Polyhydroxyalkanoate production by a novel bacterium *Massilia* sp. UMI-21 isolated from seaweed, and molecular cloning of its polyhydroxyalkanoate synthase gene

(Running title: PHA production by a bacterium isolated from seaweed)

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ABSTRACT

We successfully isolated one microorganism (UMI-21) from *Ulva*, a green algae that contains starch. The strain UMI-21 can produce polyhydroxyalkanoate (PHA) from starch, maltotriose, or maltose as a sole carbon source. Taxonomic studies and 16S rDNA sequence analysis revealed that strain UMI-21 was phylogenetically related to species of the genus *Massilia*. The PHA content under the cultivation condition using a 10-L jar fermenter was 45.5% (w/w). This value was higher than that obtained after cultivation in a flask, suggesting the possibility of large-scale PHA production by UMI-21 from starch. A major issue for the industrial production of microbial PHAs is the very high production cost. Starch is a relatively inexpensive substrate that is also found in abundant seaweeds such as *Ulva*. Therefore, the strain isolated in this study may be very useful for producing PHA from seaweeds containing polysaccharides such as starch. In addition, a 3.7-kbp DNA fragment containing the whole PHA synthase gene (*phaC*) was obtained from the strain UMI-21. The results of open reading frame (ORF) analysis suggested that the DNA fragment contained two ORFs, which were composed of 1,740 (*phaC*) and 564 bp (*phaR*). The deduced amino acid sequence of PhaC from strain UMI-21 shared high similarity with PhaC from *Ralstonia eutropha*, which is a representative PHA-producing bacterium with a class I PHA synthase. This is the first report for the cloning of the PHA synthase gene from *Massilia* species.

INTRODUCTION

Polyhydroxyalkanoates (PHAs), which are accumulated by numerous bacteria as carbon and energy storage materials under nutrient limitation and carbon excess, are aliphatic polyesters with thermoplasticity, optical activity, and biodegradability (1,2). More than 150 different monomers have been reported in PHAs (3). Based on the number of carbon atoms in their monomeric units, PHAs are classified into two groups, short-chain-length (scl) PHAs with C3 to C5 monomer units and medium-chain-length (mcl) PHAs with C6 to C14 monomer units (4). In general, scl PHAs exhibit thermoplastic properties and are mainly produced by *Ralstonia eutropha* (*R. eutropha*) strains, whereas mcl PHAs usually have elastomer- or adhesive-like properties and are synthesized by *Pseudomonas* species (5).

PHAs, which originate from renewable resources such as sugars and vegetable oils, have attracted great interest as alternatives to petrochemical-based plastics because of their material properties are similar to those of various thermoplastics and elastomers and their complete biodegradability (1,2). To date, several PHAs, such as poly(3-hydroxybutyrate) [P(3HB)], poly(3HB-*co*-3-hydroxyvalerate) [P(3HB-*co*-3HV)], and poly(3HB-*co*-3-hydroxyhexanoate) [P(3HB-*co*-3HHx)], have been commercially produced by microbial fermentation. However, a major issue regarding the industrial production of microbial PHAs is that their production cost is much higher than that of conventional petrochemical-based plastic materials. For industrial microbial production of PHAs, the cost of the fermentative substrate constitutes a significant proportion of the total cost (1,6,7). The cost of biopolymer production could be reduced by using inexpensive biomass, by-products, and waste streams as carbon sources, which can also help to convert these materials into value-added products. Accordingly, numerous research efforts have been made to exploit inexpensive fermentable raw materials as substrates for PHA production. For instance, we reported PHA

production from waste glycerol forming a biodiesel fuel production process by *Pseudomonas* sp. SG4502 (8). Gonzalez-Lopez et al. reported P(3HB) and P(3HB-*co*-3HV) production from alpechin, a wastewater from olive oil mills that is rich in sugars and phenolic compounds, by *Azotobacter chroococcum* H23 (9). Although various kinds of biomass have been used for material production by bacteria, most of them were obtained from land. Seaweed is an abundant biomass in the sea, and inedible seaweed exists in large quantities. In addition, seaweeds contain various kinds of polysaccharides such as alginic acid and starch, among others; therefore, they are a very attractive carbon source for material production by bacteria.

In this study, we attempted to screen for PHA-producing bacteria on *Ulva*, a green algae that contains starch. By using enrichment cultivation, we succeeded in isolating one microorganism, designated UMI-21, that can produce P(3HB) from starch as a sole carbon source. Based on the results of the 16S rDNA sequencing and phenotypic and phylogenetic analyses, strain UMI-21 was identified as *Massilia* sp. Furthermore, we succeeded in cloning a *pha* locus from the UMI-21 bacterium, and identified two open reading frames encoding PHA synthase (PhaC) and a repressor protein (PhaR). In addition to the production of PHA from various carbon sources, including glucose, maltose, maltotriose, and starch, the composition of the *phaC* gene cluster was also discussed in this article.

MATERIALS AND METHODS

Chemicals Peptone, yeast extract, casamino acids, glucose, corn starch, soluble starch, D-maltose, D-maltotriose, disodium hydrogen phosphate (Na_2HPO_4), potassium dihydrogen phosphate (KH_2PO_4), ammonium chloride (NH_4Cl), and magnesium sulfate heptahydrate ($MgSO_4 \cdot 7H_2O$) were purchased from Wako Pure Chemical Industries (Osaka, Japan). All other chemicals were of reagent-grade quality or better.

Media Mineral salt (MS) medium, containing 3.6 g of Na_2HPO_4, 1.5 g of KH_2PO_4, 1.0 g of NH_4Cl, 0.2 g of $MgSO_4 \cdot 7H_2O$, and 1 mL of trace element solution per liter of distilled water (10), was used for the screening. Kato *et al*. reported that a higher C/N ratio promoted PHA accumulation in *Pseudomonas* sp. (10). Therefore, NH_4Cl was decreased to 0.5 $g \cdot L^{-1}$ for PHA production. Nutrient-rich (NR) medium containing 10 g of peptone, 2 g of yeast extract, and 10 g of meat extract per liter of distilled water was used for precultivation of the bacteria. Modified MS (MSM) medium, containing 3.6 g of Na_2HPO_4, 1.5 g of KH_2PO_4, 4.0 g of NH_4Cl, 1.2 g of $MgSO_4 \cdot 7H_2O$, and 1 mL of trace element solution per liter of distilled water, was used for cultivation in a jar fermenter. R2A medium, containing 0.5 g of peptone, 0.5 g of yeast extract, 0.5 g of casamino acids, 0.5 g of glucose, 1.5 g of starch, 0.3 g of $KHPO_4$, 0.05 g of $MgSO_4 \cdot 7H_2O$, 0.3 g of sodium pyruvate, and 15 g of agar per liter of distilled water, was used for growth on plates.

Isolation of bacteria accumulating polyhydroxyalkanoate *Ulva*, a seaweed collected from the coast, was used as a source of PHA-producing bacteria. Each seaweed sample was added to 50 mL of MS medium containing 1% (w/v) starch and incubated for 3 days at 30°C with shaking at 150 $strokes \cdot min^{-1}$. After repeating the subcultures several times, each culture was streaked on MS agar medium containing 1% (w/v) starch and incubated at 30°C for 72 h. The isolated strains were tested for PHA synthesis. The cells were inoculated in 5 mL of NR medium at 30°C for 16 h. Then, the cultures were inoculated into 100 mL of nitrogen-limited MS medium containing 1% (w/v) starch in 500-mL Erlenmeyer flasks with baffles and incubated at 30°C for 72 h with shaking at 150 rpm. The cells were collected by centrifugation at 10,000 × g for 10 min. The collected cells were washed twice with distilled water and then lyophilized. The dried cell extracts were subjected to gas chromatography (GC) analysis as described in the following paragraph.

Production of polyhydroxyalkanoate The isolated PHA-producing bacterium UMI-21 was cultured

in 5 mL of NR medium at 30°C for 24 h. The cultures were inoculated into 100 mL of nitrogen-limited MS medium containing a carbon source (2% [w/v]) in 500-mL Erlenmeyer flasks with baffles, and then incubated at 30°C with shaking at 150 rpm for 72 h. Corn starch, soluble starch, glucose, maltose, and, maltotriose were used as carbon sources. Bacterial cells were collected by centrifugation. The collected bacterial cells were washed twice with distilled water, and then lyophilized. Polymer was extracted from the dried microbial cell bodies by using a Soxhlet extractor with chloroform as a solvent, and then precipitated by the addition of methanol after the solvent was removed with an evaporator. The cell dry weights, PHA yields, and PHA contents were described as average values of three independent samples with standard deviations.

To evaluate the possibility of using UMI-21 for the large-scale production of PHA from starch, the bacterium was cultivated in a 10-L jar fermenter (BMS-10NP3; ABLE & Biott Co., Ltd., Tokyo, Japan). First, UMI-21 was cultured in 250 mL of NR medium at 30°C for 24 h. Then, the starter culture was inoculated into 5 L of MSM medium containing 5% (w/v) soluble starch in a 10-L jar fermenter and incubated at 30°C with shaking at 150 rpm for 72 h. The carbon source concentration and C/N ratio in the MSM medium for jar fermenter experiment was increased to improve the cell growth and PHA content. The product was prepared as described above.

Analyses The isolated PHA-producing bacterium UMI-21 was cultivated on an R2A plate, and genomic DNA was prepared from a single colony with PrepMan Ultra Reagent (Life Technologies Japan, Tokyo, Japan). The PCR product was purified with the QIAquick Gel Extraction Kit (Qiagen Japan, Tokyo, Japan), and then directly sequenced with the ABI PRISM 3100 Genetic Analyzer (Life Technologies Japan). The strain UMI-21 16S rDNA sequence was determined by using BLAST (http://blast.ncbi.nlm.nih.gov.Blast.cgi), and the sequence was deposited in the DNA Data Bank of Japan (DDBJ; accession number AB823088).

Polymer composition and accumulation were analyzed with the GC-2010 capillary GC system (Shimadzu Co., Kyoto, Japan) equipped with a flame ionization detector and a Zebron ZB-1 GC capillary column (0.25 mm i.d. × 30 m; Phenomenex, Inc., CA, USA). A sample was prepared as follows: approximately 10 mg of polymer or 50 mg of dried microbial cell was treated with a solution containing 1.7 ml of methanol, 0.3 ml of sulfuric acid and 2.0 ml of chloroform at 100°C for 3 h to convert the constituents to their methyl esters. One milliliter of water was added to the reaction mixture, and then, the lower chloroform layer was used for GC analysis.

The ^{1}H-NMR spectra of the polymers were obtained in deuterated chloroform ($CDCl_3$) using a Bruker MSL400 spectrometer (400 MHz) at a 90° pulse with a 4-ms, 3000-Hz spectral width and a 4-s repetition rate. The chemical shifts were reported in ppm with tetramethylsilane as the internal reference.

The molecular weights of the obtained polymers were determined by using a gel-permeation chromatography (GPC) system (Shimadzu Corporation, Kyoto, Japan) equipped with tandem TSKgel Super HZM-H columns (6.0 mm i.d. × 150 mm; Tosoh Corporation, Tokyo, Japan) at 40°C. Chloroform was used as an eluate, and the calibration was performed using polystyrene samples as standards.

Cloning and sequencing of the *pha* locus from UMI-21 Two primers, phaCF1_FW (5′-ATCAACAA(G/A)T(T/A)CTAC(A/G)TC(C/T)T(C/G)GACCT-3′) and phaCR4_RV (5′-AGGTAGTTGT(T/C)GAC(C/G)(A/C)(A/C)(G/A)TAG(T/G)TCCA-3′), were used to obtain a partial DNA fragment of the PHA synthase gene from UMI-21 (11). DNA was amplified by PCR using UMI-21 genomic DNA as a template and KOD FX polymerase (Toyobo Co., Ltd., Osaka, Japan). The reaction conditions consisted of an initial 2-min denaturation step at 94°C, followed by 35 cycles of 98°C for 10 s, 50°C for 30 s, and 68°C for 1 min, with a final elongation step of 68°C for

5 min (12). The amplified 500-bp DNA fragment was purified with a gel extraction kit (Qiagen Japan). The nucleotide sequence of the fragment was determined with the ABI PRISM 3100 Genetic Analyzer. The upper (5'-region of *phaC* gene) and lower (3'-region of *phaC* and whole *phaR* genes) regions of the amplified 500-bp DNA fragment were cloned with the Takara LA PCR™ in vitro Cloning Kit (Takara Bio, Inc.) using cassettes and cassette-primers method.

RESULTS

Phenotypic and phylogenetic characterization of a bacterium producing PHA To obtain bacteria capable of producing PHA from a seaweed *Ulva*, we first performed enrichment cultivation in MS medium containing starch as a carbon source. *Ulva*, a seaweed collected from the coast, was used as a source of PHA-producing bacteria. The culture solution was spread onto an MS plate containing 1% (w/v) starch, and then incubated at 30°C for 72 h. The amount and monomer composition of the PHA accumulated in the bacterial cells were determined by GC analysis. As a result, we found a bacterium that could produce P(3HB) from starch and named the bacterium UMI-21.

We analyzed the microbiological properties of strain UMI-21, and found that strain UMI-21 is an aerobic, gram-negative, motile, non-spore-forming, rod-shaped bacillus (0.8–1.0 × 1.5–2.0 μm). The strain was able to grow at temperatures of up to 30°C and at pH 5.5–8.0 in MS medium containing 1% (w/v) starch. Anaerobic growth did not occur in the presence of nitrate. Tween-80, esculin, and starch were hydrolyzed by the bacterium, but urea, arginine, and gelatine were not hydrolyzed. Strain UMI-21 did not assimilate L-arabinose, D-mannose, D-mannitol, *N*-acetyl-D-glucosamine, potassium gluconate, *n*-capric acid, adipic acid, DL-malic acid, sodium citrate, or phenylacetic acid.

Analysis of the 16S rDNA sequence using BLAST indicated that strain UMI-21 belongs to the genus *Massilia* and that the closest phylogenetic neighbors of UMI-21 are *Massilia alkalitolerans*

(96.3% identity; accession number AY679161) and *Massilia varians* (97.8% identity; accession number AM774587). Phylogenetic analyses using Molecular Evolutionary Genetics Analysis (MEGA) software (ver. 4.0) showed that strain UMI-21 was clustered with other bacteria of the genus *Massilia* (Fig. 1). Based on the results of the 16S rDNA sequencing and phenotypic and phylogenetic analyses, strain UMI-21 was identified as *Massilia* sp. (13–18).

Polyhydroxyalkanoate productivity of strain UMI-21 To investigate the PHA productivity of strain UMI-21 from starch, the strain was cultivated under nitrogen-limiting conditions in MS medium supplemented with corn starch or soluble starch as a carbon source. The polymers that accumulated in the cell were extracted from dried cells with chloroform and were analyzed. When corn and soluble starch were used as the carbon sources, strain UMI-21 reached total biomasses of 3.95±0.32g·L^{-1} and 3.31±0.03 g·L^{-1}, respectively (Table 1). The PHA content in the cells was 30.3% (w/w) and 27.2% (w/w), respectively (Table 1). The weight-average molecular weight (M_w) and polydispersity (M_w/M_n) of the products made from corn starch were 7.6×10^5 and 2.8, respectively (Table 1). The ^{1}H NMR and GC analyses clearly indicated that the product was P(3HB) (Fig. 2).

Starch is a high molecular weight homopolymer of glucose. In general, bacteria incorporate monosaccharides and/or oligosaccharides generated by starch-hydrolysing enzymes such as amylase and gluco-amylase. To confirm the assimilation mechanism, we analyzed PHA production by UMI-21 using glucose, maltose, and maltotriose as carbon sources. When maltose and maltotriose were used as carbon sources, the same results were observed as those using starch. In contrast, both cell growth and PHA production were significantly lower when glucose was used as a carbon source than when starch was used. These results strongly suggest that strain UMI-21 degrades starch and incorporates the resulting oligosaccharides such as maltose and maltotriose. Therefore, strain UMI-21 could have a specific incorporation mechanism for maltooligosaccharides. Their molecular

weights and polydispersities were 7.6–9.5 × 10^5 and 2.6–2.9, respectively, and they were comparable to each other.

Polyhydroxyalkanoate production using a jar fermenter To evaluate the possibility of using UMI-21 for the large-scale production of PHA from starch, the bacterium was cultivated in a 10-L jar fermenter. The result of the ^{1}H-NMR analysis suggested that the polymer produced by the cultivation of UMI-21 using a jar fermenter was P(3HB). The total biomass (cell dry weight) and PHA content were 8.29 g·L^{-1} and 45.5% (w/w), respectively. These values were higher than those obtained after cultivation in a flask, suggesting the possibility of large-scale PHA production by UMI-21 from starch.

Cloning and sequencing of DNA fragments containing the PHA synthase gene (*phaC*) The PHA synthesis genes of these bacteria have not yet been identified, though PHA production from starch by *Massilia* species has been reported (19). We attempted to clone the PHA synthesis genes of UMI-21 as follows. First, approximately 500-bp DNA fragment was amplified by PCR using primers phaCF1_FW and phaCR4_RV, which were designed based on the consensus sequence of *phaC* (11). The nucleotide sequence of the fragment was determined with the ABI PRISM 3100 Genetic Analyzer and was subjected to BLAST analysis. The BLAST results indicated that the fragment was a part of a PHA synthase gene. The Takara LA PCR™ in vitro Cloning Kit was used to clone a gene fragment containing the whole *phaC* gene and surrounding regions. Finally, a 3.7-kbp DNA fragment containing the whole *phaC* gene was obtained (Fig. 3). The nucleotide sequence of the fragment was determined by primer walking (Fig. 3). The results of open reading frame (ORF) analysis suggested that the DNA fragment contained two ORFs, which were composed of 1,740 and 564 bp (DDBJ accession no. AB823089). For *phaC* gene, the putative promoter regions are underlined and the putative ribosomal binding site is boxed (Fig. 3-b).

A database search with the ORFs using Protein BLAST showed that the ORFs shared sequence similarity with PhaC and PhaR (Table 2). PHA synthases are divided into four classes (classes I to IV) based on their activities toward substrates with different carbon chain lengths and their subunit compositions (4). The deduced amino acid sequence of PhaC from strain UMI-21 shared high similarity with PhaC from *R. eutropha*, which is a representative PHA-producing bacterium with a class I PHA synthase (Table 2).

Figure 4 shows partial sequence alignments of the PHA synthases (PhaCs) from strain UMI-21, *Janthinobacterium* sp. HH01, *Herbaspirillum seropedicae*, *R. eutropha* H16, V*ariovorax paradoxus*, and *Hydrogenophaga* sp. PBC. It has been reported that all PHA synthases contain a putative lipase box, [Gly-Xaa-(Ser/Cys)-Xaa-Gly-Gly], in which the essential active site serine is replaced with a cysteine in the PHA synthase (3,20). The PhaC from strain UMI-21 also contained a putative lipase box with a cysteine residue (Fig. 4). Furthermore, Rehm *et al*. reported that the highly conserved Cys-319, Asp-480, and His-508 residues in PhaC from *R. eutropha* constituted the catalytic triad (20). Since these amino acids are conserved in the sequences deduced from *phaC* in strain UMI-21 (Fig. 4: Cys-315, Asp-461, and His-493), they may constitute a catalytic triad.

DISCUSSION

We successfully isolated a novel bacterium, strain UMI-21, that is able to accumulate PHA from starch. Taxonomic studies and 16S rDNA gene-sequence analysis revealed that strain UMI-21 was phylogenetically related to species of the genus *Massilia* (13–18). The closest species is *M. varians* CCUG 35299; PHA productivity has not been reported for this species. Moreover, strain UMI-21 could not assimilate L-arabinose, D-mannose, D-mannitol, N-acetyl-D-glucosamine, potassium gluconate, n-capric acid, adipic acid, DL-malic acid, sodium citrate, and phenyl acetate. As a result, we concluded that strain UMI-21 was a *Massilia* sp.

Massilia timonae was for the first time isolated and identified from the blood of an immunocompromised patient with cerebellar lesions by La Scola et al. (13). The genus *Massilia* belongs to the family *Oxalobacteraceae*, and 18 species of this genus have been isolated from the various environmental conditions such as air, soil, and water. Recently, Cerrone *et al*. reported that bacteria in the genus *Massilia* can accumulate PHA from starch, and *M. albidiflava*, *M. lutea*, *M. dura*, *M. plicata*, *M. brevitalea*, *M. aerilata*, and *M. aurea* were used in their experiment (19). Some strains could produce PHA, and higher PHA productivity was achieved when starch was used as the carbon source in the growth medium instead of glucose (19). These results are identical to those we observed in our experiment with UMI-21. In general, bacteria incorporate monosaccharides and/or oligosaccharides generated by hydrolysis with starch-hydrolysing enzymes such as amylase and gluco-amylase because starch is a high molecular weight polymer composed of glucose. Interestingly, more PHA was obtained when starch was used as a carbon source. Therefore, to estimate the mechanism underlying the assimilation of starch in strain UMI-21, we measured PHA production from glucose, maltose, and maltotriose. When maltose or maltotriose was used as a carbon source, PHA content increased up to 40% (w/w), suggesting that oligosaccharides are preferably ingested by the bacterium. In contrast, since cells did not grow primarily when glucose was used as a carbon source (Table 1), glucose was not ingested by the bacterium. In *Saccharomyces cerevisiae*, the Agt1 transporter has relatively high activity towards maltotriose and maltose (21), which is related to the uptake of maltose and maltotriose by the cells. A transporter specific for cyclodextrins (CDs), which are produced from maltodextrins by CD-glucanotransferases (CGTases), has been reported for bacteria such as *Klebsiella oxytoca* (22,23). Although the detailed metabolic mechanism of starch hydrolysis in strain UMI-21 is yet unknown, UMI-21 may have an uptake mechanism for maltose and maltotriose. Since glucose is a common carbon source for most microorganisms, these unique

substrate-uptake mechanisms for starch metabolism could be a strategy of winning competitors. Further studies are needed to determine the unique metabolic system for starch utilization in UMI-21.

The nucleotide sequence of more than 50 PHA synthase genes from a wide variety of bacteria has been determined. PHA synthases are classified into four classes according to their primary structures deduced from these sequences, substrate specificities, and subunit composition (4). Class I and class II PHA synthases comprise enzymes consisting of only one type of subunit (PhaC). Class III PHA synthases comprise enzymes consisting of two different types of subunits, PhaC and PhaE. Class IV PHA synthases comprise enzymes consisting of two different types of subunits. Based on the result that strain UMI-21 produced P(3HB) and the deduced amino acid sequence of PhaC shared similarity with PhaC from *R. eutropha*, which is a representative PHA-producing bacterium with a class I PHA synthase, we concluded that UMI-21 PhaC belonged to class I.

PHA synthase genes and other genes related to the metabolism of PHA are often clustered in bacterial genomes. In *R. eutropha*, the genes encoding the class I PHA synthase (*phaC*), β-ketothiolase (*phaA*), and NADP-dependent acetoacetyl-CoA reductase (*phaB*) constitute the *phaCAB* operon (24). In UMI-21, *phaR* is downstream of *phaC* instead of *phaA* and *phaB*. Very recently, the sequence of *M. timonae* CCUG 45783 (accession No. NZ_JH992933) has been determined. Although *phaR* was downstream of *phaC*, as in strain UMI-21, other genes related to PHA synthesis, such as *phaA* and *phaB*, were not found near *phaC* and *phaR*. In *R. eutropha*, PhaR is proposed to function as a repressor of *phaP* and *phaR* transcription under conditions in which P(3HB) does not accumulate (25). Once the accumulation of P(3HB) is initiated, PhaR is thought to dissociate from the promoter and bind to oligomeric or polymeric forms of $(HB)_n$. The PhaR proteins from *R. eutropha* and strain UMI-21 are approximately 80% similar. Therefore, strain UMI-21 PhaR could have a function similar to that of *R. eutropha* PhaR (26), though the existence of *phaP* genes have not

been confirmed in strain UMI-21.

Thus far, mainly land biomass has been used as a source for material production by bacteria. For PHA production, starch from plants was widely used as a carbon source (27–32). Although seaweed is eaten as a food, inedible seaweed also exists in large quantities. In addition, seaweeds contain various kinds of polysaccharides such as alginic acid, starch, and others; therefore, seaweed is a very attractive carbon source for material production by bacteria. We isolated a novel bacterium from a green algae (*Ulva*) in order to produce PHA from seaweeds containing polysaccharides such as starch. The isolated bacterium, strain UMI-21, can produce PHA by using a high molecular-weight starch as a carbon source and may have the ability to utilize other polysaccharides in addition to starch. Although further examination is needed to realise large scale PHA production using seaweed as a carbon source, detailed analyses of the assimilation mechanism for starch and other polysaccharides in strain UMI-21 could contribute to the low-cost production of PHA from seaweed. The production of PHA using starch from *Ulva* is now in progress and will be reported in the near future.

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Figure and Table Legends

Fig. 1 Phylogenetic tree of strain UMI-21 and related microorganisms. The tree is based on an alignment of the 16S rDNA genes of UMI-21 and type strains in the genera *Massilia* (14 strains), *Janthinobacterium* (2 strains), and *Oxalobacter* (1 strain).

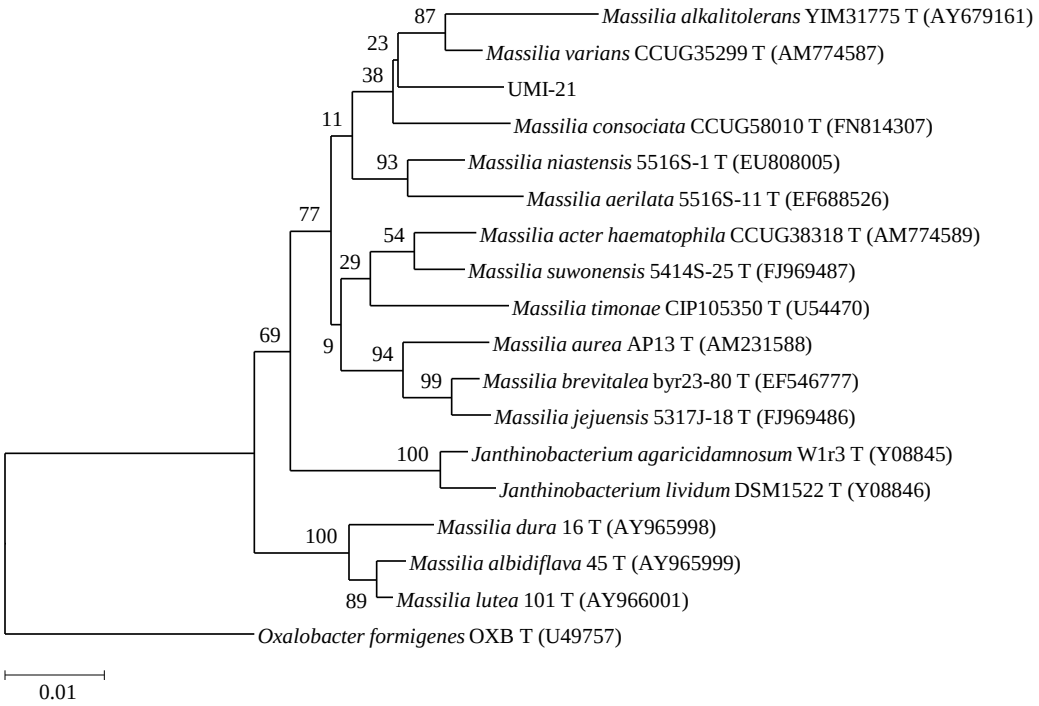
Fig. 2 ^{1}H-NMR spectrum of the polymer produced by UMI-21 from corn starch.

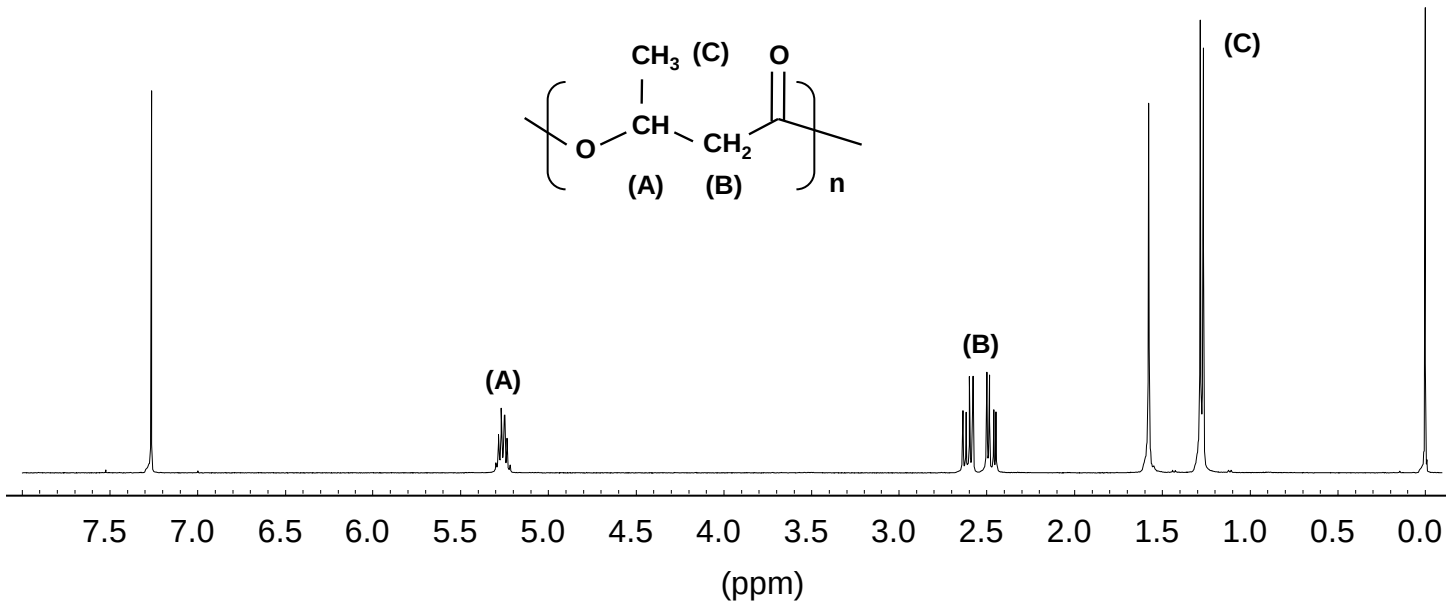
Fig. 3 (a) Organization of the UMI-21 PHA biosynthesis genes (*phaC* and *phaR*) and the DNA sequencing strategy and (b) the putative promoter regions and ribosomal binding site for *phaC* gene. The arrows show the primers and the sequenced regions. The putative promoter regions are underlined and the putative ribosomal binding site is boxed.

Fig. 4 Alignment of the partial deduced amino acid sequences of PHA synthases from strain UMI-21 and similar proteins from *Janthinobacterium* sp. HH01, *Herbaspirillum seropedicae*, *Ralstonia eutropha* H16, *Variovorax paradoxus*, *Hydrogenophaga* sp. PBC. The portions of the PHA synthases thought to comprise the catalytic domain, including the putative catalytic triad (C-D-H), are shown.

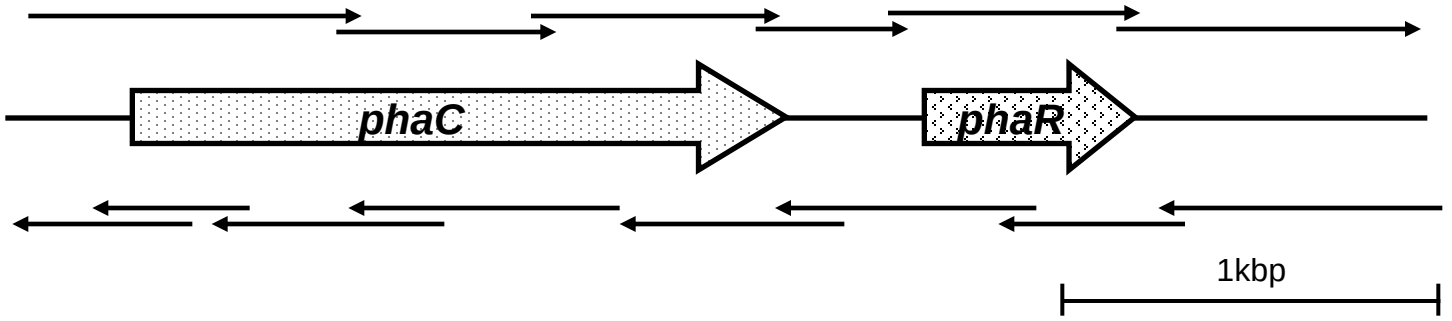
Table 1 Cell dry weights (CDWs), PHA yields, molecular weights, polydispersities, PHA contents, and monomer compositions of PHAs produced by UMI-21.

Table 2 Homology of the PHA synthesis gene products of strain UMI-21 to proteins of other bacteria.


87
Massilia alkalitolerans YIM31775 T (AY679161)
23
Massilia varians CCUG35299 T (AM774587)
38
UMI-21
11
Massilia consociata CCUG58010 T (FN814307)
93
Massilia niastensis 5516S-1 T (EU808005)
Massilia aerilata 5516S-11 T (EF688526)
77
54
Massilia acter haematophila CCUG38318 T (AM774589)
29
Massilia suwonensis 5414S-25 T (FJ969487)
Massilia timonae CIP105350 T (U54470)
69
9
94
Massilia aurea AP13 T (AM231588)
99
Massilia brevitalea byr23-80 T (EF546777)
Massilia jejuensis 5317J-18 T (FJ969486)
100
Janthinobacterium agaricidamnosum W1r3 T (Y08845)
Janthinobacterium lividum DSM1522 T (Y08846)
100
Massilia dura 16 T (AY965998)
Massilia albidiflava 45 T (AY965999)
89
Massilia lutea 101 T (AY966001)
Oxalobacter formigenes OXB T (U49757)
0.01


CH3 (C)
O
CH
O
CH2
(A)
(B)
n
(C)
(B)
(A)
7.5
7.0
6.5
6.0
5.5
5.0
4.5
4.0
3.5
3.0
2.5
2.0
1.5
1.0
0.5
0.0
(ppm)

a



b CGGCGACGATCCACATGCTTTTCTCCAAGGTTGTAGACCCCGATTCTAAACAACGGATGCTGACATGAACATGCCT

M N M P

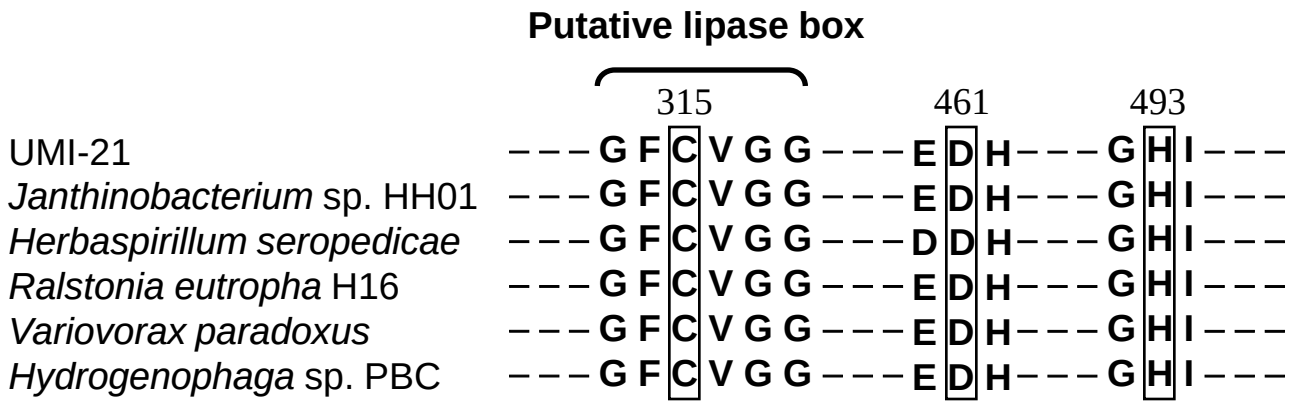

Putative lipase box
315
461
493
UMI-21 – – – G F C V G G – – – E D H – – – G H I – – –
Janthinobacterium sp. HH01 – – – G F C V G G – – – E D H – – – G H I – – –
Herbaspirillum seropedicae – – – G F C V G G – – – D D H – – – G H I – – –
Ralstonia eutropha H16 – – – G F C V G G – – – E D H – – – G H I – – –
Variovorax paradoxus – – – G F C V G G – – – E D H – – – G H I – – –
Hydrogenophaga sp. PBC – – – G F C V G G – – – E D H – – – G H I – – –

Table 1 Cell dry weights (CDWs), PHA yields, molecular weights, polydispersities, PHA contents, and monomer compositions of PHAs produced by UMI-21.

Carbon sources (2%)	CDW[a] (g/l)	PHA yield (g/l)	$M_w x10^{5}$[b]	M_w / M_n	PHA [%(w/w)%]	Momomer composition[c] (mol%)
						3HB
Corn starch	3.95±0.32	1.20±0.04	7.6	2.8	30.3±0.17	100
Soluble starch	3.31±0.03	0.90±0.06	8.7	2.9	27.2±1.66	100
Glucose	0.67±0.04	0.02±0.01	7.9	3.7	2.4±1.6	100
Maltose	3.68±0.08	1.39±0.08	7.6	2.6	37.7±1.31	100
Maltotriose	3.75±0.17	1.51±0.06	9.5	2.7	40.2±2.06	100

[a]Cell dry weight (CDW) was defined as cell components plus PHA.
[b]Molecular weights were determined by gel permeation chromatography (GPC).
[c]Monomer compositions were determined by ^{1}H-NMR and GC analyses.

Table 2 Homology of the PHA synthesis gene products of strain UMI-21 to proteins of other bacteria.

Organism	PhaC		PhaR	
	accession No.	identity (%)	accession No.	identity (%)
Janthinobacterium sp. HH01	WP_008447857.1	69	WP_008447858.1	87
Herbaspirillum seropedicae	YP_003776395.1	62	WP_017451769.1	80
Ralstonia eutropha H16	YP_725940.1	62	YP_725943.1	77
Variovorax paradoxus	WP_018902870.1	60	WP_018905029.1	62
Hydrogenophaga sp. PBC	WP_009515239.1	56	WP_009517543.1	61