Production of poly(3-hydroyxybutylate) by a novel alginolytic bacterium *Hydrogenophaga* sp. strain UMI-18 using alginate as a sole carbon source

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A novel alginolytic bacterium *Hydrogenophaga* sp. strain UMI-18 that produces poly(3-hydroyxybutylate) (PHB) in the alginate-mineral salt (AMS) medium containing 1% (w/v) sodium alginate as a sole carbon source was isolated from a decayed brown seaweed litter. The yield of PHB produced by strain UMI-18 was 1.1 ± 0.15 g/L of AMS and the PHB content in dried cell pellet was 58 ± 4% (w/w). Glucose, fructose, galactose, mannose, mannitol, sucrose and lactose were also available for the production of PHB by strain UMI-18. The yield of PHB in 1% (w/v) carbohydrate media reached 2.03—2.24 g/L for glucose and fructose, 0.75—1.64 g/L for alginate, galactose, mannitol and sucrose, and ~0.15 g/L for lactose. The PHB produced by strain UMI-18 showed a glass-transition temperature (T_g) at 4°C, a melting temperature at 175°C, and an average molecular mass of 860 kDa. Draft genome analysis of the strain UMI-18 revealed that an alginate-assimilating gene cluster is located in contig 8 comprising 453,520 bp and the PHB-synthesis gene cluster is in contig 15 comprising 653,793 bp.

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[**Key words:** Poly(3-hydroyxybutylate); Alginate; Alginate-assimilating gene cluster; Poly(3-hydroyxybutylate)-synthesis gene cluster; *Hydrogenophaga* sp. UMI-18]

Bacterial polyhydroxyalkanoates (PHAs) are known as intracellular storage materials that are produced under limited nutrition conditions (1–3). They are also recognized as environmentally friendly bioplastics because of their biodegradability and carbon neutral properties. Chemical structure of monomer units of PHAs are diverse, i.e., more than 150 kinds of monomer hydroxyalkanoate units with different chain structures and branch lengths have been found (4,5). Based on the number of carbon atoms contained in the monomer, PHAs have been classified to short-chain-length PHAs (scl-PHAs) comprising monomers of 3–5 carbon-atoms and medium-chain-length PHAs (mcl-PHAs) comprising monomers of 6–14 carbon atoms. Generally, scl-PHAs exhibit hard and brittle properties due to their crystalline structure, while mcl-PHAs exhibit elastic and adhesive properties due to their lower crystalline configuration (6–8). The biodegradability of bacterial PHAs is an advantageous property over petroleum-derived plastics, which are causing various environmental problems and the deterioration of the ecosystem (9–12). Furthermore, biocompatibility of PHAs makes them as materials for medical applications (13).

Up to now, many PHA-producing bacteria have been investigated and the majority of the PHA produced were identified as poly(3-hydroxybutyrate) (PHB). Consequently, physicochemical properties and biosynthesis pathway of the bacterial PHB have been extensively studied (14–21). In the industrial production of

PHB, carbohydrates and lipids from terrestrial plants are generally used as carbon sources (10,22). The use of terrestrial biomass for the production of bioplastics, however, have been apprehensive for causing food *vs.* material conflicts as in the case of bioethanol production using starch and molasses (23,24). On the other hand, most of seaweed biomass in the marine environment are still underutilized and expected as a carbon source for biomaterial production. Particularly brown algae, which contains approximately ~20% alginate and ~10% mannitol (w/w, in dry weight), are attractive as a marine biomass for the production of various industrial materials (24,25). Actually, ethanol fermentation of alginate and mannitol was realized by using genetically modified microbes (26,27). Production of PHB were also succeeded by several PHB-producing bacteria isolated from marine environment. Namely, *Massilia* sp. strain UMI-21 and *Burkholderia* sp. strain AIU M5M02 isolated from seaweeds were used for the production of PHB using starch and mannitol as carbon sources, respectively (28,29).

Alginate is one of the most abundant algal polysaccharide. It has been used as a viscosifier and gelling agent (25,30,31). Brown algae, *Macrocystis* sp., *Saccharina* sp., and *Ascophyllum* sp. are good sources for alginate production (30). Other brown algae are also available as alginate sources. Despite the abundance of alginate, this polysaccharide has not yet been used for the bacterial PHA production. This may be due to the rare occurrence or the uncultivability of alginolytic-PHA producing bacteria. Isolation of this kind of bacteria will make brown algae a promising carbohydrate source for PHA production. Thus, under these circumstances, we had attempted to isolate alginolytic PHA-producing bacteria from

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marine environment, and recently succeeded to isolate an algino-lytic bacterium, *Hydrogenohaga* sp. strain UMI-18 from a decayed seaweed litter. This strain was found to be capable of producing PHA in a medium containing alginate as a sole carbon source.

In the present study, we report the production of PHA by the strain UMI-18 in an alginate-mineral-salt (AMS) medium and other carbon source media. We also report some physicochemical properties of the PHA and the co-occurrence of alginate-assimilating and PHB-synthesis gene clusters in the strain UMI-18 genome.

MATERIALS AND METHODS

Materials   Sodium alginate (*Macrocystis pyrifera* origin, 300–400 cp) was purchased from Fujifilm Wako Pure Chemical Industry Ltd. (Tokyo, Japan). Poly-tetrafluoroethylene (PTFE) membrane filter (Omnipore, 0.45 μm pore size, 47 mm diameter) was from Merch KGaA (Dermstadt, Germany) and the PHB used as a standard material was from Sigma–Aldrich (St. Louis, MO, USA). Other reagents were purchased from Fujifilm Wako Pure Chemical Industry Ltd.

Screening of alginolytic PHB-producing bacteria   Five grams of decayed brown seaweed litter (comprising mainly of *Sargassum* sp.), which was collected in the shore of Hakodate, Hokkaido, Japan in May 2014, was added to 50 mL of 1% (w/v) alginate-containing mineral salt medium (AMS medium) (mineral salt (MS) medium: 3.6 g/L Na_2HPO_4, 0.75 g/L KH_2PO_4, 0.5 g/L NH_4Cl, 0.2 g/L $MgSO_4 \cdot 7H_2O$ and 0.1 mL of trace element solution (9.3 g/L $FeCl_3$, 7.8 g/L $CaCl_2$, 0.22 g/L $CoCl_2 \cdot 6H_2O$, 0.16 g/L $CuSO_4 \cdot 5H_2O$, 0.12 g/L $NiCl_2 \cdot 6H_2O$, 0.11 g/L $CrCl_3 \cdot 6H_2O$ in 0.1 N HCl)), and incubated at 25°C for a week with shaking at 150 rpm in a BR-43FL shaker (Taitec, Tokyo, Japan). The enriched culture was diluted 10–100 times with MS medium, and each 50 μL aliquot was spread on 1.5% agar plates containing AMS medium and 1 μg/mL Nile-red and cultivated at 25°C for 5 days. The colonies showing red fluorescence upon irradiation at 312 nm were selected as PHA-synthesizing bacteria candidates. Bacterial isolates were identified by the sequence analysis for 16S ribosomal RNA (rRNA) gene and by the morphological and biochemical analyses at Techno Suruga Laboratory Co. Ltd. (Shizuoka, Japan).

Preparation of PHA from alginolytic bacteria   Alginolytic PHA-producing bacteria were inoculated to 25 mL of AMS medium and precultured at 30°C with shaking at 150 rpm until OD_{600} reached 1.2. The preculture was transferred to 1 L of AMS medium and further cultivated at 30°C for 72 h. Bacteria were then harvested by centrifugation at $4000 \times g$ for 20 min and the pellet was added to 1 L of nitrogen-limited AMS medium (NH_4Cl concentration was reduced to 1/10 of the AMS medium) and cultivated at 30°C to induce PHA production. After 72-h cultivation, bacteria were collected by centrifugation at $8000 \times g$ for 5 min and rinsed with distilled water and lyophilized. The dried cell pellet was suspended in 10 mL chloroform and incubated at 60°C for 3 h to extract PHA. The extract was cooled at room temperature and filtered through PTFE membrane. To the filtrate, 10 volume of methanol was added to precipitate PHA. The precipitates were collected by centrifugation at $8000 \times g$ for 5 min and dried in vacuo. The PHA was further purified by repeating methanol precipitation from chloroform solution. The average yield of PHB and its content in the dried cell pellet were estimated from the triplicate experiments and shown with standard deviations.

Characterization of PHA produced by alginolytic bacteria   Chain structure of the PHA produced by the alginolytic bacteria was first investigated by the crotonic acid method (32). Briefly, 0.01 mL of PHA (1 mg/mL in chloroform) was added to 10 mL of concentrated sulfuric acid and incubated at 100°C for 10 min. After cooling at room temperature, UV absorption spectrum was recorded with U-3010 spectrophotometer (Hitachi High Tech Science, Tokyo, Japan). Formation of crotonic acid from PHB was assessed by detecting maximum absorption at 235 nm. Structure of PHA was also analyzed by ^{1}H NMR with ECP-400 NMR spectrometer (400 MHz; JOEL, Tokyo, Japan) at the Global Facility Center of Hokkaido University, Japan. Differential scanning calorimetry (DSC) profile of PHA was determined with a DSC1 calorimeter (Mettler Toledo, Tokyo, Japan). Temperature scanning was carried out at 10°C/min from −50°C to 200°C with a constant nitrogen flow at 50 mL/min. Viscosity of PHA in chloroform was determined with an Ostwald viscometer (flow time for solvent; 16 s). Briefly, PHA was dissolved in chloroform at 60°C to make 1.0–4.0 mg/mL and the flow time of the PHA solution was measured at 30°C to determine the intrinsic viscosity [η]. Molecular weight of PHA was estimated using the formula of Mark–Houwink–Sakurada: $[\eta] = KM^{\alpha}$ with K and α values, 1.18×10^{-5} mL/mg and 0.78, respectively (33).

Bacterial PHA production using different carbon sources   The alginolytic bacterium was precultured in an AMS medium until OD_{600} reached 1.2. Then, 2 mL of the preculture was transferred to a 100 mL of MS medium containing 1% (w/v) glucose, fructose, galactose, mannose, xylose, mannitol, sucrose, lactose, starch or cellulose. The medium was incubated at 30°C with 150 rpm shaking. For every 12 h, 1 mL of culture was aseptically withdrawn to measure cell density with OD_{600}. The amount of PHB produced was also evaluated every 24 h by the crotonic acid method (32). Briefly, 1 mL of culture was centrifuged at $12,000 \times g$ for 2 min and the bacterial pellet was rinsed with distilled water and lyophilized. PHB was extracted from the pellet at 60°C for 3 h with 1 mL of chloroform. The extract (5 μL) was then added to 5 mL of concentrated sulfuric acid and incubated at 100°C for 10 min. The amount of PHB was determined by measuring absorbance at 235 nm of the crotonic acid derived from the PHB by using a calibration curve drawn with known amount of commercial PHB (Sigma–Aldrich). At the end of cultivation, i.e., at 120-h cultivation, the remaining media were separately centrifuged at $12,000 \times g$ for 5 min, and the dried cell weights and PHB yields were determined to estimate PHB contents in the dried cells. The average values of OD_{660} of the media and PHB yields in triplicate experiments were shown with standard deviations.

Genome analysis for the alginolytic PHA-producing bacterium   Genomic DNA of the bacterium was extracted using ISOHAIR DNA extraction kit (Nippon Gene, Tokyo, Japan). Genome sequence was analyzed with Illumina Hiseq X
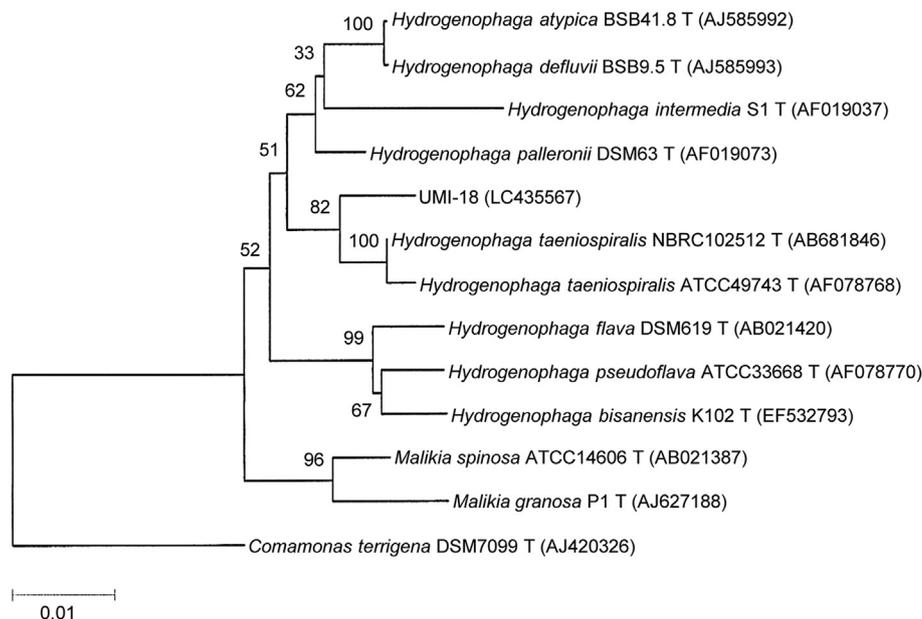

FIG. 1. Phylogenetic tree of the 16S rRNA sequences of *Hydrogenophaga* sp. strain UMI-18 and other related bacteria. The tree was generated using neighbor-joining method. *Comamnas terrigena* DSM7099T was used as an out group. Numbers at tree branches indicate the bootstrap values using 1000 replicates.

Sequencer (Illumina, Inc., San Diego, CA, USA) at Hokkaido System Science Co. Ltd. (Sapporo, Japan). The whole genome was assembled using the Platform for Assembling Nucleotide Sequences (PLATANUS) (http://platanus.bio.titech.ac.jp). Then, the genes in the assembled genome were annotated using Microbial Genome Annotation Pipeline (MiGAP) (https://www.migap.org) and Basic Local Alignment Search Tool (BLAST) (https://blast.ncbi.nlm.nih.gov/Blast.cgi).

RESULTS

Isolation and characterization of alginolytic PHA-producing bacteria   We could isolate 4 strains of alginolytic PHA-producing bacteria by the screening of ∼2000 colonies. Among the four isolates, strain UMI-18 seemed to be the most promising bacterium because it showed the highest growth rate and PHA yield in the AMS medium. Therefore, we focused on the strain UMI-18 as an alginolytic PHA-producing bacterium in this study. BLAST search for its 16S rRNA (DDBJ accession number LC435567) indicated that this strain belongs to the genus *Hydrogenophaga* (Fig. 1). Its 16S rRNA gene sequence showed the highest sequence similarity (98.8%) to that of *Hydrogenophaga taeniospralis* NBRC strain 102512 (GenBank accession number AB681846). Strain UMI-18 was gram-negative and rod-shaped ($0.7-0.8 \times 1.2-1.5$ μm) and formed smooth, tallow, circular convex and entire edged colony with the size less than 1 mm. It was positive in catalase and oxidase tests and negative in acid and gas production from glucose and in oxidation and fermentation tests. These properties along with other biochemical characteristics (Supplementary Table S1) indicated that strain UMI-18 belongs to the genus *Hydrogenophaga*. Thus, in this study, we concluded that the strain UMI-18 is a *Hydrogenophaga* sp.

Characterization of PHA produced by the strain UMI-18   Dried cell pellet (1.9 ± 0.3 g) of strain UMI-18 was obtained from 1 L of the nitrogen limited AMS culture by 72-h cultivation and 1.1 ± 0.15 g of PHA was extracted from the pellet. The yield of PHA from the pellet was 58 ± 4% (w/w). The PHA was regarded as polyhydroxybutyrate (PHB) because crotonic acid-like material showing a maximal absorbance at 235 nm was generated by the sulfuric acid treatment (Supplementary Fig. S1). Its UV absorption spectrum was indistinguishable from the spectrum of crotonic acid derived from the commercial PHB (Sigma−Aldrich). Chemical structure of the PHA was also analyzed by ^{1}H NMR. As shown in Fig. 2A, peak signals were seen at 1.27−1.28 ppm, 2.44−2.63 ppm and 5.22−5.30 ppm, which are ascribable to methyl, methylene and methine groups of PHB, respectively. These characteristics in ^{1}H NMR spectrum were practically the same as those of PHB reported previously (21,29). On the bases of the crotonic-acid analysis and ^{1}H NMR spectrometry, the PHA produced by strain UMI-18 was regarded as PHB. According to the DSC analysis, glass-transition temperature (T_g) and melting temperature (T_m) values for the PHB were determined as 4°C and 175°C, respectively (Fig. 2B). By the viscosity measurement, an average molecular weight of the PHB was determined as 860 kDa with an average degree of polymerization 10,000.

Production of PHB by the strain UMI-18 in different carbon-source media   To examine if strain UMI-18 can use carbon sources other than alginate for the production of PHB, the strain was cultivated in MS medium containing 1% (w/v) glucose, fructose, galactose, mannose, xylose, mannitol, sucrose, lactose, starch or cellulose, and the growth rate and PHB production in these media were examined. As shown in Fig. 3A, OD$_{600}$ of the culture reached 6.9 ± 0.1 in AMS medium at 48 h and then gradually decreased to 5.2 ± 0.1 at 120 h. This decrease was considered to be due to the alkalization of the AMS medium by the consumption of acidic substrate, i.e., alginic acid, in the medium. Actually, pH of the AMS medium was increased to >9

after 48-h cultivation. Such significant pH change was not observed in other media containing neutral carbohydrate substrates. Strain UMI-18 could grow in the media containing glucose, fructose, galactose, mannose, mannitol, sucrose and lactose (Fig. 3A). The maximum OD$_{600}$ (and appropriate cultivation time) for each carbon source was as follows: glucose, 15.6 ± 0.5 (84 h); fructose, 14.1 ± 0.1 (84 h); galactose, 9.7 ± 0.1 (96 h); mannose, 6.3 ± 0.1 (120 h); mannitol, 8.9 ± 0.2 (120 h); sucrose; 7.5 ± 0.1 (120 h); lactose, 1.4 ± 0.1 (120 h). On the other hand, no growth was observed in the medium containing starch, cellulose or xylose. These results suggest that strain UMI-18 does not possess amylase and cellulase and also xylose assimilating enzymes. According to the growth rates, glucose and fructose appeared to be the most preferable substrates for strain UMI-18, and alginate was also regarded as a good substrate comparable with galactose, mannitol and sucrose. Mannose and lactose were considered to be a potential carbon source; however, a long lag phase was observed before a log phase.

As shown in Fig. 3B, the yield of PHB reached 1.25 ± 0.04 g/L in AMS medium at 48-h cultivation and then decreased to 0.75 ± 0.06 at 120 h. This decrease was also considered to be due
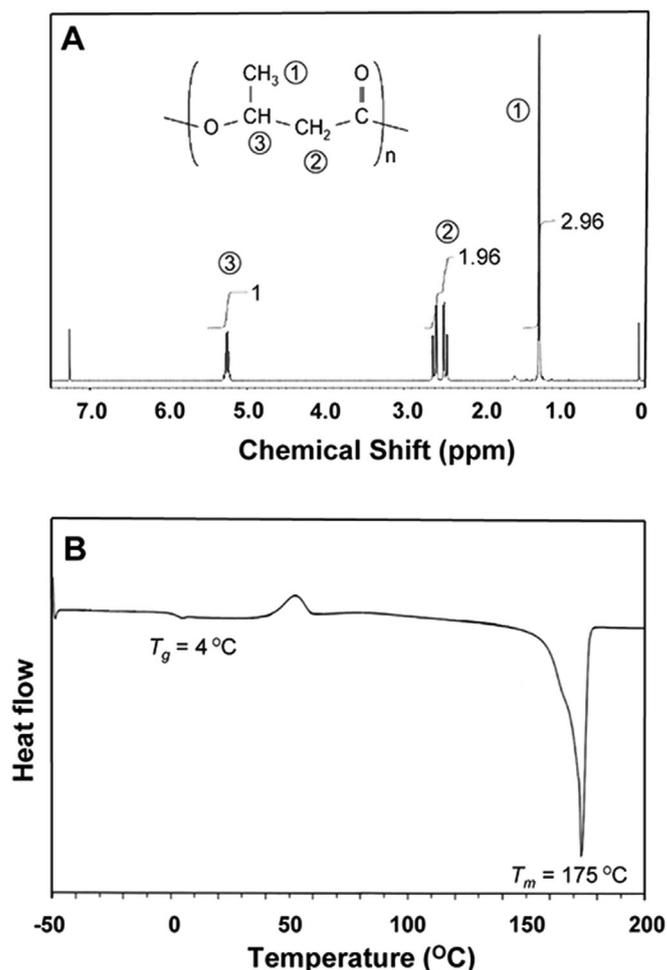

FIG. 2. NMR and DSC analyses for PHA from strain UMI-18. (A) ^{1}H NMR spectrum of PHA from strain UMI-18. PHA from strain UMI-18 was dissolved in deuterated chloroform to make 5 mg/mL and the ^{1}H NMR was measured at 27°C with EPC-400 NMR spectrometer (400 MHz; JOEL). Numbers in circles indicate the respective proton atoms and their corresponding signals, respectively. The integral values for respective signals are also shown. (B) DSC thermogram of PHB from strain UMI-18. DHC profile of the PHB was recorded under nitrogen gas flow of 50 mL/min and temperature increasing rate of 10°C/min using DSC1 calorimeter (Mettler Toled). T_g, glass transition temperature. T_m, melting temperature.
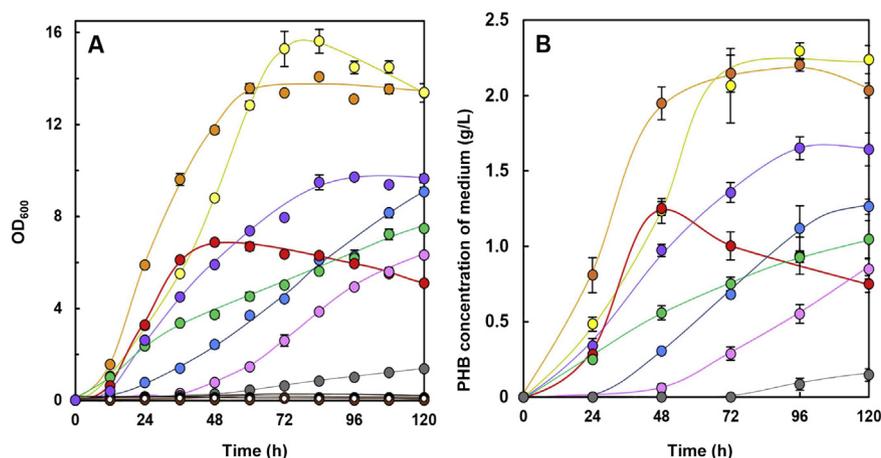
FIG. 3. Growth of strain UMI-18 and production of PHB in MS media containing different carbon sources. (A) Growth curves of strain UMI-18. Strain UMI-18 was grown at 30°C in MS media containing 1% different carbohydrates, i.e., alginate (AMS medium, red), glucose (yellow), fructose (orange), galactose (purple), mannose (pink), mannitol (blue), xylose (white), sucrose (green), lactose (gray), starch (brown), and cellulose (black). (B) Production of PHB by strain UMI-18. One milliliter of each culture medium was withdrawn and centrifuged at appropriate time intervals and PHB was extracted from the cell pellet after lyophilization. The amount of PHB extracted was determined by crotonic acid method. Colors of symbols are the same as in panel A.

to the alkalization of the medium, which may lead the bacteria to death phase. The maximal yields of PHB (at appropriate cultivation time) with different carbon-source media were as follows: glucose, 2.30 ± 0.06 g/L (96 h); fructose, 2.20 ± 0.04 g/L (96 h); galactose, 1.65 ± 0.08 g/L (96 h); mannose, 0.85 ± 0.07 g/L (120 h); mannitol, 1.26 ± 0.05 g/L (120 h); sucrose, 1.05 ± 0.12 g/L (120 h); and lactose, 0.15 ± 0.04 g/L (120 h). Then, dried cell weights, PHB yields and PHB contents at 120-h cultivation in the different carbon sources were determined (Table 1). Cultivation in glucose and fructose media resulted in the highest PHB yields (2.03−2.24 g/L) and high PHB contents (71.4−71.8 % (w/w)) in the dried cells. While, alginate, galactose, mannitol, mannose and sucrose media resulted in moderate PHB yields (0.75−1.64 g/L) and PHB contents (43.2−68.5% (w/w)). Lactose medium resulted in the lowest PHB yield (0.15 g/L) and PHB content ($\sim$22.0% (w/w)). Thus, glucose and fructose were confirmed to be the preferable carbon sources for the production of PHB, and alginate was also a good carbon source comparable with galactose, mannitol, mannose and sucrose.

Analysis for strain UMI-18 genome The assembled genome of the strain UMI-18 comprised 39 contigs with 1004−653,793 base pairs. Putative alginate lyase genes for HyAly-I, -II, -III and -IV, which were tentatively classified under polysaccharide-lyase families (PL, http://www.cazy.org/) −17, −7, −15, and −5, respectively, were located in one alginate-assimilating-gene cluster found in contig 8 (453,520 bp) (Fig. 4A). In this cluster, genes encoding alginate-metabolic enzymes that are essential for assimilating 4-deoxy-L-erythro-5-hexoseulose uronic acid (DEH) to pyruvate and glyceraldehyde-3-phosphate were also located. Two genes encoding short chain dehydrogenases (SDR) (DEH-

reductase, HyDehR-I and II), one encoding 2-keto-3-deoxy-D-gluconate kinase (KDG kinase, HyKdgK) and one encoding 2-keto-3-deoxy-6-phosphogluconate aldolase (KDPG aldlase, HyKdpgA) were also found. Several transporter genes (HyABC transporter-a, -b, -c and -d; HyMFS transporter; Hyporin) that are necessary in incorporating alginate oligosaccharides and DEH into the cell were also found in the same gene cluster. Occurrence of these genes provides the genetic basis of the alginate-assimilating capability of strain UMI-18. Further, three enzymes responsible for PHB-synthesis, i.e., β-ketothiolase (HyPhaA), NADPH-dependent acetoacetyl CoA reductase (HyPhaB) and Class I PHA synthase (HyPhaC), were found in contig 15 (653,793 bp) to be assembled in a single cluster (Fig. 4B). The presence of alginate-assimilating gene cluster and PHB-synthesis gene cluster in its genome supports the ability of strain UMI-18 to assimilate alginate and produce PHB.

DISCUSSION

In the present study, we could successfully isolate the alginolytic PHB-producing bacterium strain UMI-18 from the decayed brown seaweed litter. According to its 16S rRNA gene sequence and its physiological properties, strain UMI-18 was regarded as a novel strain of *Hydrogenophaga* (Fig. 1). Many species of *Hydrogenophaga* have been reported to produce PHA using different carbon sources, that is, *Hydrogenophaga* sp. produced PHA using sucrose as a carbon source (34), while *Hydrogenophaga pseudoflave*, which is a closely related species to strain UMI-18 produced PHB using glucose, fructose, galactose, xylose, mannose and lactose (35,36). *Hydrogenophaga palleroni* produced poly(3-hydroxybutyrate-co-hydroxyvarate) P(3HB-co-3HV) using volatile fatty acids as carbon source (37). Similarly, *Malikia spinose* and *Malikia granosa*, which are closely related to *Hydrogenophaga*, were also reported to synthesize PHA (38). However, there has been no report on the *Hydrogenophaga* that uses alginate as a carbon source for the production of PHA. Similarly to *H. pseudoflave* (35,36), strain UMI-18 assimilated glucose, fructose, mannose and lactose (Fig. 3A); however, it did not grow in xylose medium unlike *H. pseudoflave*. Thus, strain UMI-18 was regarded as the novel *Hydrogenophaga* sp. that can assimilate alginate producing PHB.

The strain UMI-18 produced 1.1 ± 0.15 g of PHB in 1 L AMS medium. The amount of PHB accounts for $58 \pm 4\%$ (w/w) of the total

TABLE 1. Dried cell weights, PHB yields and PHB contents of strain UMI-18.

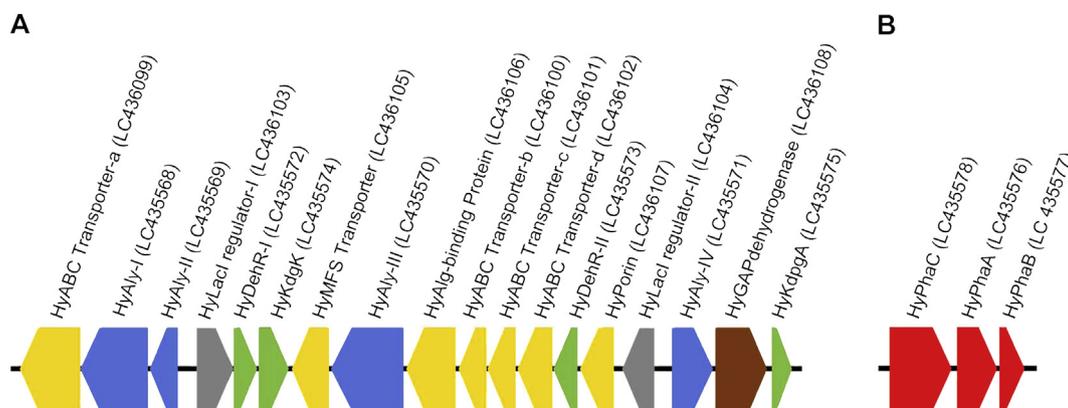| Carbon sources (1% (w/v))	Dried cell weight (g/L)	PHB yield (g/L)	PHB content (% (w/w))
Alginate	1.73 ± 0.24	0.75 ± 0.06	43.2 ± 3.21
Glucose	3.13 ± 0.15	2.24 ± 0.09	71.4 ± 2.99
Fructose	2.83 ± 0.15	2.03 ± 0.05	71.8 ± 1.74
Galactose	2.40 ± 0.25	1.64 ± 0.11	68.5 ± 4.54
Mannitol	2.27 ± 0.18	1.26 ± 0.05	55.7 ± 2.27
Mannose	1.73 ± 0.15	0.85 ± 0.07	49.0 ± 3.85
Sucrose	1.97 ± 0.10	1.05 ± 0.12	53.2 ± 6.27
Lactose	0.67 ± 0.12	0.15 ± 0.04	22.0 ± 6.15

FIG. 4. Schematic representation of the alginolytic gene cluster and the PHB synthesis gene cluster of strain UMI-18. (A) Arrangement of each gene in the alginolytic gene cluster of 24.4 kbp. Colors used for are; alginate lyase genes (blue), DEH-metabolic enzyme genes (green), transporter genes (yellow), transcription regulator gene (gray) and GAP dehydrogenase gene (brown). (B) Arrangement of the PHB synthesis genes in the cluster comprising 4.0 kbp. Nucleotide sequences of the genes are available in DDBJ/EMBL/GenBank with the accession numbers following gene annotations.

TABLE 2. Properties of PHB produced by *Hydrogenophaga* sp. strain UMI-18 and other PHB-producing bacteria.

Strain	Carbon source	T_g[a] (ºC)	T_m[b] (ºC)	M_w[c] (KDa)	Reference
Hydrogenophaga sp. strain UMI-18	Alginate	4	175	860[d]	Present study
Azotobacter chroococcum	Sucrose	2.6−4.1	177.6−179.8	1740−2378[d]	Savenkova et al. [33]
Hydrogenophaga pseudoflava	Lactose	N.D.[e]	178.9	827[f]	Koller et al. [36]
Hydrogenophaga sp.	Sucrose	2.32	173.31	N.D.[e]	Tanamool et al. [34]
Cupriavidus necator	Glycerol	3.8	175.1	890[f]	Tanadchangsaeng and Yu [39]
Massilia sp. strain UMI-21	Starch	N.D.[e]	N.D.[e]	760[f]	Han et al. [28]
Burkholderia sp. strain AIU M5M02	Mannitol	N.D.[e]	N.D.[e]	1860[f]	Yamada et al. [29]
Bacillus megaterium	Fructose	6	176	N.D.[e]	Pradhan et al. [40]

[a] T_g, glass transition temperature.
[b] T_m, melting temperature.
[c] M_w, molecular weight.
[d] Calculated by viscosimetric measurements.
[e] N.D., not determined.
[f] Measured by size exclusion chromatography.

dried cell weight. This strain can use glucose, fructose, galactose, mannose, mannitol, sucrose and lactose for the production of PHB. Among these carbohydrates, glucose and fructose were the most preferable substrates. Alginate was also considered to be a good substrate; however, alkalization of the alginate medium during cultivation appeared to repress the growth of strain UMI-18. Thus, it seemed important to maintain pH of the medium for the production of PHB in high yield. General physicochemical properties of the PHB, e.g., T_g, T_m and molecular weight, were comparable to those of other PHBs reported in *Massilia* sp. strain UMI-21 [28], *Burkholderia* sp. strain AIU M5M02 [29], *Azotobacter chroococcum* [33], *Hydrogenophaga* sp. [34], *Hydrogenophaga pseudoflava* [36], *Cupriavidus necator* [39], and *Bacillus megaterium* [40] (Table 2).

The alginate-assimilating gene cluster and PHB-synthesis gene cluster were identified in the draft genome of strain UMI-18 (Fig. 4A, B). It appeared to have an alginate-metabolic pathway similar to those of *Flavobacterium* sp. strain UMI-01 [41,42] and *Sphingomonas* sp. strain A1 [27]. In this pathway, alginate is initially degraded to monosaccharide (DEH) by the synergistic action of endolytic and exolytic alginate lyases in the periplasmic space and cytosol. Then, the DEH is reduced to KDG by DEH reductases in cytosol and the KDG is phosphorylated to KDPG by KDG kinase. Finally, KDPG is split to pyruvate and glyceraldehyde-3-phosphate by KDPG aldolase, and they are metabolized through the central metabolic pathway. In strain UMI-18, a part of the pyruvate derived from alginate is considered to be used for PHB synthesis. Actually, the enzymes responsible for the production of PHB from pyruvate, i.e., β-ketothiolase, NADPH-dependent acetoacetyl-CoA synthetase and

Class I PHA synthase, are encoded in the genome of strain UMI-18 as HyPhaA, HyPhaB and HyPhaC genes, respectively (Fig. 4B). The deduced amino-acid sequences of HyPhaA, HyPhaB and HyPhaC genes showed 93%, 97% and 84% identities to those of putative PhaA (GenBank accession number WP_066088257), PhaB (GenBank accession number WP_066088255) and PhaC (GenBank accession number WP_066088259) of *Hydrogenophaga crassostreae*, respectively, while 72%, 73% and 61% identities with those of PhaA (GenBank accession number WP_010810132), PhaB (GenBank accession number WP_010810131) and PhaC (GenBank accession number WP_011615085) of *C. necator*, respectively.

We further investigated by BLAST search if alginate lyase genes are present in some members of the family *Comamonadaceae*, which include *Hydrogenophaga* sp. and *Makilia* sp. However, no species possessing the alginate lyase genes was found. Thus, strain UMI-18 was considered to be a novel alginolytic species of the genus *Hydrogenophaga*. It may be possible to consider that the alginolytic gene cluster of the strain UMI-18 was acquired through horizontal gene transfer from other alginolytic bacteria. BLAST search revealed that the deduced amino-acid sequences of alginate lyases HyAly-IV and HyAly-III of strain UMI-18 show 50% and 57% sequence identity to A1-III (PL-5 alginate lyase) (GenBank accession number BAB03312.1) and A1-IV (PL-15 alginate lyase) (GenBank accession number BAB03319.1) of *Sphingomonas* sp. strain A1, respectively. HyAly-I and HyAly-II were regarded as PL-17 and PL-7 alginate lyases, respectively. It is necessary to characterize these alginate lyases to reveal their roles in the complete depolymerization of alginate.

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