Isolation of poly[D-lactate (LA)-*co*-3-hydroxybutyrate)]-degrading bacteria from soil and characterization of D-LA homo-oligomer degradation by the isolated strains

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Running title: Poly(D-lactate-*co*-3-hydroxybutyrate)-degrading bacteria

Abbreviation: PHA, Polyhydroxyalkanoates; PDLA, poly(D-lactic acid); DP, degree of polymerization; PHBH or P(3HB-*co*-3HHx), poly(3-hydroxybutyrate-*co*-3-hydroxyhexanoate).

Abstract

P[D-lactate (LA)-*co*-3-hydroxybutyrate (3HB)] is an artificial polyhydroxyalkanoate (PHA) containing unusual D-LA units. In this study, the P(D-LA-*co*-3HB)-degrading bacterial group in the soil was analyzed and the bacterial degradation of the D-LA clustering structure in the copolymer were evaluated by using chemically synthetic D-LA homo-oligomers. A total of 216 soil samples were screened on the basis of clear zone formation on agar plates containing emulsified P(64 mol% D-LA-*co*-3HB). The 16S rRNA analysis of the isolated bacteria resulted in the identification of eight *Variovorax*, three *Acidovorax*, and one *Burkholderia* strains, which are closely related to previously identified natural PHA-degrading bacteria. These bacteria nearly consumed the P(D-LA-*co*-3HB) emulsion in the liquid culture; however, a small amount of the D-LA fraction remained unconsumed, which should be attributable to the D-LA-clustering structure in the copolymer. Cultivation of the isolated bacteria with the D-LA homo-oligomers revealed that the oligomers with a degree of polymerization (DP) ranging from 10 to 30 were partly consumed by six *Variovorax* and one *Acidovorax* strains. In contrast, the

oligomers with DP ranging from 20 to 60 were not consumed by the isolated bacteria. These results indicate that D-LA homo-oligomers with DP higher than approximately 20 are hardly degraded by the soil bacteria. Molecular dynamic simulation of the D-LA homo-oligomers indicated that the upper limit of DP is likely to be determined by the conformational structure of the oligomers in water. The information obtained in this study will be useful for the molecular design of biodegradable D-LA-containing polymers.

Keywords: polyhydroxyalkanoate, bioplastic, biodegradable plastic, PDLA

Highlights (85 characters)

- 11 P(D-lactate-*co*-3-hydroxybutyrate)-degrading soil bacteria were isolated.
- D-LA homo-oligomers with approx. DP ≥ 20 were not consumed by the isolates.
- The conformations of D-LA homo-oligomers were estimated by MD simulation.

1. Introduction

Polyhydroxyalkanoates (PHAs) are bacterial polyesters produced from renewable resources. Currently, a PHA copolymer, poly(3-hydroxybutyrate-*co*-3-hydroxyhexanoate) [P(3HB-*co*-3HHx) or PHBH], is commercially manufactured as a commodity plastic by Kaneka Corp. (Japan). PHAs have attracted increasing attention because they exhibit superior biodegradability in various natural environments and controlled aerobic and anaerobic conditions [1,2]. In contrast, poly(L-lactic acid), the most widespread chemically synthesized bio-based plastic, is a compostable plastic that is degraded efficiently only under managed conditions [3]. There is limited information on the biodegradability of poly(D-lactic acid) (PDLA) (see Discussion).

A broad range of bacteria and fungi have been isolated from various environments as degraders of naturally occurring PHAs such as P(3HB) and PHBH by using clear zone formation on emulsified PHA-containing agar plates [1]. Techniques such as denaturing gradient gel electrophoresis and metagenomics have revealed that the microbial community varies depending on environmental factors such as soil, freshwater, and sea in different areas [4–9]. For example, PHA-degrading soil bacteria have been identified to belong to the genera *Acidovorax*, *Acinetobacter*, *Arthrobacter*, *Bacillus*, *Burkholderia*, *Cytophaga*, *Cupriavidus*, *Mycobacterium*, *Nocardiopsis*, *Pseudomonas*, *Rhizobium*, *Variovorax*, *Stenotrophomonas*, *Xanthomonas*, and *Zoogloea* and bacterium Ellin [1,6–8].

In 2008, artificial PHAs containing unusual D-lactate (LA) units were synthesized using the engineered PHA synthase PhaC1$_{Ps}$STQK, lactate-polymerizing enzyme (LPE) [10]. The artificial polymer, P(D-LA-*co*-3HB), possesses favorable physical properties such as semitransparency and flexibility [11,12]. P(D-LA-*co*-3HB) shares part of its structure with PDLA, and, therefore, the biodegradability of the copolymer has been of interest.

The screening of soil bacteria that can degrade P(67 mol% D-LA-*co*-3HB) resulted in the isolation of a Betaproteobacteria (Burkholderiales) bacterium, *Variovorax* sp. C34 [13]. PHA depolymerases (PhaZs) play a central role in PHA biodegradation [14]. PhaZ derived from *Variovorax* sp. C34 ($PhaZ_{Vs}$, Accession#, BAR87946.1) is capable of hydrolyzing all four types of ester bonds in P(67 mol% D-LA-*co*-3HB), i.e., the linkages of 3HB-3HB, 3HB-LA, LA-3HB, and LA-LA. However, the enzyme did not hydrolyze high-molecular-weight PDLA. The assay using D-LA homo-oligomers indicated that hydrolysis of the LA-LA linkage in the D-LA homopolymer by PhaZs was limited by the degree of polymerization (DP) of the substrate, and an inverse relationship was observed between the DP and hydrolysis efficiency [15]. The results prompted us to investigate the bacterial group that can degrade P(D-LA-*co*-3HB) and whether they exhibit the DP-dependent degradation of the D-LA homo-polymer.

The aim of this study was, therefore, to isolate the P(D-LA-*co*-3HB)-degrading bacterial group in the soil and evaluate their capacity of consuming D-LA homo-oligomers with different DPs, which were prepared via the finely controlled chemical polymerization. Using the candidate isolates, we discuss the biodegradability of the target polymers in terms of the hydrolysable DP of D-LA homo-oligomer together with a molecular dynamic study.

2. Materials and Methods

2.1. P(D-LA-*co*-3HB) production

P(D-LA-*co*-3HB) was biosynthesized using batch jar fermentation. The polymers with 64 and 67 mol% LA were obtained in two batches. Seed culture of the recombinant *E. coli* strain JWMB1 (a dual-gene-knockout mutant Δ*pflA*Δ*dld* of parent strain BW25113) harboring pTV118N*pctphaC*1(ST/FS/QK)AB [16] was prepared in 100 mL of Luria broth (LB) containing 100 mg/L ampicillin and 100 mg/L kanamycin in a flask for 7 h at 30 °C with reciprocal shaking at 120 rpm. The seed culture was used to inoculate 4 L of LB, including 20% xylose, 100 mg/L ampicillin, and 100 mg/L kanamycin in a 5 L jar fermenter (MDL-500; Marubishi Co., Ltd., Japan) and cultivated at 30 °C with agitation at 500 rpm and aeration at 1.5 vvm (volume of air/volume of jar/minute) for 48 h. The cells were harvested and lyophilized, and the polymer was extracted with chloroform at 60 °C for 2 days. The remaining cell debris was removed by using a PTFE filter with a pore size of 0.20 μm. The flow-through fraction was evaporated, and the precipitant was rinsed with hexane. The obtained polymer was weighed after overnight incubation to remove the solvents. The LA fraction of the obtained P(LA-*co*-3HB) was determined on the basis of the ^{1}H NMR spectrum, as described previously [17].

2.2. Preparation of D-LA polymer and oligomers

PDLA ($M_n = 3.8 \times 10^4$, $M_w/M_n = 1.96$) was chemically synthesized [13]. Two types of D-LA homo-oligomers with DP (LA units) of approximately 10–30 and 20–60 (Figure S2), designated as DLA20 and DLA40, respectively, were chemically synthesized as follows: In an argon-filled glove box, 3.47 mmol of 3-phenyl-1-propanol and D-lactide (38.9 mmol and 78.4 mmol for DLA20 and DLA40, respectively) were dissolved in 78.4 mL of dry dichloromethane (CH_2Cl_2). Then, 380 µmol and 755 µmol of 1,8-diazabicyclo[5.4.0]-7-undecene was added to the CH_2Cl_2 solution to initiate the polymerization of DLA20 and DLA40, respectively. After 2 min, the polymerization was quenched by the addition of benzoic acid (excess). The mixture was purified by reprecipitation from the CH_2Cl_2 solution into MeOH/*n*-hexane = 9/1 (v/v) to obtain PDLA as a white solid.

To measure the DP ranges of the D-LA homo-oligomers, 5 mg of the obtained oligomers (DLA20 and DLA40) was dissolved in 1 mL of tetrahydrofuran (THF) for MALDI-TOF-MS analysis. Two milligrams of *trans*-2-[3-(4-*tert*-butylphenyl)-2-methyl-2-propenylidene]malononitrile was dissolved in 50 µL of 0.1 w/v% trifluoroacetic acid in THF as the matrix solution for PLA. The polymer and matrix were mixed at a ratio of 1:4, 1:1, or 4:1 and applied to MALDI-TOF-MS (Microflex, Bruker Daltonics) with a linear mode setting, positive ion detection, 30% laser power, mass ranges from 0 to 3100 *m/z* and 1500 to 5000 *m/z* for DLA20 and DLA40, respectively. The MS intensities were integrated from several spots.

2.3. Preparation of polymer and oligomer emulsions

The polymer and oligomer emulsions were prepared using sonication with a modified method [13]. One hundred milligrams of P(LA-*co*-3HB) polymer was dissolved in 1 mL CH_2Cl_2 and heated at 60 °C for 5 min. The polymer solution was combined with 25 mL of water and emulsified by sonication (40 W) on ice for 60 s. One milliliter of Plysurf® A210G (DKS Co. Ltd. Kyoto, Japan), an anionic surfactant (phosphate ester) commonly used for bioplastic degradation assay, was added to prevent demulsification, and, then, CH_2Cl_2 was evaporated with gentle stirring at 60 °C for 1 h.

The PHBH emulsion was prepared as follows. One hundred milligrams of PHBH, which was kindly provided by Kaneka Corporation, Tokyo, Japan, was dissolved in 4 mL CH_2Cl_2 and heated at 60 °C for 30 min. The polymer solution was combined with 75 mL of water and emulsified by sonication on ice for 60 s. Five hundred microliters of Plysurf® was added, and CH_2Cl_2 was evaporated with gentle stirring at 60 °C overnight.

Fifty milligrams of D-LA oligomer was dissolved in 1 mL CH_2Cl_2 and heated at 60 °C for 30 min. The oligomer solution was combined with 25 mL of water, and the emulsion was prepared using the same protocol as that for PHBH.

2.4. Isolation and identification of bacterial strains

The previously prepared soil enrichment culture [13] was spread onto the P(D-LA-*co*-3HB) plate. Bacteria that formed a clear zone on the P(D-LA-*co*-3HB) plate within a week of cultivation were further isolated as single colonies. The isolated bacteria were identified on the basis of the partial 16S rRNA sequence. The 16S rRNA gene fragment was amplified directly from the colonies by using a pair of primers, 1100F (5′-CAACGAGCGCAACCCT-3′) and 1492R (5′-GGTTACCTTGTTACGACTT-3′), and KOD FX Neo DNA polymerase (Toyobo, Japan). The sequences of the partial rRNA were deposited into NCBI (LC513944-LC513958). BLASTn was used, and a phylogenetic tree was constructed with high homology sequences from other strains by MAFFT alignments [18] and RaxML [19].

2.5. Culture conditions

For screening with the P(D-LA-*co*-3HB) plate, 1 g of the emulsified P(D-LA-*co*-3HB) polymer was added to 1 L of a modified yeast extract (YE) medium containing 1 g $(NH_4)_2SO_4$, 0.25 g yeast extract, 0.1 g NaCl, 0.2 g $MgSO_4$ $7H_2O$, 0.1 g $CaCl_2$ $2H_2O$, 0.01 g $FeSO_4$ $7H_2O$, and 0.8 mg $MnSO_4$ $5H_2O$ in 10 mM KH_2/K_2HPO_4 (pH 7.0) including 1.5% agar. To measure P(D-LA-*co*-3HB) degradation ability, the isolated bacteria were inoculated in 2 mL of YE broth containing 1 g/L emulsified P(D-LA-*co*-3HB) polymer in a test tube and cultivated at 30 °C at 180 rpm for 7 days. Non-inoculated culture YE broth and *Variovorax* sp. C34 (LC513959) culture served as negative and positive controls, respectively. To evaluate D-LA homo-oligomer-degrading ability, the isolated bacteria were also used to inoculate 2 mL of YE broth containing 0.5 g/L emulsified DLA20 and DLA40 as well as 0.5 g/L PHBH in a test tube and cultivated at 30 °C at 180 rpm for 7 days.

2.6. GC analyses

After cultivation, the whole culture was frozen in liquid nitrogen for 15 min and lyophilized for 24 h. To quantify the residual P(D-LA-*co*-3HB), ethanolysis of the whole culture was performed, and the concentrations of LA-ethyl and 3HB-ethyl were determined using gas chromatography

(GC). Five hundred microliters of 15% v/v H_2SO_4 in ethanol and 250 μL chloroform were added to the dried sample of the whole cultures and heated at 100 °C for 120 min, with a 30 min interval for vortexing. The samples were cooled down to room temperature, and 5 mL of ice-chilled ultrapure water was added and vigorously agitated by vortexing. The samples were centrifuged at 4 °C and 1,690 × *g* for 15 min (see the maximum gravity of the tubes supplied by the manufacturer), and the lower chloroform layer was dried by passing it through Na_2SO_4 and incubation with molecular sieves (4A 1/16, Wako) for at least for 30 min. GC-2010 (Shimazu) was coupled with a flame ionization detector equipped with an InterCap1 column (0.25 mm I.D. × 30 m, df = 0.25 μm; GL Sciences) and hydrogen generator OPGU-2200 (STEC). Gas flow and temperature conditions during the measurements were as follows: N_2, 43.6 mL/min; He, 30.0 mL/min; H_2, 40.0 mL/min, air, 400.0 mL/min; detector temperature, 310 °C; injector temperature, 205 °C; gas temperature, 100 °C; and column oven, 40–300 °C. Poly(50 mol% D,L-lactide-*co*-glycolide) ($M_w = 3 \times 10^4$–6×10^4; Sigma-Aldrich) and P(3HB) were used as the standard curves for LA and 3HB, respectively.

2.7. Molecular dynamic (MD) simulation of D-LA homo-oligomers

Initial molecular structures and atomic charges of the D-LA homo-oligomers with different DPs (7, 17, and 30) were obtained using the semi-empirical quantum mechanical AM1-bcc method [20]. Each optimized D-LA homo-oligomer was solvated by thousands of water molecules (TIP3P model) under periodic boundary conditions. The D-LA homo-oligomer and water molecules were further energy-minimized at 0 K and heated up to 300 K. The heated system was equilibrated for 120 ps. The equilibrated system was used for molecular dynamic simulation for 10–20 ns under the constant number-pressure-temperature conditions. The obtained trajectory data were used for analysis of the radius of gyration (R_g) to catch up the degree of extension of the oligomer chains. The AMBER (ver. 14) program [21] was used for the MD simulation.

3. Results and Discussion

3.1. Bacterial screening by using the P(D-LA-*co*-3HB) plate

Of a total of 216 soil samples, 12 bacteria were isolated as single colonies forming clear zones on the plate containing emulsified P(64 mol% D-LA-*co*-3HB) (Fig. S1). The isolated bacteria were identified on the basis of the homology search for the 16S rRNA region (Table 1). Seven *Variovorax* sp. and three *Acidovorax* sp., classified as Betaproteobacteria (Burkholderiales), were identified. In addition, another Betaproteobacteria (Burkholderiales), *Burkholderia* sp., and a *Brevibacillus* sp. (Firmicutes) were isolated. These are similar to previously identified

PHA-degrading bacteria from soil [1,7,8], which suggests that P(D-LA-*co*-3HB)-degrading bacteria are closely related to natural PHA-degrading bacteria.
The 216 enriched cultures were also used to inoculate liquid medium containing the emulsified PDLA, but no apparent PDLA degradation was observed for all samples (data not shown).

3.2. Evaluation of P(64 mol% D-LA-*co*-3HB) degradation by the isolated bacteria

The consumption of P(D-LA-*co*-3HB) was evaluated using the ethanolysis-based method. Ethanolysis converts the polymer, oligomer, and monomer into ethyl esters of LA and 3HB. Therefore, a decrease in the concentration of the ethanolysis products is direct evidence of the consumption of the target components, which means mineralization and/or assimilation of the polymer when the test is performed under aerobic conditions.

First, *Variovorax* sp. C34 was investigated to verify the method. The cells were cultivated in the liquid medium containing 1 g/L P(67 mol% D-LA-*co*-3HB) emulsion. The whole cultures were harvested periodically, and the amounts of remaining LA and 3HB units in the soluble and insoluble fractions of the culture were measured using GC (Fig. 1). The soluble fraction did not contain LA and 3HB, indicating that the soluble monomer/oligomer fractions of LA and 3HB were rapidly consumed by the bacterium. The 3HB units in the insoluble fraction also completely disappeared at 2 days, indicating the rapid consumption of 3HB units. In contrast, the decrease in LA content in the insoluble fraction considerably reduced after 4 days, and a trace amount of LA units was detected after 14 days of cultivation. This indicates that P(67 mol% D-LA-*co*-3HB) contains a small number of the persistent D-LA-clustering structure and is consistent with our previous result that $PhaZ_{Vs}$ hydrolyzes the D-LA oligomer with a DP = 30 and lower [15]. These results indicate that the ethanolysis-based method successfully detected the consumption of P(D-LA-*co*-3HB) by the bacterium.

Next, the P(D-LA-*co*-3HB)-consuming capacity of the newly isolated bacteria was tested. The concentrations of the remaining LA and 3HB units in the polymer were measured at 7 days (Fig. 2). All isolates, except for *Brevibacillus* sp. B16i, significantly consumed P(64 mol% D-LA-*co*-3HB) (Fig. 2A). Approximately 10% of the polymer consumption by *Brevibacillus* sp. B16i was attributable to the consumption of the D-LA fraction in the polymer (Fig. 2B). The isolates belonging to the genera *Variovorax and Acidovorax* consumed the polymer at a relatively more rapid extent than *Burkholderia* sp. A44 did, and they exhibited the same level of consumption of P(64 mol% D-LA-*co*-3HB) as that reported previously for *Variovorax* sp. C34 (Fig. 2A). Notably, a trace amount of LA units (less than 1 w/v%) was detected even from the cultures of the strongest isolates *Acidovorax* sp. A23i and *Variovorax* sp. C5i, which would be attributable to the presence of the D-LA-clustering structure in the copolymer. A positive relationship was observed between the decreases in the polymer content and LA fraction (Fig. 2A and 2B).

3.3. Characterization of D-LA homo-oligomer degradation by the isolated bacteria

It was previously demonstrated that $PhaZ_{Vs}$ was able to hydrolyze D-LA homo-oligomers with a DP of approximately 30 and lower, whereas $PhaZ_{Af}$ from *Alcaligenes faecalis*, which is a well-known P(3HB)-degrading bacterium, hydrolyzed D-LA homo-oligomers with a DP of approximately 10 and lower [15]. Therefore, the upper DP limit of D-LA homo-oligomer hydrolysis by PhaZs and/or other degrading enzyme(s) secreted from bacteria could be a major factor that determines the bacterial degradability of P(D-LA-*co*-3HB) because the structure of the copolymers partially contains the D-LA homo-oligomeric sequences. Therefore, the upper limit of DP was directly assessed using chemically synthesized D-LA homo-oligomers with different DPs.

The 12 isolates and *Variovorax* sp. C34 were cultivated in media containing emulsified DLA20 (DP ranging from 10 to 30) and DLA40 (DP ranging from 20 to 60). The amount of LA units in the culture containing DLA20 and DLA40 was measured at 7 days (Fig. 3A and Fig. 4A). When D-LA homo-oligomers were used as the sole carbon source, no cell growth or oligomer consumption was observed for all the strains (data not shown). Therefore, PHBH emulsion was added to the culture to support cell growth and induce the expression of PhaZ. The 3HB units were nearly fully consumed under most of the conditions (Fig. 3B and Fig. 4B). In addition, part of the LA units was also consumed, indicating that LA-LA linkages in the D-LA homo-oligomers were partially hydrolyzed. More than 100% LA was detected in several cultures, which was probably due to LA fermentation from 3HB. *Brevibacillus* sp. B16 consumed neither PHBH nor DLA20, whereas *Burkholderia* sp. A44 efficiently consumed PHBH but did not consume DLA20. *Acidovorax* spp. efficiently consumed PHBH and hardly consumed DLA20. DLA20 was partially consumed by several *Variovorax* isolates, whereas DLA40 was slightly consumed by only *Variovorax* sp. C34. These results indicate that the P(D-LA-*co*-3HB)-consuming bacteria can be divided into three groups: group 1 bacteria, such as *Burkholderia* and *Acidovorax* spp., hardly consume D-LA homo-oligomers. Group 2 bacteria include only *Variovorax* spp. that partly consume DLA20 but not DLA40, which indicates that these *Variovorax* spp. can consume D-LA homo-oligomers with DP of less than approximately 20. Group 3 bacteria, including *Variovorax* sp. C34, partly consume DLA20 and DLA40, which is consistent with the results of a previous study in which $PhaZ_{Vs}$ from *Variovorax* sp. C34 hydrolyzed D-LA homo-oligomers with a DP of approximately 30 [15]. Notably, the group 3 bacteria were found to be rare in the soil environment investigated in this study.

3.4. MD simulation of D-LA homo-oligomers

The D-LA homo-oligomers with different DPs and high-molecular-weight PDLA contain the same ester bond of LA-LA linkages. Therefore, the limited consumption of D-LA homo-oligomers depending on their DP with a threshold of approximately 20 was presumably due to the conformation of the oligomers. To examine the hypothesis, MD simulation of the D-LA homo-oligomers with different DPs was performed (Fig. 5). The radius of gyration (R_g) value of the D-LA homo-oligomers with a DP of 7 in water corresponded to that of the extended form. In contrast, the D-LA homo-oligomers with a DP of 30 mostly adopted the aggregated globular conformation. The conformation of the D-LA homo-oligomers with a DP of 17 fluctuated between the extended and aggregated forms. These results suggest that the aggregated structure of the D-LA homo-oligomers was hardly consumed by the bacteria; therefore, the upper limit of DP for biological consumption, which was approximately 20, is likely to be determined by the conformational structure of the D-LA homo-oligomer.

3.5. Phylogenetic relationships of the isolated bacteria

The phylogenetic tree of the identified bacteria and several type strains based on 16S rRNA sequences is shown in Fig. 6. *Acidovorax* sp. is closely related to *Variovorax* sp. [22] and *Burkholderia* sp. is distantly related to *Variovorax* sp., indicating the relationship between the distance in the phylogenetic tree and P(D-LA-*co*-3HB)-consuming capacity of the bacteria. Among *Acidovorax* spp., A23i and A41i were categorized into the clade of the type strain of *Acidovorax facilis*, and both isolates preferably consumed 3HB units in the polymer (Fig. 2B). In contrast, A15i was categorized into the clade of the type strain of *Acidovorax delafieldii* and consumed both D-LA and 3HB simultaneously (Fig. 2B). Overall, *A. facilis* spp. (A23i and A41i) consumed P(D-LA-*co*-3HB) faster than *A. delafieldii* sp. (A15i) (Fig. 2A). *A. delafieldii* has been reported to degrade PHBH much faster than *A. facilis* [5]. These results suggest that preferential degradation of P(D-LA-*co*-3HB) and PHBH by *A. delafieldii* and *A. facilis* could be different.

The distribution of P(D-LA-*co*-3HB)-degrading bacteria was based on the phylogenetic tree. The P(D-LA-*co*-3HB)-degrading *Acidovorax* strains isolated in this study were related to *Acidovorax* sp. strain DB5 (Fig. 6), which is a natural PHA-degrading soil bacterium from Malaysia [23]. *Variovorax* sp. A35i was categorized into the clade of the type strain of *Variovorax paradoxus* B4 and *Variovorax* sp. TS13, which were isolated from soils in the United States [22], Germany, and Japan [24,25], respectively. The second group, including

A32i4, C4i, and C34, was categorized into the clade of the type strain of *Variovorax boronicumulans*, a species with a high similarity to *V. paradoxus*, isolated from soil in Japan [26] and *V. paradoxus* 5C-2 isolated from soil in Russia [27,28]. The third group, including A32i1, C11i, A5i, and C5i, has 100% identity to *Variovorax* sp. InS341 isolated from soil in Antarctica. Therefore, the related species of *Acidovorax* spp. and *Variovorax* spp. involved in P(D-LA-*co*-3HB) degradation are likely distributed in the soils of a wide range of areas.

Extracellular PhaZs have two types of catalytic domains, types 1 and 2 (CD1 and CD2, respectively), on the basis of the amino acid sequences [29,30]. PhaZ$_{Vs}$ of *Variovorax* sp. C34 is categorized to have CD2, and PhaZ$_{Af}$ of *Alcaligenes faecalis* T1, CD1 [15]. On the basis of the homology search of the genome sequences of the related strains mentioned in Fig. 6, *Variovorax* and *Acidovorax* possess two types of PhaZs with CD1 and CD2 [accession number QFZ86010.1 (CD1) and QFZ87174.1 (CD2) in *Variovorax paradoxus* strain 5C-2 and AOG22341.1 (CD1) and AOG21354.1 (CD2) in *Acidovorax* sp. RAC01], whereas *Burkholderia* and *Brevibacillus* have only PhaZs with CD1 (AIO28731.1 in *Burkholderia cepacia* strain ATCC 25416, and VEF92371.1 in *Brevibacillus brevis* strain NCTC2611). Indeed, previous identification and characterization of several PhaZs were consistent with the results of the genome analyses. For example, PhaZ$_{Ap}$ of *Acidovorax* sp. TP4 (Accession#, BAA35137.1) [31] is known to have CD2, whereas extracellular PhaZ$_{Bc}$ from *Burkholderia cepacia* DP1 (Accession# AYO89594.1) [32] is categorized to have CD1. These results suggest that PhaZ with CD2 could contribute to the bacterial degradation of P(D-LA-*co*-3HB), although further studies will be needed.

Besides Betaproteobacteria, *Brevibacillus* sp. B16i, which has 99.7% identity to *Brevibacillus parabrevis* Sh1 (Table 1, Fig. 6), formed a clear zone on the emulsified P(D-LA-*co*-3HB) plate, although the consumption ability of the copolymer was much lower than that of the other isolated bacteria (Fig. 2A). It should be noted that the strain consumed only the D-LA fraction but not the 3HB fraction (Fig. 2B and C). This may suggest that the strain used other esterases to consume the D-LA fraction. However, *Brevibacillus* sp. B16i is unlikely to be a PDLA-degrading bacterium because no D-LA homo-oligomer consumption by the strain was observed (Fig. 3). To our best knowledge, no bacterium that consumes high-molecular-weight PDLA has been reported to date. *Bacillus stearothermophilus* reportedly decreased the molecular weight of PDLA at 60 °C, and its growth was promoted by the presence of PDLA [33]. However, the contribution of non-enzymatic degradation was not excluded, and, therefore, enzymatic degradation of PDLA was not convincingly demonstrated.

4. Conclusion

We successfully isolated 11 P(D-LA-*co*-3HB)-degrading bacteria. Most of the isolated bacteria were *Variovorax* and *Acidovorax* related to previously identified natural PHA-degrading soil bacteria. The genera *Variovorax* and *Acidovorax* presumably possess two types of PhaZs in their genomes, and PhaZ with CD2 could be involved in P(D-LA-*co*-3HB) degradation. The isolates partially consumed DLA20 (DP ranging from 10 to 30) and hardly consumed DLA40 (DP ranging from 20 to 60), suggesting that the D-LA-clustering structure with a DP of approximately 20 and higher in the copolymer is not ubiquitously degraded in the soil. MD simulation of the D-LA homo-oligomers indicated that the upper limit is likely to be determined by the conformational structure of the oligomers in water. These results suggest that the copolymers containing no D-LA-clustering structure, such as P(30 mol% D-LA-*co*-3HB), can be widely degraded in the environments. This information is useful for the molecular design of biodegradable D-LA-containing polymers.

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Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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Table 1. The identified species that formed clear zone on agar plates of emulsified P(64 mol% D-LA-*co*-3HB).

	Name	16S rRNA Accession #	Top BLASTn hit description_accession #	E-value	Ident. (%)
A5i	*Variovorax* sp.	LC513944	*Variovorax* sp. strain InS341_MN315416.1	0	100
A15i	*Acidovorax* sp.	LC513945	*Acidovorax delafieldii* strain AB5_MN449446.1	0	99.7
A23i	*Acidovorax* sp.	LC513946	*Acidovorax* sp. RAC01_CP016447.1	0	99.7
A32i1	*Variovorax* sp.	LC513948	*Variovorax* sp. strain InS341_MN315416.1	0	100
A32i4	*Variovorax* sp.	LC513949	*Variovorax paradoxus* strain 5C-2_CP045644.1	0	99.7
A35i	*Variovorax* sp.	LC513951	*Variovorax* sp. strain TS13_MN715854.1	5E-134	98.6
A41i	*Acidovorax* sp.	LC513952	*Acidovorax* sp. RAC01_CP016447.1	0	100
A44i	*Burkholderia* sp.	LC513953	*Burkholderia cepacia* strain MHGNU B106_MK779204.1	4E-155	100
B16i	*Brevibacillus* sp.	LC513955	*Brevibacillus parabrevis* strain Sh1_MF276892.1	2E-174	99.7
C4i	*Variovorax* sp.	LC513956	*Variovorax paradoxus* strain 5C-2_CP045644.1	0	99.7
C5i	*Variovorax* sp.	LC513957	*Variovorax* sp. strain InS341_MN315416.1	0	100
C11i	*Variovorax* sp.	LC513958	*Variovorax* sp. strain InS341_MN315416.1	0	100

Figures

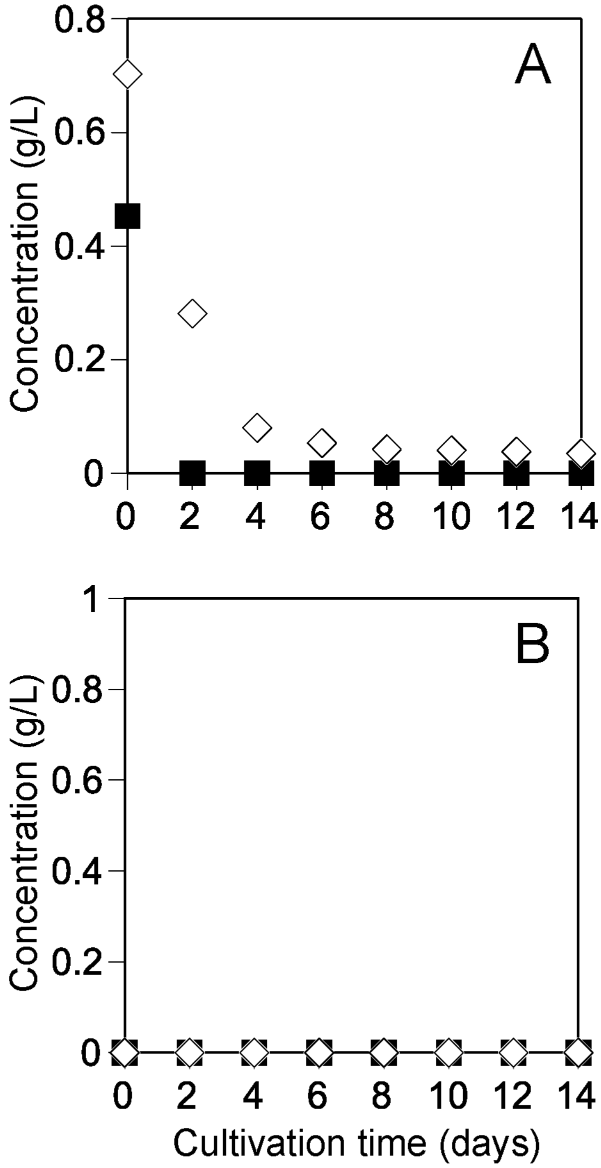

Fig. 1. Time-course of P(67 mol% D-LA-*co*-3HB) consumption by *Variovorax* sp. C34. LA (diamond) and 3HB (square) concentrations in the precipitant (A) and supernatant (B) were measured using gas chromatography.

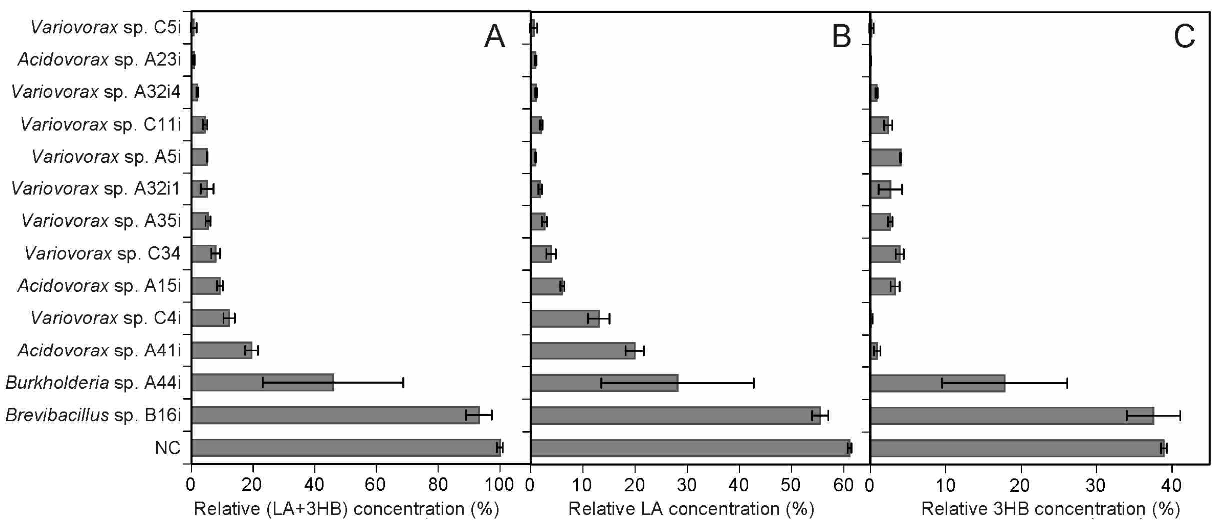

Fig. 2. The remaining LA and 3HB in the cultures of the isolates on P(64 mol% D-LA-*co*-3HB) after 1 week of cultivation. The amounts of the total polymer (A), LA fraction (B), and 3HB fraction (C) are shown as concentrations relative to those of the negative control culture (NC) with no inoculation. Detailed information on the isolates is provided in Table 1. The data are shown as mean ± standard deviation values of biological triplicates.

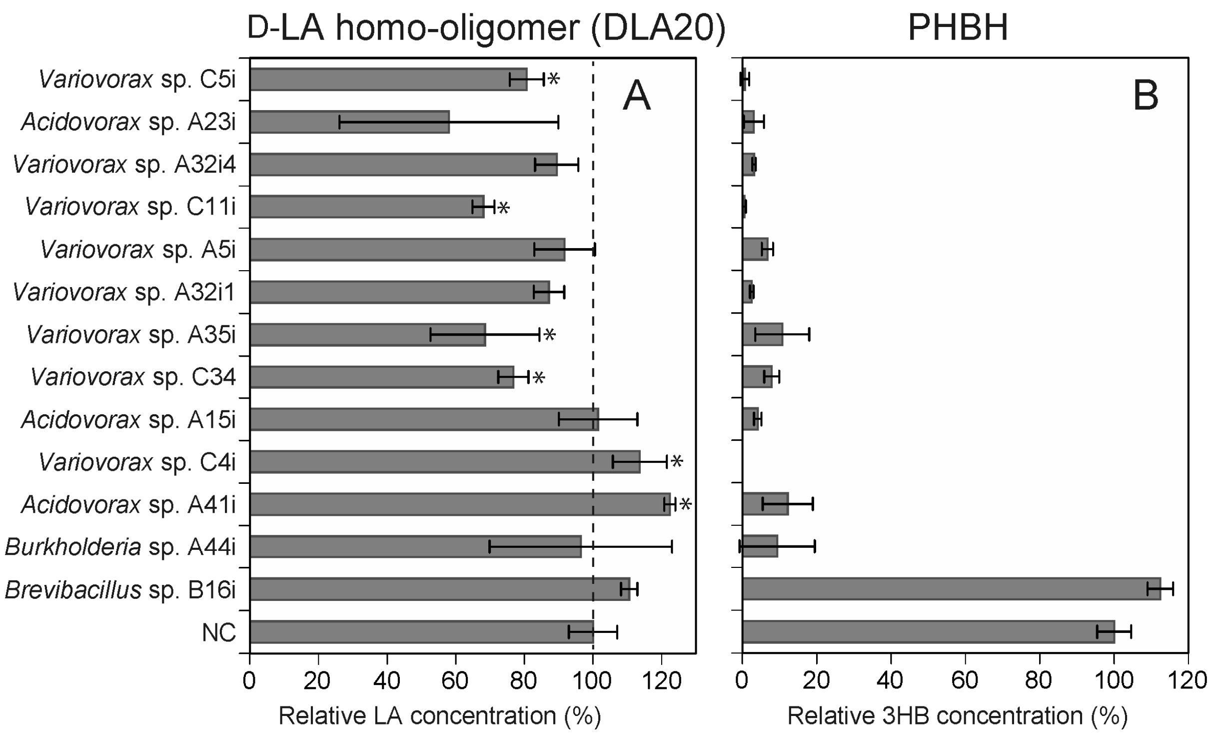

Fig. 3. The remaining LA and 3HB in the cultures of the isolates on D-LA homo-oligomers (DLA20) and PHBH after 1 week of cultivation. The amounts of LA (A) and 3HB (B) fractions are shown as concentrations relative to those of the negative control culture (NC) with no inoculation. Detailed information on the isolates is provided in Table 1. The data are shown as

mean ± standard deviation values of biological triplicates. *Student's *t*-test was performed ($p <$ 0.05).

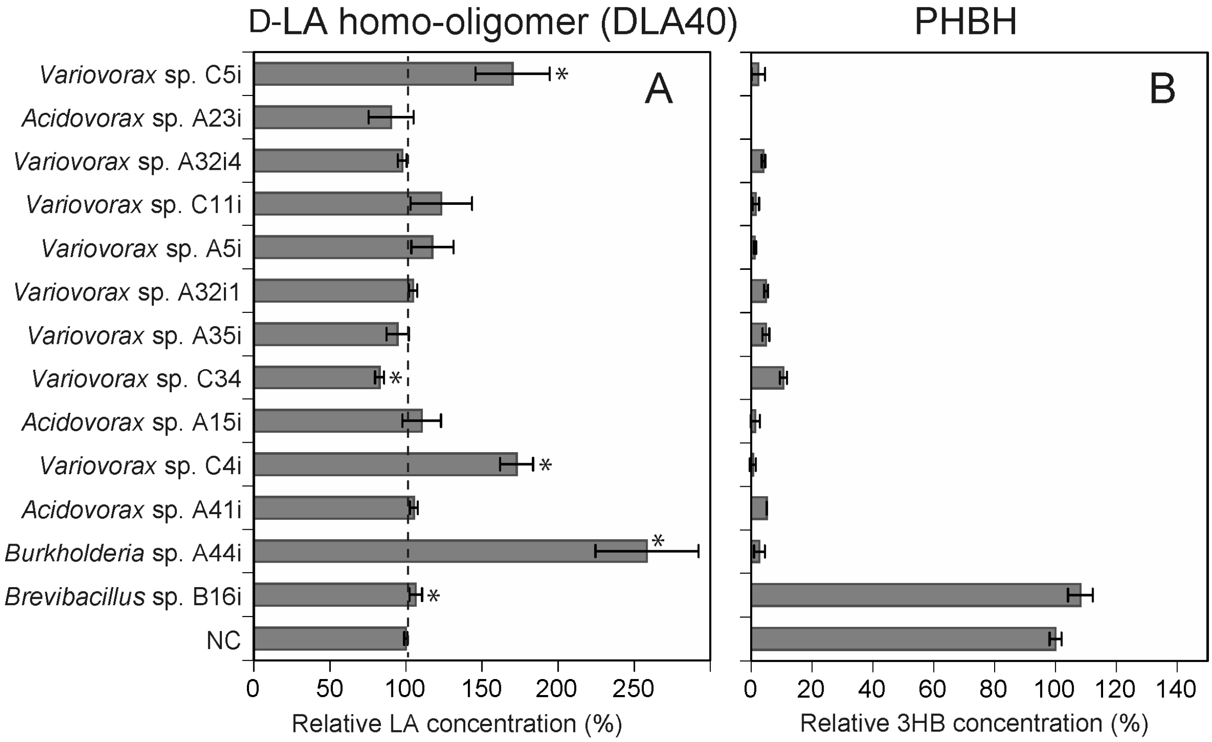

Fig. 4. The remaining LA and 3HB in the cultures of the isolates on D-LA homo-oligomers (DLA40) and PHBH after 1 week of cultivation. The amounts of LA (A) and 3HB (B) fractions are shown as concentrations relative to those of the negative control culture (NC) with no inoculation. Detailed information on the isolates is provided in Table S1. The data are shown as mean ± standard deviation values of biological triplicates. *Student's *t*-test was performed ($p < 0.05$).

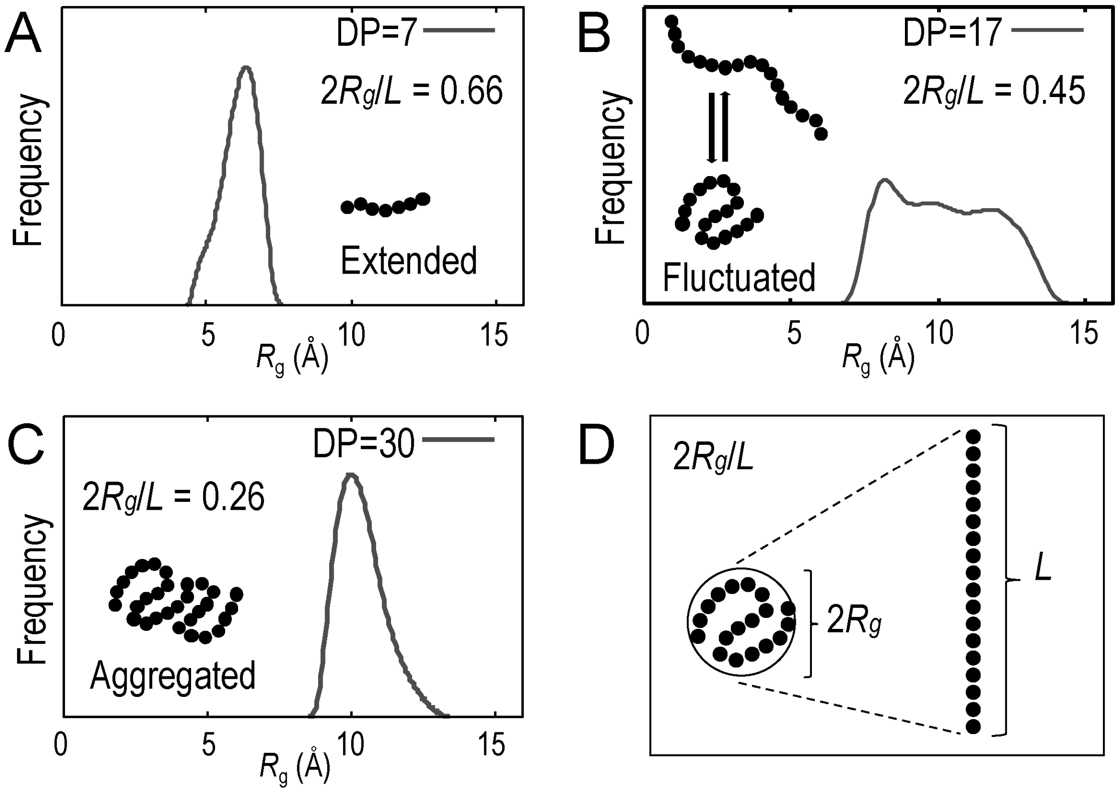

Fig. 5. Molecular dynamic simulation of D-LA homo-oligomers with different degrees of polymerization (7, 17, and 30) in water at 300 K (A, B, and C, respectively). The radius of gyration (R_g) and length (L) are defined as shown in (D).

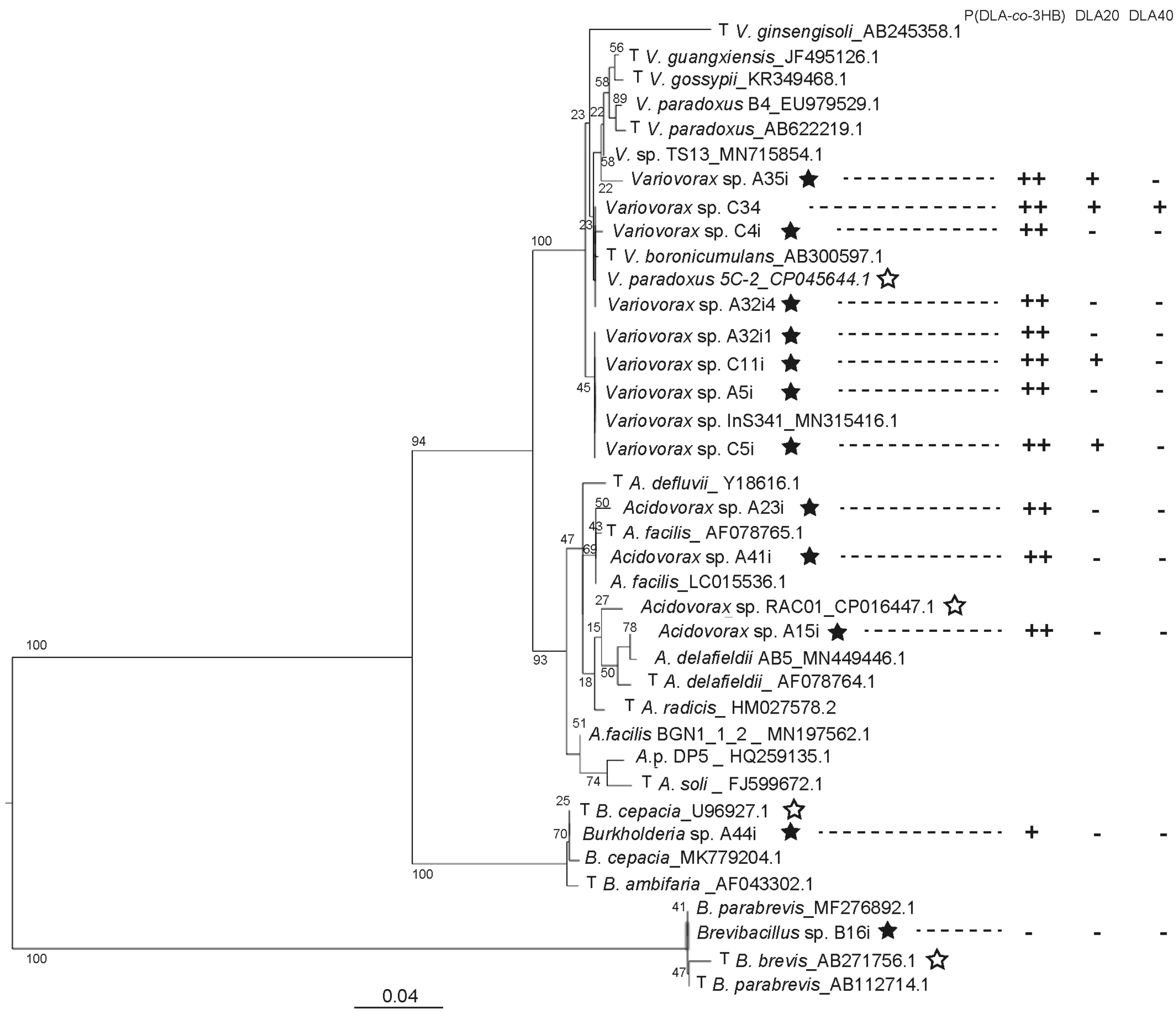

Fig. 6. Phylogenetic tree of 16S rRNAs of P(D-LA-*co*-3HB)-degrading bacteria identified in this study, relevant type strains, and BLASTn best hit species. Accession numbers of 16S rRNA nucleotide sequences are provided after each species name. Black stars indicate isolates in this study, and their consumption capability of P(D-LA-*co*-3HB) and D-LA oligomers (DLA20 and DLA40) is drawn in the right panel. White stars indicate the species used for the genome analyses.