 min | 828.9 ± 32.0 | 36.9 ± 3.5 | 4.45 ± 0.34 |
| | 3 h | 801.1 ± 56.0 | 430.4 ± 14.3 | 53.82 ± 2.11 |
| | 6 h | 745.6 ± 61.6 | 372.7 ± 16.6 | 50.15 ± 3.53 |
| | 9 h | 833.3 ± 43.3 | 160.6 ± 11.4 | 19.26 ± 0.37 |

ND, not detected.

430.4 ± 14.3 mg $L^{-1}$ (53.82% ± 2.11%). Interestingly, the accumulated PHB began to decrease during the incubation period (Figs. 2 and 3; Table 1). In fact, PHB contents within the cells after induction for 9 h were 21.85% ± 2.82% and 19.26% ± 0.37% under autotrophic and heterotrophic conditions, respectively (Table 1).

## DISCUSSION

In this paper, we report that strain TH-1 has several sets of PHB-synthesizing genes. The distinctive characteristic of the genetic composition of strain TH-1 is the existence of three copies of *phbC* genes. To the best of our knowledge, only *R. eutropha*, *Pseudomonas oleovorans*, and *Pseudomonas aureofaciens* have been reported to have two copies of the *phbC* gene in their genome (5,21). The existence of multiple sets of PHB synthetic genes may be associated with the rapid metabolic response of strain TH-1, as can be seen in Fig. 2.

Here, we report that PHB accumulation began in strain TH-1 under nitrogen limitation under both autotrophic and heterotrophic conditions. Under autotrophic conditions, some cyanobacteria and bacteria produce PHAs (Table 2). Among them, a cyanobacterium *Synechococcus* sp. MA19 accumulates PHA up to 62% (w/w) of the dry cells in 260 h-cultivation with cell density of 2.3 (g $L^{-1}$) (8), with a PHA productivity of 0.002 g-PHA/g-cell·h. Also, *Alcaligenes eutrophus* ATCC 17697$^T$ produces PHA up to 82% (w/w) of dry cells after 40 h of cultivation, with a PHA productivity of 0.021 g-PHA/g-cell·h (12). Also, *Ideonella* sp. strain O-1 has the highest PHA productivity with 0.029 g-PHA/g-cell·h (15). In comparison, under autotrophic conditions, strain TH-1 produces PHB up to 39 % (w/w) of dry cells after 16 h of cultivation, with a PHB productivity of 0.024 g-PHB/g-cell·h. Although the PHB content in strain TH-1 is lower than *A. eutrophus* and the productivity is a little lower than *Ideonella*, strain TH-1 can be a suitable host to autotrophically

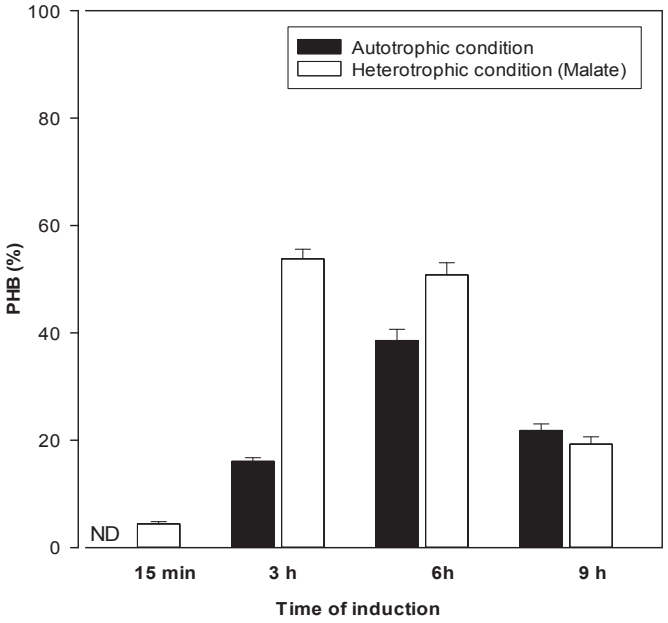


FIG. 3. Percentage of PHB accumulation after induction under autotrophic or heterotrophic conditions. Percentage of PHB was calculated using PHB content (weight) within the dried cells (weight). ND, not detected.

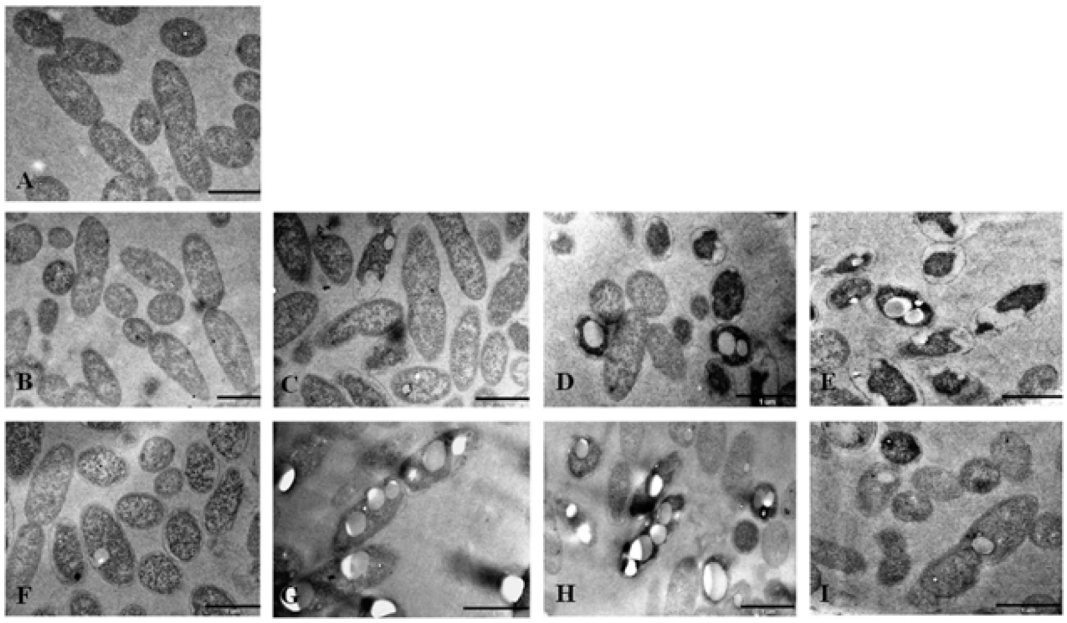


FIG. 2. Time course of PHB granule formation in strain TH-1. (A) Negative control: cells were grown under sufficient nutrition conditions. (B–E) Time course after incubation under autotrophic conditions: (B) 15 min, (C) 3 h, (D) 6 h, and (E) 9 h. (F–I) Time course after incubation under heterotrophic conditions with malate: (F) 15 min, (G) 3 h, (H) 6 h, and (I) 9 h. White areas within the bacteria are PHB inclusions. Scale bars: 1 μm.

**TABLE 2.** The production of PHB from $CO_2$ using a chemolithoautotrophic cultivation system.

| Strain | $H_2$:$O_2$:$CO_2$ (vol %) | Limitation | Cell concentration (g/l) | PHB concentration (g/l) | Cultivation period (h) | PHB productivity (g-PHB/g-cell·h) | Culture method | PHB content within cells (%) | Reference |
|---|---|---|---|---|---|---|---|---|---|
| *Hydrogenophilus thermoluteolus* TH-1 | 75:10:15 | N | 0.7 | 0.27 | 16 | 0.024 | Batch | 39 | This study |
| *Synechococcus* sp. MA19 | NA | P | 2.3 | 1.43 | 260 | 0.002 | Batch | 62 | 8 |
| *Alcaligenes eutrophus* H 16 | 60:10:20 | N | 18 | 14 | 70 | 0.011 | Air lift | 78 | 10 |
| *Alcaligenes eutrophus* ATCC 17697$^T$ | 75:15:10 | $O_2$ | 60 | 36 | 60 | 0.010 | Continuous | 60 | 11 |
| *Alcaligenes eutrophus* ATCC 17697$^T$ | 86.5:4.9:9.8 | $O_2$ | 26 | 22 | 40 | 0.021 | Batch | 82 | 12 |
| *Ralstonia eutropha* B-5786 | 60:20:10 | N | 30 | 22 | 70 | 0.010 | Continuous | 75 | 13 |
| *Ralstonia eutropha* ACM 1296 | 70:20:10 | $O_2$ | 16 | 6 | 40 | 0.009 | Continuous | 38 | 14 |
| *Ideonella* sp. strain O-1 | 70:10:10 | N | 7 | 5 | 24 | 0.029 | Continuous | 78 | 15 |

NA, not applicable.

produce PHB, i.e., from $CO_2$. Further research based on the fermentation technology is now underway.

Under heterotrophic conditions, PHB production was observed immediately after induction for 15 min (Fig. 2F). This implies that the cells have an efficient system to switch metabolism. Therefore, it would be useful and interesting to characterize the system regulating PHB formation, for which a project is also underway in our laboratory.

Rehm et al. reported that the Phasin proteins affect PHA granules size. Genome analysis revealed that strain TH-1 has 2 genes for phasin (HPTL1334 and HPTL0593), whose products would support PHA assembly (19). Therefore, these phasin genes may play an important role in PHB accumulation. During the experiment, we found that the amount of PHB granules started to decrease after 6 and 9 h of incubation under heterotrophic and autotrophic conditions, respectively. In contrast, TH-1 genome contains no phaZ (PHB depolymerase). Recently, the presence of thiolysis activity was reported for PHA synthase. PHA can be degraded thiolysis with CoASH (22,23). In case of strain TH-1, either of the polyhydroxyalkanoate synthases might be active in thiolysis or some other enzyme(s) may catalyze PHB degradation, which could be resolved by future studies.

Supplementary data to this article can be found online at https://doi.org/10.1016/j.jbiosc.2018.11.011.

## ACKNOWLEDGMENTS

This work was partly supported by a research grant from The Iwatani Naoji Foundation. The authors would like to thank Enago for the English language review.

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