ISSN 0026-2617, Microbiology, 2006, Vol. 75, No. 5, pp. 593–598. © Pleiades Publishing, Inc., 2006.
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EXPERIMENTAL ARTICLES

Degradation of Polyhydroxyalkanoates and the Composition of Microbial Destructors under Natural Conditions

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Received May 17, 2005; in final form, March 6, 2006

Abstract—The degradation dynamics of polyhydroxyalkanoates of different composition has been studied in an eutrophic storage reservoir for two seasons. It has been shown that the biodegradation of polymers under natural conditions depends not only on their structure and physicochemical properties but also, to a great extent, on a complex of weather–climatic conditions affecting the state of the reservoir ecosystem. The molecular genetic analysis of 16S rRNA has revealed bacterial species (clones) probably involved in the degradation of polyhydroxyalkanoates in a model storage reservoir.

DOI: 10.1134/S0026261706050092

Key words: polyhydroxyalkanoates, biodegradation, identification, 16S rRNA.

The capacity for degradation in the environment (river and sea waters, soil, sludge, and compost) is one of the most valuable characteristics of polyhydroxyalkanoates (PHA). PHA are degraded completely (to carbon dioxide and water) under aerobic conditions and with methane formation in anaerobic conditions. Effective PHA destructors include various bacteria from widespread soil and water genera (*Pseudomonas*, *Alcaligenes*, *Comamonas*, *Streptomyces*, *Ilyobacter*) [1–3], as well as fungi (*Ascomycetes, Basidiomycetes, Deuteromycetes, Mastigiomycetes, Myxomycetes*) [4].

Potential microbial PHA destructors are generally isolated by inoculation of microbiological samples on agar plates or latex medium based on PHA particles or granules as the only carbon and energy source. Microbial exodepolymerases hydrolyze PHA to soluble products forming lysis zones on the plate surface; colonies from these zones have been selected for further studies. Recently, molecular genetic methods have been used for the identification of PHA destructors. A novel tropical marine bacterium, *Pseudomonas* sp. NRRL B-300083, degrading a hydroxybutyrate and hydroxyvalerate (PHB/PHV) copolymer in seawater has been identified by 16S rRNA gene sequence analysis [5]. Several strains of denitrifying bacteria *Comamonas testosterone* hydrolyzing polyhydroxybutyrate (PHB) and PHB/PHV copolymer have been isolated from activated sludge [6].

The expanding outlook for PHA application makes research into the degradability of these polymers in the environment highly relevant. Data on PHA degradation by pure microbial cultures in model laboratory conditions do not allow us to form an accurate understanding of the pattern of bioplastic degradation in multicomponent ecosystems under various and changing environmental conditions. The few available works show that PHA degradability is influenced by many factors, including the specificity and activity of extracellular depolymerases, properties of the polymer (stereoconfiguration of its molecules, crystallinity, molecular weight), as well as temperature, salinity, acidity of the environment, etc. [4].

The goal of this work was to study PHA degradation dynamics in a small eutrophic storage reservoir and to perform molecular genetic identification of the microorganisms involved in this process.

MATERIALS AND METHODS

The technique for the study of the degradation of polyhydroxyalkanoates in a natural reservoir. The experiments were performed during the summer field seasons of 1999–2000 in a small recreational eutrophic reservoir on the Bugach River (a secondary tributary of the Yenisei River), 0.32 km^2, up to 8 m deep, on the outskirts of Krasnoyarsk (56°03' N 92°43' E). In summer, the reservoir is susceptible to cyanobacterial (blue–green algal) blooms. Two types of PHA samples synthesized by the bacterium *Ralstonia eutropha* B5786 according to the technology developed at the Institute of Biophysics (Siberian Department of the Russian Academy of Sciences) were used in the work: a PHB homopolymer (molecular weight 340 kDa, crystallinity, 76%) and a PHB/PHV copolymer with 14 mol % of

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hydroxyvalerate (140 kDa and 52%, respectively). Preweighted polymer samples as film discs (40 mm in diameter, 0.07–0.10 mm thick) were placed into net nylon traps at the depth of 1 m. The rate of sample degradation was assessed by weight decrease. The discs were removed from the traps at regular intervals, washed with distilled water, dried, and weighed. Simultaneously, the state of the reservoir ecosystem was analyzed; the water temperature and the total quantity of cyanobacterial biomass were determined.

The methods of isolation of aquatic bacteria involved in polyhydroxyalkanoate degradation. The bacteria involved in the bioplastic degradation were isolated from the reservoir ecosystem by exposure of PHB/PHV films in the reservoir for 42 days (September 5 to October 17, 2000) in 2 l transparent plastic bottles, at the depth of 0.5 m (the control bottle without PHA was exposed to the same conditions). The bottlenecks were closed with mill gauze to provide oxygen access and to restrict the participation of zooplankton in the degradation of polymer films. The experimental and control water samples, wash-outs from the films, and the water sample taken from the reservoir on the last day of the experiment were treated by denaturing gradient gel electrophoresis (DGGE) in order to separate the DNA fragments of equal size with different nucleotide sequences [7].

Water samples were pre-filtered through 2.5-μm membrane filters to remove cyanobacteria. The total quantity of bacteria in the samples after preliminary filtration was estimated by epifluorescent microscopy with fluorescamine staining [8]. Bacterial plankton was collected by filtration on 0.2-μm membrane filters; then the filters were placed into sterile tubes. The bacteria were washed off the filters with 2 ml of sterile solution (10 mM Tris–HCl (pH 7.6), 100 mM NaCl, and 2 mM EDTA) and centrifuged; the supernatant was removed and the pellets were stored at –70°C until DNA isolation.

Total DNA isolation. DNA for amplification with polymerase chain reaction (PCR) was isolated by cell lysis with guanidine isothiocyanate followed by DNA sorption on a glass sorbent [9]. The quality and quantity of the isolated high-molecular DNA was analyzed by horizontal electrophoresis in 1% agarose gel.

PCR amplification. The 16S rRNA gene fragments, 586 bp, were amplified from bacterial plankton total DNA using PCR with universal primers GC341F and 926R [10]. PCR was performed on a Biometra TPersonal (Biometra, Germany) in the following temperature-time mode: the first cycle at 94°C, 30 s; 65°C, 35 s; 72°C, 40 s; then the annealing temperature was decreased by 1°C/cycle for 9 cycles; 20 cycles at 94°C, 30 s; 56°C, 35 s; 72°C, 40 s; and the final polymerization at 72°C, 5 min. The contamination was monitored by the negative control reaction with the addition of sterile water as a template. PCR products were analyzed by electrophoresis in 1.2% agarose gel. PCR products were desalinated by gel filtration on AutoSeq G-50 columns (Amersham Pharmacia Biotech, USA); 800 ng of the DNA thus obtained was used for DGGE analysis.

Denaturing gradient gel electrophoresis. DGGE was performed on a DCode Universal Mutation Detection System (BioRad, USA) in 6% polyacrylamide gel with the denaturing factor gradient of 40 to 70% (the 100% denaturing factor is a mixture of 7 M urea solution and 40% deionized formamide). The electrophoresis was carried out at 60°C in a single-strength TAE buffer at 100 V for 17 h. On completion of the electrophoresis, the gel was stained with ethidium bromide in order to obtain digital UV images (302 nm) on an AlphaImager (Alpha Innotech Corp., USA). The images were processed by the densitometry analysis program 1D-Multi in the AlphaEase 5.5 software package for quantitative assessment of the dynamics of DNA content in the individual bands. DNA bands were excised from the gel with a sterile scalpel and placed into the microtubes with 50 μl of sterile water for passive elution.

The cloning and sequencing of the 16S rRNA Genes. The eluates of the PCR products were reamplified and cloned by A/T cloning at the site EcoRV (with completed cohesive 3'T-ends) of the polylinker of plasmid pBluescript II (Stratagene, USA). The competent cells of *E. coli* XL-1 Blue were used to obtain the recombinants. The nucleotide sequences of the resulting inserts were determined on an ALFexpress II automatic sequencer (Amersham Pharmacia Biotech, USA), Device Sharing Center, Science and Education Center "Yenisei" (Krasnoyarsk State University, Krasnoyarsk), using a Thermo Sequenase Cy5 Dye Terminator Kit (Amersham Pharmacia Biotech, USA) according to the manufacturer's instructions. Universal plasmid primers M13 were used for the sequencing. The obtained nucleotide sequences were compared with the sequences from GenBank and EMBL databases using the BLAST online service [www.ncbi.nlm.nih.gov/blast].

RESULTS AND DISCUSSION

The process of degradation of PHA samples in the storage reservoir was studied over the course of several field seasons and the microorganisms involved in PHA destruction were identified. In the summer months of 1999, PHB films were degraded almost uniformly during the whole period under study (42 days) (Fig. 1c). During this time, the weight of the films decreased by 43.5% of the initial value and the specific rate of degradation, μ, was 0.011 mg day^{-1}. The PHB/PHV films were destroyed much more quickly (Fig. 1a and 1b), with the marked stages of the dynamics as follows: no reliable changes in the film weight were detected during the first 14 days; afterwards, intense destruction of the material occurred. In the first experiment (Fig. 1a), covering the period of June 16 to July 21, the films were

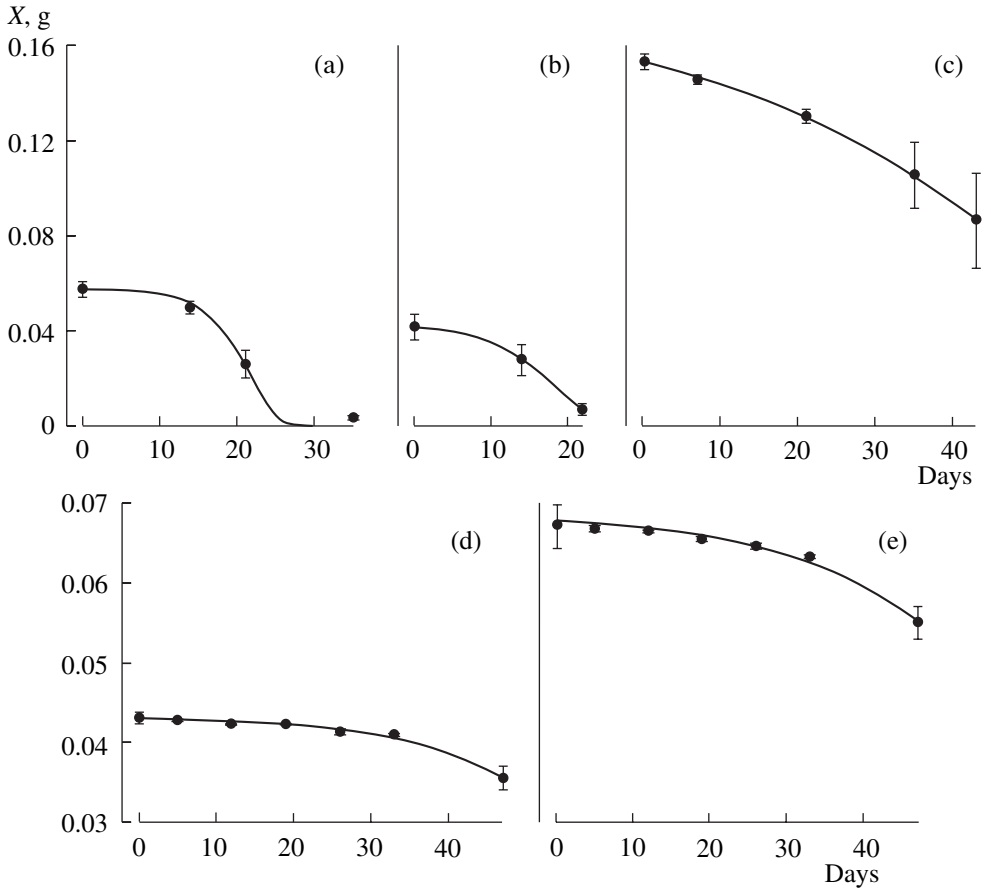

Fig. 1. Degradation of PHA films in the storage reservoir on the Bugach River and its approximation by Equation (1) (solid line): (a) PHB/PHV, beginning of the experiment, 16.06.1999; (b) PHB/PHV, 21.07.1999; (c) PHB, 30.06.1999; (d) PHB, 18.05.2001; (e) PHB/PHV, 18.05.2001.

almost completely destroyed (residual weight: 0.7% of the initial value) after a two-week lag phase, over the subsequent 21 days in July when the water temperature significantly increased; the mean μ value was 0.129 mg days^{-1}. The dynamics of PHB/PHV destruction in the second experiment (July 21 to August 12) was similar (Fig. 1b). However, the specific rate of film destruction in August was higher: 0.174 mg days^{-1}.

The observed difference in the rates of PHB and PHB/PHV degradation is in agreement with the existing concept that the homogenous PHB with higher crystallinity and melting point is less accessible to degradation by microorganisms and enzymes than the less crystalline PHB/PHV and well conforms to our previous results on the destruction of analogous samples of bioplastics under laboratory conditions [11]. The rate of bioplastic degradation in the reservoir was higher by an average of 20–30% as compared with the degradation of similar samples under laboratory conditions.

In 2000, the rates of polymer degradation were comparable with those of the previous season. However, quite a different picture was observed during the field season of 2001 (May 18 to July 4) (Fig. 1d, 1e). The biodegradation of PHA films of both types occurred with practically the same rates at extremely low μ values. In contrast to the previous seasons (1999 and 2000), the average rate of degradation for the period was 0.004 mg days^{-1} for both polymers. It should be noted that this field season was characterized by an unusually warm spring. In the middle of May, the air temperature was up to 25.9°C and the water temperature in the reservoir was 13.4°C (Fig. 2). This resulted in the heavy growth of cyanobacteria (Fig. 3). Probably, as a consequence, the growth of the microorganisms involved in PHA degradation was inhibited. The total concentration of bacterial plankton during the period of cyanobacterial bloom was considerably lower than usual: 6×10^5 cells/ml (Fig. 3).

Experimental data on the dynamics of bioplastic degradation in the reservoir were theoretically analyzed using a set of models [12, 13]. The best statistically reliable results were obtained with the model describing the active consumption of the studied (tested) substrate by bacteria growing on another substrate; in this case,

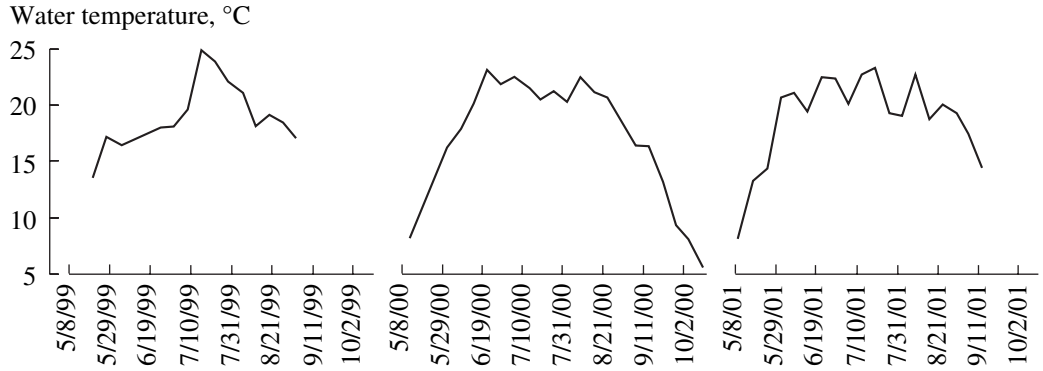

Fig. 2. The dynamics of water temperature in the Bugach reservoir, 1999–2001. The figures 5/8/99 on the X axis indicate May 8, 1999, etc.

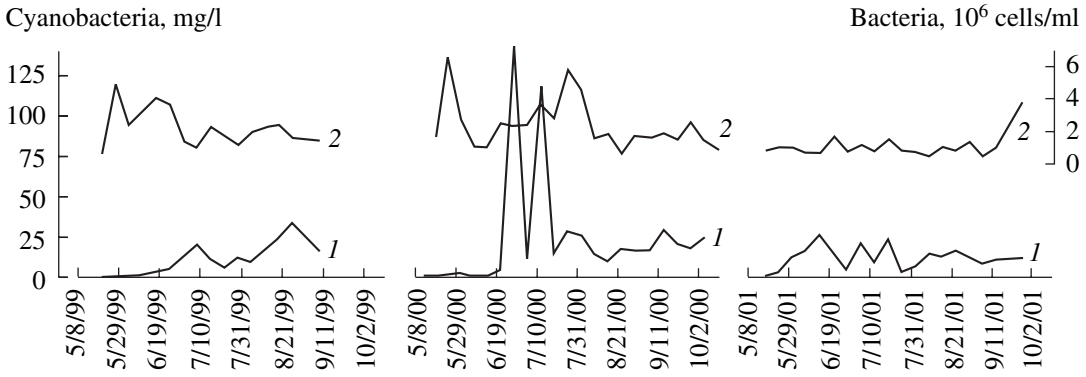

Fig. 3. The dynamics of cyanobacterial biomass (*1*) and total quantity of bacterial plankton (*2*) in the Bugach reservoir, 1999–2001. The markings on the X axis are similar to those on Fig 2.

their growth was exponential and the concentration (availability) of test substrate was low:

$$x(t) = x(0)e^{-(k_1/R)[\exp((Rt)-1)]}, \quad (1)$$

where $x(t)$ is the polymer concentration at a given time t; R is the maximum specific growth rate under the given conditions; $k_1 = V_{max}B(0)/K_m$; V_{max} is the maximum specific reaction rate under the given conditions; $B(0)$ is the initial magnitude of a population; and K_m is the constant of half-saturation. The model type was selected according to the results of the field season 2001, including more than five points (Table 1).

As is evident from the presented data, in 1999 the half-life period of PHB was almost two times lower as compared with PHB/PHV, whereas in 2001 (Fig. 1) these values were close. Thus, PHA biodegradation in the environment depends not only on the structure and physicochemical properties of the polymer, but also is substantially determined by the complex of weather–climatic conditions influencing the state of the reservoir ecosystem. The effect of specific factors (e.g., temperature) on the polymer degradation rate has not yet been revealed. However, it has been demonstrated that the specific degradation rate can vary from 0.004 to

Table 1. The parameters of approximation of the kinetics of PHA biodegradation in the Bugach storage reservoir

Date	PHA	*n*	*RSS*	*F*	k_1 (days^{-1})	R (days^{-1})	*HL*, days
16.06–21.07.1999	PHB/PHV	4	1.474×10^{-5}	243.4	0.000549	0.2880	20.5
21.07–12.08.1999	PHB/PHV	3	3.501×10^{-7}	1742.6	0.005590	0.1880	17.0
30.06–12.08.1999	PHB	5	2.506×10^{-6}	3687.1	0.004960	0.0396	47.4
18.05–6.07.2001	PHB	7	5.860×10^{-7}	341.8	0.000449	0.0743	63.9
18.05–6.07.2001	PHB/PHV	7	15.569×10^{-7}	373.7	0.000738	0.0619	65.9

Note: *n*, number of experimental points; *RSS,* residual squares total; *F*, Fisher criterion; k_1 and *R*, constants of Equation (1); *HL*, half life.

0.174 mg day^{-1} depending on the weather–climatic conditions and the reservoir ecosystem state.

The analysis of bacterial plankton species composition during the period of observation yielded the following results. The total quantity of bacterial plankton was: 1.1×10^6 cells/ml in the control sample, 2.3×10^6 cells/ml in the sample with the films, 16.1×10^8 cells/ml in the wash-offs from the films, and 0.9×10^6 cells/ml in the sample from the reservoir. The species composition of microorganisms isolated from the samples of the water to which the PHB/PHV films had been exposed and from the wash-offs had qualitative and quantitative differences in terms of the character of distribution of DNA bands on DGGE from the control PHA-free water sample and from the bacterial plankton samples taken from the experimental reservoir in October 2000 (Fig. 4).

The control sample had a strong resemblance to the bacterial plankton sample from the reservoir. The differences between them are probably due to the fact that the control sample was essentially isolated from the inflow of organic substrate during the experiment. Apparently, sp-19, sp-24, and sp-35 species, which occurred in both samples in equal quantities, were less fastidious. In experimental water samples, sp-5, sp-16, sp-22, and sp-25 dominated in the presence of the polymer. The weight of the films in the experimental sample decreased by 0.1233 g, or by 7.2%, during the period of observation (42 days), while the specific rate of polymer degradation was 0.002 mg day^{-1}.

One can mention a significant (twofold or more, according to the densitometry data) increase of the relative share of sp-5, sp-16, and sp-25 in the wash-offs from the films as compared with the water to which they were exposed. This characteristic of the dynamics suggests that the above species mainly have an attached mode of existence, effecting the primary degradation of the polymer on film surfaces by extracellular depolymerases. At the same time, the reversed dynamics of sp-18 and sp-22 supports the assumption that these species are free-living plankton bacteria, which probably have no extracellular depolymerases and which utilize the products of primary copolymer degradation: tetra-, tri-, di-, and monomers of β-hydroxybutyric and β-hydro-xyvaleric acids [14].

The nucleotide sequences of 16S rRNA gene fragments excised from the corresponding regions of DGGE gels were determined in order to identify the species (clones) sp-5, sp-16, sp-18, sp-19, sp-22, sp-24, sp-25, sp-34, and sp-35 (Fig. 4). The sequences were deposited in the EMBL/GenBank online database of under the following numbers: AJ583816, AJ583805, AJ583806, AJ583807, AJ583808, AJ583809, AJ583810, AJ583813, and AJ583814. The results of nucleotide sequence analysis are presented in Table 2. Out of the four species found only in the presence of PHB/PHV, two (sp-16 and sp-25) had more than 97% similarity with closely related bacteria. It is known that

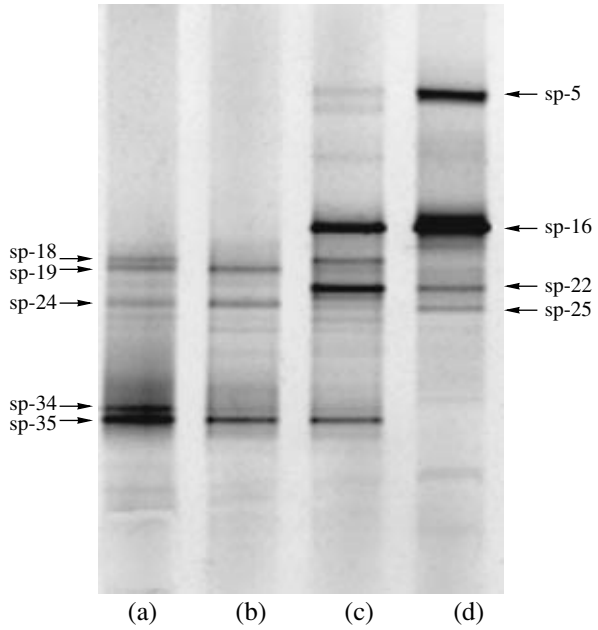

Fig. 4. DGGE of the amplified 16S rRNA gene fragments from bacterial plankton samples from the Bugach reservoir, October 2000 (a), and from the experiment on degradation of PHB/PHV heteropolymer films: (b) the control without the polymer, (c) the water to which the polymer films were exposed, and (d) the wash-off from the films.

the *Pseudomonas* and *Variovorax* bacteria, which are close in terms of the 16S rRNA sequence to the species isolated from the reservoir, are able to utilize PHB and PHB/PHV [15–17]. Most *Leptothrix* bacteria (the closest relative of clone sp-22) are able to accumulate and, as a consequence, to utilize PHB (see Bergey's Manual of Systematic Bacteriology). The bacteria of *Bacteroidetes* group (sp-5) participate in PHA degradation rather rarely [17]. However, the capacity for PHB accumulation has not been demonstrated for any cultured genus of this phylogenetic cluster (see Bergey's Manual of Systematic Bacteriology). This fact is in agreement with the existing concept that the species with PHA-degrading exodepolymerases are not necessarily capable of the synthesis of these polymers, while endodepolymerases are characteristic only of PHA-producing microorganisms [4].

Thus, molecular genetic methods have been used to identify the species (clones) of bacteria which are probably able to utilize PHA in a freshwater ecosystem. For the first time, the kinetics of PHA biodegradation in a natural water reservoir has been determined and its significant variability subject to environmental conditions has been shown.

ACKNOWLEDGMENTS

The work was supported by the Ministry of Education of the Russian Federation and US Civilian Research and Development Foundation (CRDF) (grant

Table 2. The comparative analysis of nucleotide sequences of 16S rRNA gene fragments obtained from the DGGE bands of the amplified samples of bacterial plankton from the experiment on degradation of PHB/PHV films, 2000

Clone	Length, bp	Closest relative	Similarity, %	Phylogenetic group
sp-5	578	Nonculturable *Haliscomenobacter* sp.	95.3	*Bacteroidetes*
sp-16	585	*Pseudomonas putida,* strain KT2440	98.1	*γ-Proteobacteria*
sp-18	585	Nonculturable bacterium, clone SJA-109	96.6	*β-Proteobacteria*
sp-19	586	Nonculturable bacterium, clone WYO9bC	87.8	*γ-Proteobacteria*
sp-22	582	*Leptothrix* sp. L18	96.9	*β-Proteobacteria*
sp-24	343	Nonculturable bacterium, clone Spb54	97.7	*β-Proteobacteria*
sp-25	418	Nonculturable *Variovorax* sp., clone KL-93-1-6	97.1	*β-Proteobacteria*
sp-34	562	Nonculturable bacterium, clone s41	98.2	Not determined
sp-35	567	Nonculturable bacterium, clone CL500-95	97.0	*Actinobacteria*

no. RUX0-002-KR-06). At the stage of discussion, the work was supported by individual grants for young scientists from Krasnoyarsk Regional Science Foundation, Grants no. Y1-B-02-08 and Y3-B-02-12 for young scientists, the Program "Fundamental Research and Higher Education" (Ministry of Education of Russian Federation and CRDF), and RFBR Grant no. 05-04-48270, as well as Interdisciplinary Project of Siberian Division of Russian Academy of Sciences no. 24.

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