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EXPERIMENTAL ARTICLES

Biodegradation of Polyhydroxyalkanoates (PHAs) in the South China Sea and Identification of PHA-degrading Bacteria

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Abstract—The biodegradation patterns of two types of PHA, a 3-hydroxybutyrate (3-PHB) polymer and a 3-hydroxybutyrate and 3-hydroxyvalerate (3-PHB/3-PHV) copolymer, were studied in tropical marine environments (Dam Bay, South China Sea, Nha Trang, Vietnam). No reliable differences in the degradation of 3-PHB and 3-PHB/3-PHV were revealed. It was shown that the degradation process depended mainly on the shape of a polymer product and its production method: the degradation of polymer films was found to be more active than that of molded solids. A decrease in the molecular mass of both types of PHA was detected in the course of the degradation of PHA samples. However, the degree of PHA crystallinity did not change; that is, the levels of degradation of both the amorphous and crystalline phases of PHA were almost the same. Among microbial PHA degraders, three bacterial strains, *Bacillus* sp. IBP-V002, *Enterobacter cloacae* sp. IBP-V001, and *Gracilibacillus* sp. IBP-V003, were identified based on the results of morphological, biochemical, and molecular phylogenetic analyses. The ability of the representatives of the genera *Gracilibacillus* and *Enterobacter* to degrade PHA was revealed for the first time.

Keywords: polyhydroxyalkanoates, PHA, biodegradation in marine environments, microbial PHA degraders.
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Polyhydroxyalkanoates (PHAs) are polyesters of microbial origin that, under natural conditions, are decomposed by PHA-degrading microorganisms with extracellular PHA depolymerases [1]. Microbial PHA degraders include various microorganisms that degrade these polymers in soils [2–3], compost [4], and in freshwater [5–9] and marine environments [10–12]. While the data on PHA degradation under natural conditions are scarce, there is still less information concerning PHA biodegradation in marine environments. In one of the first such studies, which was carried out in the Sea of Japan, a decrease in the molecular mass of PHA was reported. It was shown that this decrease was seasonal and depended on the water temperature [13]. The study of PHA degradation in the coastal zone of Puerto Rico showed that polymer degradation depended on the exposure site (sea, mangrove coast, or reef zone) and was determined by the number of microorganisms forming biofilms on the surfaces of polymer samples; the rate of polymer degradation did not exceed 0.1% of the initial polymer weight per day [14]. Other authors reported more rapid PHA degradation in the Baltic Sea, where the weight loss reached 60% over the 6-week period of observations [12].

The bacteria *Pseudoalteromonas* sp. NRRL B-30083 [15], *Marinobacter* sp. NK-1 [10], and *Alcaligenes faecalis* AE122 [16], as well as the actinomycetes *Nocardiopsis aegyptia* [11] and *Streptomyces* sp. SNG9 [17], were revealed among the marine microbial degraders of PHA. They were found to be able to secrete exodepolymerases and to utilize the homogeneous 3-PHB polymer and PHB/3-PHV copolymers.

The goal of the present work was to study the degradation of PHAs of different chemical structure in tropical marine ecosystems, as well as to identify microbial degraders of these polymers.

MATERIALS AND METHODS

Polyhydroxyalkanoate samples in the form of film disks (73 ± 5 mg, 30 mm in diameter, 0.1 mm thick) and molded solids (330 ± 25 mg, 10 mm in diameter, 0.5 cm high) were studied. Two types of polyhydroxyalkanoates were used, a 3-hydroxybutyrate (3-PHB) polymer and a 3-hydroxybutyrate and 3-hydroxyvalerate (3-PHB/3-PHV) copolymer; the amount of 3-

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hydroxyvalerate was 11 mol % [18]. To determine the chemical structure of PHA, the samples were subjected to methanolysis and analyzed on an Agilent 5975 Inert gas chromatograph–mass spectrometer (United States).

The process of PHA biodegradation was studied at a test site located in Dam Bay (South China Sea, Nha Trang, Vietnam). Sterile preweighed samples were placed into traps made of fine-meshed mill gauze, which, at a depth of 120 cm, were then secured in stainless steel stationary cassettes with nylon threads. The experiment was carried out during the field season of 2009 (March 11 to August 17). Changes in the sample weight were monitored taking into account the physicochemical state of seawater. Three samples of each type were retrieved from the water every 20 days, cleaned using mechanical or enzymatic techniques to remove the biofilms, rinsed with distilled water, and dried. The water temperature and reaction rate were measured using an HI 98127–HI 98128 pH meter (Hanna Instruments, Italy); dissolved oxygen and salinity were measured with a portable HI 9142 dissolved oxygen meter (Hanna Instruments, Italy) and a handheld S/Mill-E 2442-W10 salinity refractometer (Japan), respectively. A decrease in the sample weight, changes in the molecular mass and molecular mass distribution of the polymers, as well as changes in the ratios between their ordered (crystalline) and disordered (amorphous) phases, were considered indicators of PHA degradation.

Analysis of the physicochemical properties of polymers. Polymer samples (initially and during the observations) were weighed on Class IV laboratory scales (Mettler, United States). X-ray structure analysis of PHA was carried out by X-radiography using a D8 ADVANCE X-Ray Diffractometer (Bruker, Germany) equipped with a Super Speed VANTEC detector. The operation parameters were as follows: scan steps 0.016°, 200-s exposure to measure the X-ray intensity at a point, and 40 kV ± 40 μA. The molecular mass and molecular mass distribution of PHA relative to polystyrene standards (Fluka, Switzerland, Germany) were determined by gel permeation chromatography using the Waters Breeze HPLC System (Waters, United States). The weight-average molecular mass (M_w) of the polymers and their polydispersity (which makes it possible to determine the ratio between fragments with different polymerization capacities) were determined. Electron microscopic observations were carried out using an FEI Company Quanta 200 scanning electron microscope equipped with a nitrogen-free GENESIS XM 2 60 Imaging SEM X-ray microanalysis system with an APOLLO 10 silicon drift detector (EDAX, United States).

Microbiological analyses. To detect PHA-degrading microorganisms, samples collected on July 27, 2009 (after 140-day exposure), i.e., during the period of intense polymer degradation, were studied. The PHA samples were removed from the traps under sterile conditions. To remove nonadhered microbial cells, the surface was washed with sterile seawater; microbiological samples for inocula were scraped from the biofilm formed on the polymer surface. At the same time, plating of seawater samples was carried out.

Quantitative enumeration of bacterial cells in seawater and on the surface of PHA samples was carried out by plating serial dilutions on Youschimizu–Kimura (Y–K) medium containing the following (g/l): peptone, 5; yeast extract, 2; glucose, 1; K_2HPO_4, 0.2; $MgSO_4 \times 7H_2O$, 0.1; agar, 18; tap water, 500 ml; and seawater, 500 ml; pH 7.8–8. Enumeration of fungal cells was carried out on Sabouraud dextrose agar and Czapek medium containing the following (g/l): sucrose, 20; $NaNO_3$, 2; K_2HPO_4, 1; $MgSO_4 \times 7H_2O$, 0.5; KCl, 0.5; agar, 20; $CaCO_3$, 3 g; tap water, 500 ml; and seawater, 500 ml. Before dispensing, the melted Czapek medium was supplemented with sterile lactic acid (4 ml/l). The inoculated petri dishes were incubated at 28–30°C. Enumeration of bacterial and fungal cells was carried out after 1–3 and 4–5 days of incubation, respectively. To obtain pure microbial cultures, eight to ten morphotypes of each isolate were selected for subculturing and inoculated on petri dishes with relevant media. The morphology of bacterial cells was studied in gram-stained preparations. The phenotypic properties of microorganisms were studied using conventional microbiological techniques [19–20]; the morphology of vegetative cells; spore formation; motility; reaction to Gram staining; requirement for growth factors; capacity for anaerobic growth; presence of nitrate reductase; and ability to produce acids from glucose, lactose, sucrose, maltose, and mannitol, as well as catalase, oxidase, amylase, proteinase activities, were determined. The taxonomic position was determined according to *The Prokaryotes* [21].

To detect PHA-degrading microorganisms, the method of clear zones [22] was used; the samples were plated on a mineral agarized medium with the polymer (PHA) as a sole carbon source. The growth of microorganisms exhibiting PHA depolymerase activity was accompanied by formation of clear zones surrounding their colonies on the agar surface. These microorganisms were selected for identification performed by sequencing of the 16S rRNA genes of the obtained isolates.

DNA analysis. DNA was extracted using the Aqua-Pure Genomic DNA Isolation kit (Bio-Rad; United States) according to the manufacturer's protocol. PCR amplification of the 16S rRNA gene was carried out with the universal eubacterial primers 27f (5'-AGAGTTTGATCCTGGCTCAG-3') and 1492r (5'-GGTTACCTTGTTACGACTT-3'), corresponding to *E. coli* positions 8–27 and 1510–1492, respectively. The polymerase chain reaction (PCR) was performed on a Mastercycler Gradient amplifier (Eppendorf, Germany) according to the standard procedure. With-

Table 1. Hydrochemical parameters of seawater in Dam Bay

Date of sampling	Water temperature, °C	pH	Salinity, ‰	Oxygen, mg/ml
11.03.2009	27.3	7.4	32	8.3
30.03.2009	28.6	7.5	34	7.4
20.04.2009	29.0	7.0	34	6.5
07.05.2009	27.1	7.1	33	6.1
28.05.2009	29.5	7.1	33	6.9
17.06.2009	28.7	7.2	35	5.4
07.07.2009	27.7	7.2	35	6.1
27.07.2009	30.4	7.4	34	5.7

out additional purification, the PCR products were cloned into the pCR4-TOPO vector (Invitrogen, United States) in *E. coli* TOP10. Plasmid DNA was isolated from the selected clones using the PureLink Quick Plasmid Miniprep Kit (Invitrogen, United States) according to the manufacturer's protocol. Bidirectional sequencing was carried out on an ALFexpress II automatic sequencer (Amersham Pharmacia Biotech Ltd., United States) using the universal primers T3 and T7 and the Thermo Sequenase Cy5 Dye Terminator sequencing kit.

Phylogenetic analysis. Comparison of the 16S rRNA gene sequences determined in this work with the sequences in the GenBank, EMBL, and DDBJ databases was performed using the NCBI BLAST software package (http://www.ncbi.nlm.nih.gov/BLAST/). Phylogenetic analysis was performed using the Jukes and Cantor one-parameter model with the neighbor-joining method implemented in the TREECON v. 1.3b software package [23]. The 16S rRNA gene fragments obtained in this work were deposited in GenBank under accession numbers HM021764, HM021765, and HM021766.

Statistical analysis. Each measurement was made in triplicate on at least three samples. The standard deviation, mean-square deviation, and confidence interval were calculated for the significance level $\alpha = 0.05$.

RESULTS AND DISCUSSION

Degradation of PHA samples. During the whole period of observation, the hydrochemical properties of seawater remained practically unchanged (Table 1). The average water temperature was 28.75 ± 1.65°C. The pH of the water varied insignificantly (7.0–7.5) and was close to neutral. Salinity ranged from 32 to 35°; during the period of observation, its average value was 34%. The concentration of dissolved oxygen ranged from 5.4 to 8.3 mg/ml.

The weight loss dynamics of different PHA samples is shown in Figure 1. The degradation of polymer films was more pronounced. These samples were manufactured with minimal impact on the polymer and had large surface areas. After 160-day exposure in seawater, the residual weight of the 3-PHB and 3-PHB/3-PHV films was 58 and 54%, respectively; that is, the rates of polymer biodegradation were practically the same.

A different weight loss dynamics was observed during degradation of PHA molded solids (Fig. 1). During the first 80 days of exposition, the weight of the samples virtually did not change. The phase of polymer degradation occurred later (days 80–160 of exposure); by the end of the experiment, the residual weights of the 3-PHB and 3-PHB/3-PHV molded solids were 62 and 87%, respectively; however, these differences were not reliable enough. The two-stage degradation of PHA molded solids (with smaller surface areas and, consequently, smaller contact areas between the polymer and water phases) may be due to the fact that adhesion of the microorganisms to a polymer surface and adaptation of microorganisms to PHA as a substrate took much longer. Phasic polymer biodegradation might have occurred. Initially, the depolymerizing enzymes of PHA-degrading microorganisms induced PHA depolymerization and splitting of the polymer chain; then, products of polymer biodegradation were utilized, resulting in changes in the molecular mass of the polymer samples and causing a decrease in the total weight of the samples.

The obtained results do not allow us to assume that 3-PHB and 3-PHB/3-PHV differ in the rate of their degradation in seawater and that the rate of the copolymer degradation is higher than the rate of the homopolymer 3-PHB degradation. These data differ from the results of our previous investigations of PHA degradation in model soil microcosms [24] and natural water ecosystems, where the copolymer 3-PHB/3-PHV samples were degraded more rapidly than the 3-PHB samples; these differences were more pronounced as the concentration of 3-hydroxyvalerate in the copolymer increased [7–8]. Similar results obtained by other authors [13–14] demonstrated that the copolymer 3-PHB/3-PHV samples were degraded much faster than 3-PHB samples. However, in [16], it was demonstrated that the rates of 3-PHB degradation by the *Alcaligenes faecalis* culture were much higher than those of 3-PHB/3-PHV degradation. Under laboratory conditions, on media with suspension of marine sediments, it was recently demonstrated during exposure of PHA films obtained by polymer extrusion that the decrease in the molecular mass of 3-PHB samples in contact with marine sediments was more pronounced than in the case of copolymer 3-PHB/3-PHV samples [12]. The recorded differences and peculiarities of the biodegradation of different PHA samples manufactured by different techniques are most probably due to the different surface areas and surface properties of the samples. Therefore, it would be appropriate to answer the question as to what happened to the polymers during their degradation.

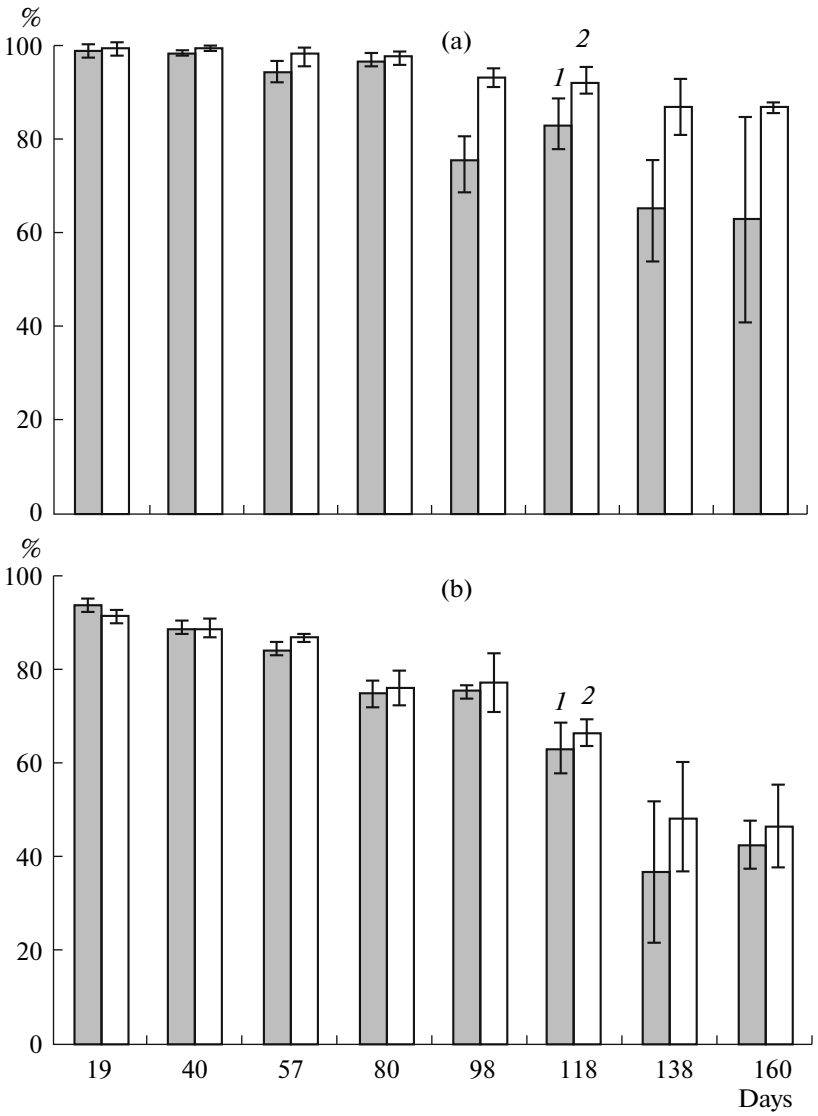

Fig. 1. Weight loss dynamics of different PHA samples after exposure in the South China Sea (March to August, 2009): molded solids (a) and films (b).

Changes in the PHA properties during biodegradation. The samples were studied by X-ray structure analysis in order to determine the effect of polymer degradation on the ratio between the ordered and disordered phases, i.e., to determine the degree of PHA crystallinity. Some authors suggest that the amorphous (disordered) phase of polymers is more susceptible to attacks by depolymerizing enzymes than is the ordered phase [25]. If so, the degree of PHA crystallinity should increase in the process of PHA biodegradation and during the much faster degradation of the amorphous phase. The initial degrees of 3-PHB and 3-PHB/3-PHV crystallinity were virtually the same (71% and 69% for polymer films, 70 and 71% for molded solids) (Table 2). Analysis of the X-ray spectra of the degraded PHA samples retrieved from seawater after 160 days of exposure did not reveal any changes in their degree of crystallinity. This finding suggests that, during PHA biodegradation in tropical marine environments, both phases (amorphous and crystalline) were degraded (washed out); as a result, the integral indicator of the polymer structure (degree of crystallinity) remained unchanged.

Polymer properties are largely dependent on the molar mass, especially on the weight-average molecular mass (M_w). During polymer degradation, a decrease in the M_w values of all samples was observed (Table 2). The most pronounced decrease (57% of the initial value) was detected in the case of copolymer molded solids; in other cases, this value ranged between 16 and 26%. The polydispersity indices of all degraded samples increased; this suggests that the amount of fragments with different polymerization capacities increased as well.

Table 2. Comparative analysis of the intact PHA samples and the samples in the process of polymer degradation in seawater

Parameter	Initial	After 160-day exposure	Initial	After 160-day exposure
	3-PHB	3-PHB	3-PHB/3-PHV	3-PHB/3-PHV
Degree of crystallinity (films), C_x, %	71	70	69	69
M_w (films), kDa	1401 ± 106	1040 ± 33	1318 ± 85	1114 ± 80
Polydispersity index (films)	2.00 ± 0.37	2.26 ± 0.20	2.01 ± 0.23	2.00 ± 0.22
Degree of crystallinity (molded solids), C_x, %	70	71	71	69
M_w (molded solids), kDa	1359 ± 63	1090 ± 87	1427 ± 46	610 ± 25
Polydispersity index (molded solids)	1.83 ± 0.11	2.57 ± 0.51	2.06 ± 0.12	2.41 ± 0.11

As the polymer samples degraded and their weight decreased, large pores and perforations appeared on the initially intact surfaces; in the course of the experiment, the sizes and numbers of these pores and perforations increased (Fig. 2).

Detection of microbial PHA degraders. During the period of investigation, the total numbers of heterotrophic bacteria and microscopic fungi in the seawater of Dam Bay growing on the Y–K medium were 1.6×10^3 and 1.0×10^2 CFU/ml, respectively. The results obtained correspond with the data presented in [26–27]. (The authors of these works have been performing microbiological analyses of the samples collected in the near-shore waters of Vietnam, including Dam Bay, for several years.) After 160 days of exposure, biofilms formed on the surfaces of the polymer samples under study. A total of 58 strains of heterotrophic bacteria and 16 strains of microscopic fungi were isolated in pure cultures from these biofilms. The greatest species diversity was found among gram-positive bacteria. Gram-negative rods prevailed, with their percentage reaching 60–65%.

Bacterial strains belonging to the genera *Pseudomonas, Pseudoalteromonas, Corynebacterium, Staphylococcus, Planococcus, Micrococcus, Arthrobacter, Cellulomonas, Enterobacter, Bacillus*, and *Gracilibacillus* were identified on the basis of their morphological, cultural, and biochemical properties. The taxonomic composition of microscopic fungi was represented by *Aspergillus, Penicillium, Trichoderma, Verticillium, Mucor*, and *Malbranchea*, identified on the basis of the morphology of their mycelium and spore-bearing structures.

To isolate PHA-degrading bacteria, biofilm samples were plated onto the diagnostic mineral agar medium with polymer powder as the sole carbon and energy source. A considerable number of isolated bacteria were incapable of PHA biodegradation. It was demonstrated that six bacterial isolates formed clear zones on the diagnostic medium with polymer (depolymerase activity) (Fig. 3). On the basis of similar morphotypes, three strains were chosen among six isolates capable of polymer degradation (Table 3); these strains were identified on the basis of their morphological, cultural, and biochemical properties, as well as their 16S rRNA gene sequences.

Strain IBP-V001 produced smooth semitransparent colonies on the seawater-containing RPA medium. In smears, straight, small, non-spore-forming, gram-negative, motile rods were detected; they were facultatively anaerobic and catalase- and oxidase-positive. The strain fermented glucose, maltose, and mannitol with formation of acid and gas; it utilized lactose and sucrose and reduced nitrate. The strain did not exhibit amylase and protease activities. Strains IBP-V002 and IBP-V003 were able to produce endospores. On agarized media, they produced opaque colonies. Strain IBP-V002 produced pigmented, pale pink colonies. Strains IBP-V002 and IBP-V003 exhibited similarity with respect to other properties: they were catalase- and oxidase-positive, possessed amylase and protease activities, and were not able to reduce nitrate. The strains fermented glucose with formation of acid. Strain IBP-V003 was not able to utilize lactose, maltose, mannitol, and sucrose. On the basis of its phenotypic properties, strain IBP-V001 was assigned to the family *Enterobacteriaceae*, whereas strains IBP-V002 and IBP-V003 were assigned to the family *Bacillaceae*.

Comparison of the obtained nucleotide sequences of the 16S rRNA genes of the isolated PHA degraders with the sequences within the GenBank database showed high homology with the sequences of some cultivable strains. Strains *Enterobacter* sp. BSRA2 and *Enterobacter cloacae* subsp. *dissolvens* were found to be closest to strain IBP-V001 (99.5% similarity), strains *Bacillus* 64 and CNJ905 PL04 were closest to strain IBP-V002 (99.7–97.8% similarity), and strains *Gracilibacillus* DstIV-1 and DstIII-1 were the closest relatives of strain IBP-V003 (98.2% similarity). Taking into consideration the recommended 16S rRNA gene sequence similarity thresholds of 91.5 and 95.0% (for determination of the species and generic affiliation,

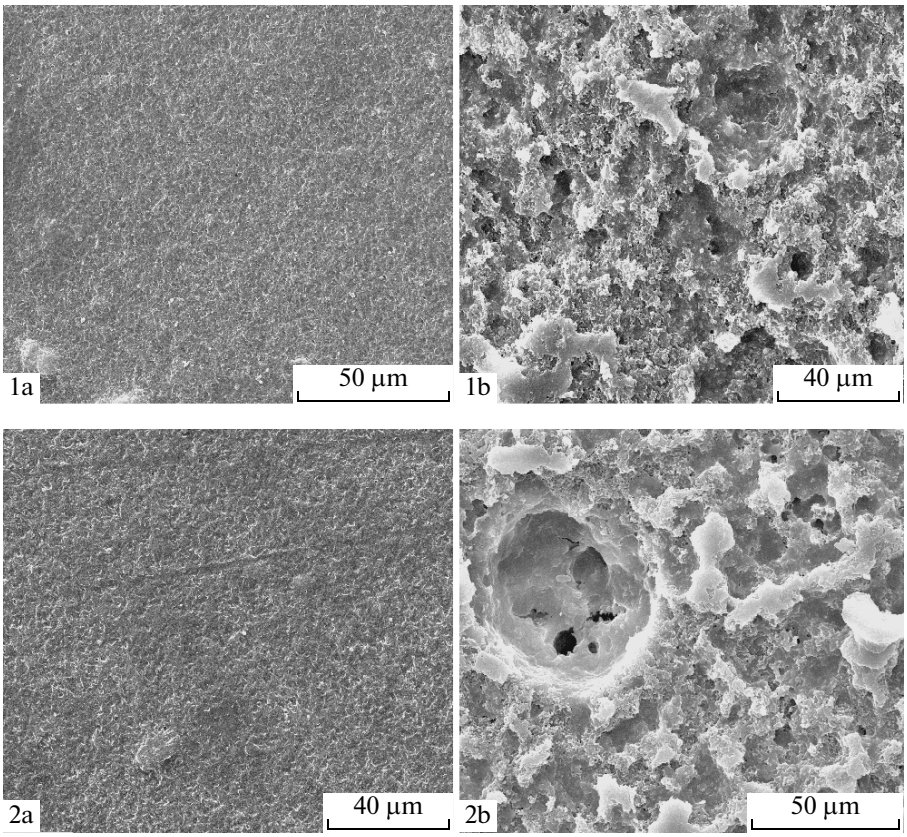

Fig. 2. Electron microphotographs of the PHA films: intact 3-PHB samples (1a), 3-PHB samples after 160-day exposure (1b), intact 3-PHB/3-PHV samples (2a), and 3-PHB/3-PHV samples after 160-day exposure (2b).

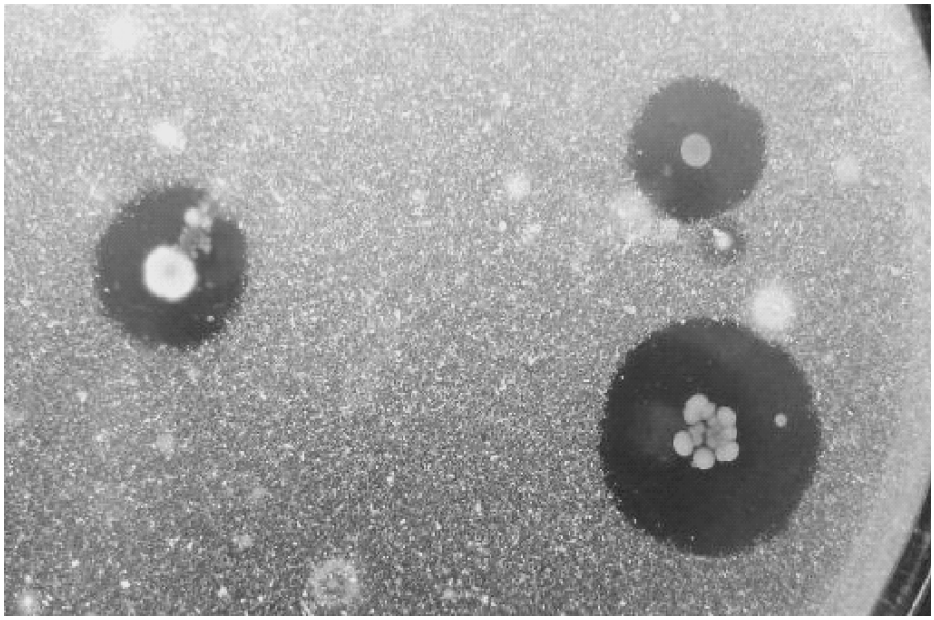

Fig. 3. Depolymerase activity of PHA-degrading bacteria (clear zones on the diagnostic medium).

Table 3. Phenotypic properties of the PHA-degrading bacteria isolated from the biofilms formed on the surfaces of polymer samples exposed to marine environments

Properties	Strains of microorganisms		
	Enterobacter cloacae IBP-V001	*Bacillus* sp. IBP-V002	*Gracilibacillus* sp. IBP-V003
Gram staining	–	+	+
Motility	+	+	–
Spore formation	–	+	+
Pigment	–	Pink	–
Catalase	+	+	+
Oxidase	–	+	+
Nitrate reductase	+	–	–
Amylase	–	+++	+++
Protease	–	+++	++
Acid production from:			
glucose	+, gas	+	+
lactose	–, gas	–	–
sucrose	–, gas	–	–
maltose	+, gas	+	–
mannitol	+, gas	+	–

Note: +, detected; –, not detected; for enzymatic activity: +, weak; ++, moderate; +++, strong.

respectively) [28–29], the isolated PHA degraders were identified as *Enterobacter cloacae* sp. IBP-V001, *Bacillus* sp. IBP-V002, and *Gracilibacillus* sp. IBP-V003. It should be noted that PHA depolymerase activity has not been previously demonstrated for the representatives of the genera *Gracilibacillus* and *Enterobacter*. The results of the comparison of the obtained 16S rRNA gene sequences (1442 bp) of strains IBP-V001, IBP-V002, and IBP-V003 with the sequences of the closest cultivable strains from the GenBank database are shown in Fig. 4. In the obtained phylogenetic tree, the strains fell into three clusters with strong bootstrap support; clusters 1 and 2 are occupied by gram-positive bacteria of the family *Bacillaceae*, whereas monophyletic cluster 3 belongs to gram-negative bacteria of the species *Enterobacter cloacae (Enterobacteriaceae*). The first cluster formed by bacteria of the genus B*acillus* consists of two subclusters, 1A (a group of *Bacillus* sp. strains) and 1B (*Bacillus firmus* strains). The second polygenetic cluster includes *Gracilibacillus* strains, which form three subclusters.

Thus, the PHA biodegradation patterns in tropical marine environments were studied. As a result, it was demonstrated that, under the given conditions, this process depends on the shape of a polymer product and its production method rather than on the chemical composition of the polymer. The degradation of polymer films was found to be more active than the degradation of molded solids. A decrease in the molecular mass of 3-PHB and 3-PHB/3-PHV was detected in the course of the sample degradation; i.e., splitting of the polymer chain occurred. The PHA polydispersity index reliably increased. The degree of crystallinity of both types of PHA did not change; that is, the levels of degradation of both the amorphous and crystalline phases of PHA were almost the same. Based on the results of morphological and molecular genetic analyses, the PHA-degrading microorganisms isolated from seawater were identified as *Enterobacter cloacae* sp. IBP-V001, *Bacillus* sp. IBP-V002, and *Gracilibacillus* sp. IBP-V003. Data on the PHA depolymerase activity of the representatives of the genera *Gracilibacillus* and *Enterobacter* are lacking in the literature.

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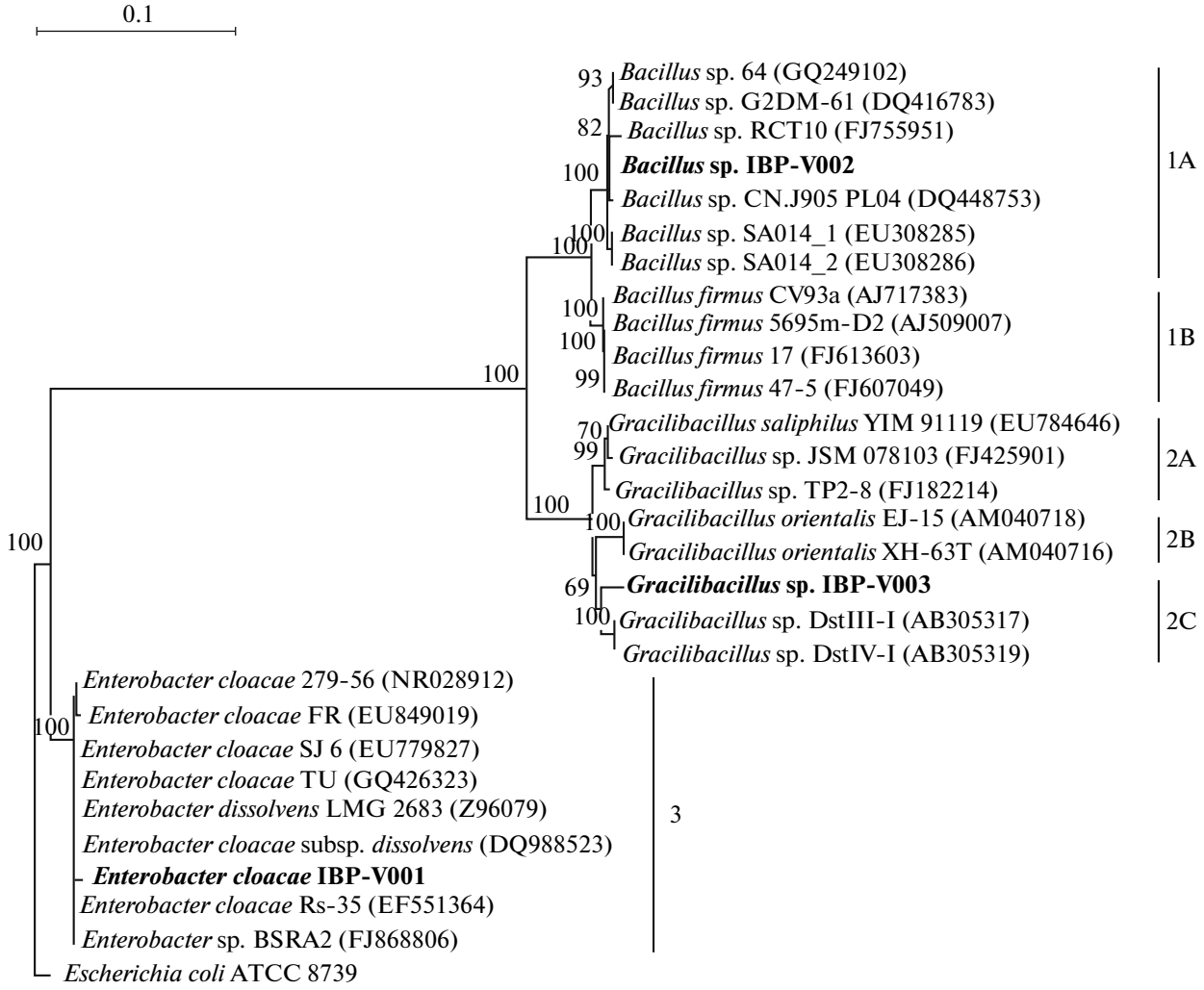

Fig. 4. Phylogenetic position of the studied PHA-degrading bacteria (in bold) based on the comparison of 1442 16S rRNA gene sequences using the neighbor-joining method. Scale bar, one nucleotide substitution for each ten sequences. The numerals show the results of bootstrap analysis (only bootstrap values above 65% were considered significant).

tists at Russian institutions of higher education (contract no. 11.G34.31.0013).

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