ISSN 0026-2617, Microbiology, 2013, Vol. 82, No. 5, pp. 637–641. © Pleiades Publishing, Ltd., 2013.
Original Russian Text © T.V. Grigoryeva, A.V. Laikov, A.A. Rizvanov, O.N. Ilinskaya, R.P. Naumova, 2013, published in Mikrobiologiya, 2013, Vol. 82, No. 5, pp. 635–639.

EXPERIMENTAL ARTICLES

Composition of the Oil-Slime Microbial Community as Determined by Analysis of the 16S rRNA Gene

T. V. Grigoryeva[1], A. V. Laikov, A. A. Rizvanov, O. N. Ilinskaya, and R. P. Naumova

[a] *Kazan (Volga region) Federal University, Russia*

Received August 14, 2012

Abstract—Analysis of the 16S rRNA genes of the cultured microorganisms of industrial oil-slime revealed predominance (~85–90%) of the *Gammaproteobacteria* in the community of aerobic heterotrophs and specific oil-slime degraders. Relation of the isolated strains with members of the genera *Pseudomonas, Stenotrophomonas,* and *Enterobacter* was established. Analysis of the same gene in the total DNA from the oil-slime revealed greater microbial diversity (~20 operative taxonomic units determined by T-RFLP) than in the cultured part of the community, which included ~12 different colony types. Three major restriction fragments were found, with their total area ~50%. These results demonstrated the low morphological and phylogenetic diversity of the oil-slime bacterial community.

Keywords: industrial oil-slime, bacterial community, 16S rRNA, T-RFLP

DOI: 10.1134/S0026261713050044

Due to high levels of toxicity and pollution, oil-slimes are dangerous industrial wastes. They are formed in the course of mechanical treatment of wastewaters of the oil and gas industry and are stored in special collectors as concentrated sediments. The autochthonous microflora of these wastes is of interest due to the requirements of decontamination and processing, as well as to the basic issues of biodiversity in anthropogenic environments. Oil-slime stored for decades is known to contain viable microorganisms. Their number may be is high as 10^6–10^8 CFU/g [1, 2]. Most isolates obtained from oil-slimes were found to have biotechnologically important properties, including multiple resistance, ability to degrade various hydrocarbons, and stimulation of plant growth [2, 3]. Available data on the microflora of industrial slimes do not provide information concerning the structure of the microbial community and its genetic diversity. Molecular biological techniques make it possible to characterize the composition of a bacterial community from oil-slime and to determine the microorganisms which are most resistant to combined chemical contamination.

The goal of the present work was to analyze the 16S rRNA gene sequences in the total DNA from oil-slime and to reveal the dominant members of the cultured part of the microbial community.

[1] Corresponding author; e-mail: tata.grigoryeva@gmail.com

MATERIALS AND METHODS

Hydrocarbon slime from the Kazanorgsintez chemical company was the subject of investigation. It contained up to 250 g/kg of chloroform-extractable matter and up to 350 g/kg of bichromate-oxidized matter. The samples from the slime stored for 10 years were collected from 1–1.5 m depth. The numbers and diversity of the microbial community were determined by plating on nutrient agar (NA) and mineral medium with oil-slime (20 g/L) as the sole carbon and energy source.

Morphological characteristics of the colonies were used to assess the diversity of the cultured microbial community. The dominant aerobic heterotrophs and specific degraders were isolated in pure cultures for further characterization. The isolates of heterotrophs and degraders were marked as G and D, respectively. Species identification of the predominant isolates was carried out by analyzing their 16S rRNA sequences. Total bacterial diversity was assessed by the number of terminal restriction fragments of the 16S rRNA gene (terminal restriction fragment length polymorphism, T-RFLP) amplified with the total DNA from the slime.

Genomic DNA of pure cultures was isolated by phenol–chloroform extraction [4]. Total DNA from slime samples was isolated using the PowerSoil DNA Isolation Kit (MO BIO, United States) according to the manufacturer's recommendations. DNA concentration in the solution was determined on a NanoDrop 1000 spectrophotometer (Thermo Fisher Scientific,

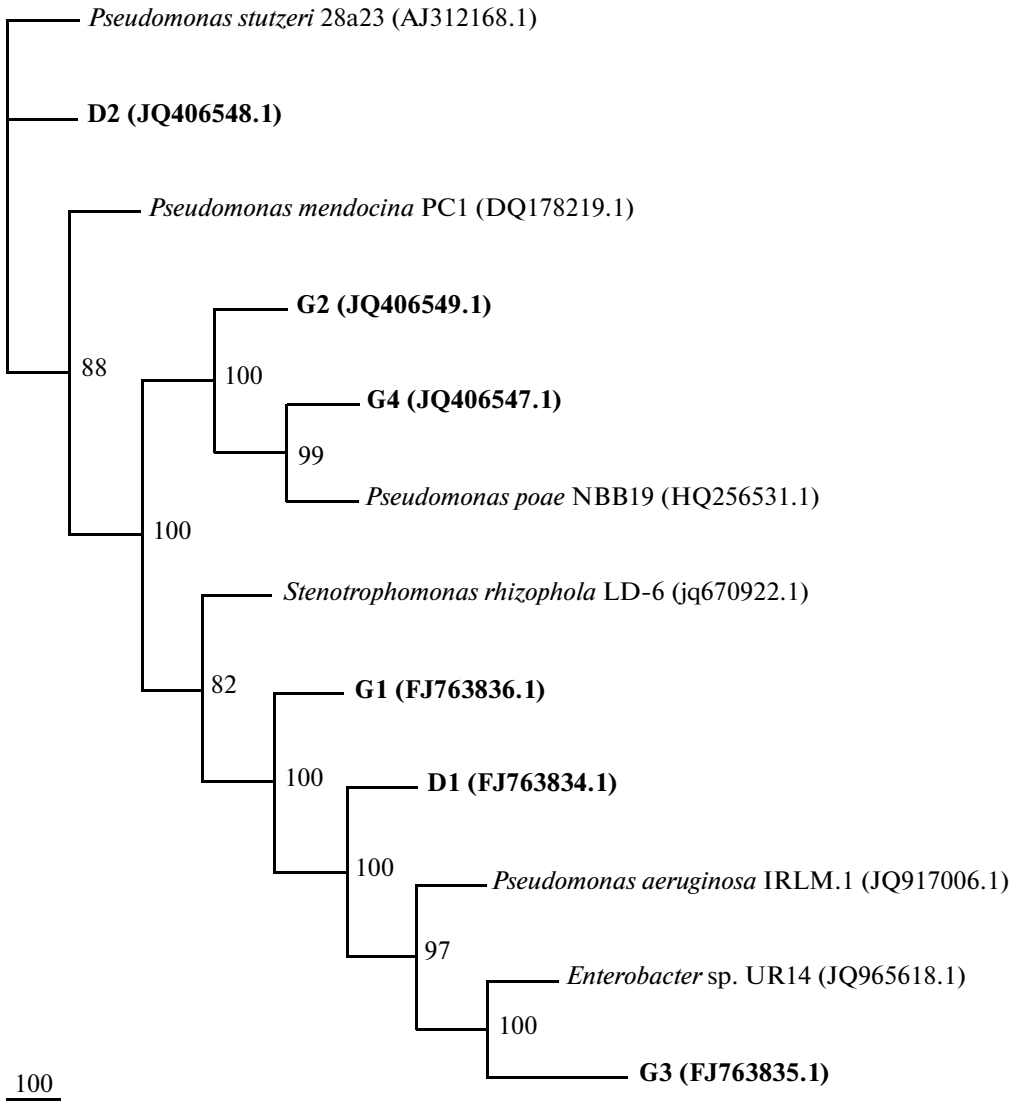

Fig. 1. Phylogenetic tree of the 16S rRNA genes showing the taxonomic position of the dominant isolates from the oil-slime microbial community.

United States). The samples were stored at –20°C. The 16S rRNA gene fragments were amplified with the universal primers 8F (5'-AGAGTTTGATCCTG-GCTCAG-3') and 1492R (5'-ACGGCTACCTTGT-TACGACTT-3') [5].

For T-RFLP analysis, the 1492R primer labeled with 5-carboxytetramethylrhodamine (TAMRA, Syntol, Russia) at the 5' terminus was used [6]. Polymerase chain reaction (PCR) was carried out on an MJ Mini Gradient Thermal Cycler (Bio-Rad, United States) in the following mode: initial denaturation for 5 min at 95°C; 35 cycles of 30 s at 95°C, 30 s at 53°C, and 2 min at 72°C; and final elongation for 7 min at 72°C. Sequencing of the 16S rRNA gene fragments and T-RFLP analysis were carried out by Syntol (Russia) on an automatic capillary sequencer. The *Msp*I restriction endonuclease was used for T-RFLP analysis. The marker enzyme kit used was GS600LIZ (Applied Biosystems, United States); Peak Scanner 1.0 software package (Applied Biosystems, United States) was used to analyze the results.

The 16S rRNA gene sequences were compared to the sequences from NCBI database using the BLAST software package (http://blast.ncbi.nlm.nih.gov). The sequences were aligned using the CLUSTAL W function in the BIOEDIT 7.0.4 software package (http://jwbrown.mbio.ncsu.edu/BioEdit/bioedit.html) [7]. The phylogenetic tree was constructed by the maximum economy method using the PHYLIP 3.5 software package (http://evolution.genetics.washington.edu/phylip/getme.html). The TreeView software

Identification of the dominant members of the cultured part of the oil-slime microbial community by 16S rRNA gene sequencing of pure cultures

Strain	Colony morphology	Share in the cultured communities, %	Phylogenetic group	Most closely related valid species, GenBank	Similarity of the 16S rRNA genes, %
G1	Round, white, smooth, glossy, even edge	34	*Gammaproteobacteria*	*Stenotrophomonas rhizophila* LD-6 (JQ670922.1)	99
G2	Irregular, flat, smooth, glossy, transparent	34	*Gammaproteobacteria*	*Pseudomonas mendocina* PC1 (DQ178219.1)	99
G3	Round, smooth, glossy, transparent, with a gray tint, even edge	20	*Gammaproteobacteria*	*Enterobacter* sp. UR14 (JQ965618.1)	100
G4	Round, smooth, glossy, transparent, with a brown tint, even edge	6	*Gammaproteobacteria*	*Pseudomonas poae* NBB19 (HQ256531.1)	100
Infrequently occurring G		6	ND	ND	ND
D1	Round, creamy, smooth, glossy, even edge	70	*Gammaproteobacteria*	*Pseudomonas aeruginosa* IRLM.1 (JQ917006.1)	100
D2	Round, crumpled, yellow-brown, wavy edge	23	*Gammaproteobacteria*	*Pseudomonas stutzeri* 28a23 (AJ312168.1)	99
Infrequently occurring D		7	ND	ND	ND

G and D designate aerobic heterotrophs and specific degraders, respectively. ND stands for no data.

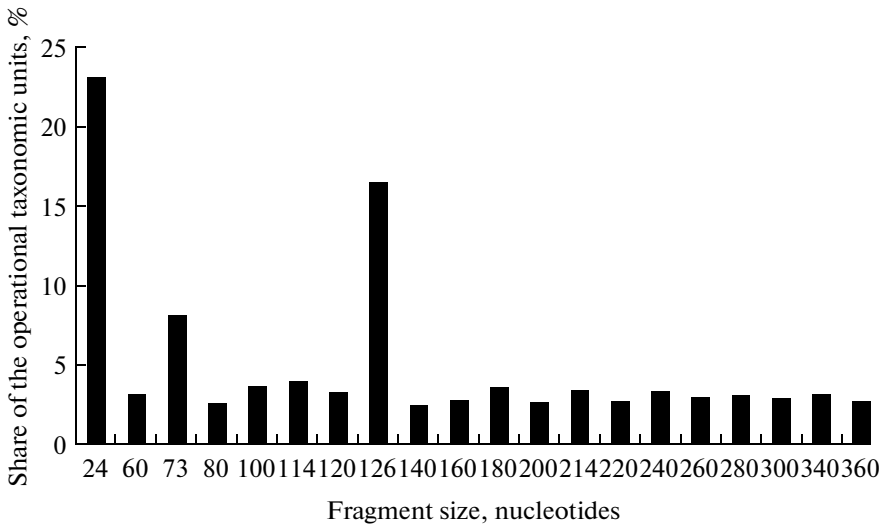

Fig. 2. Diversity of the operational taxonomic units obtained by restriction analysis of the 16S rRNA gene amplified with the total DNA from the oil-slime.

package (http://taxonomy.zoology.gla.ac.uk/rod/treeview.html) was used for visualization.

RESULTS AND DISCUSSION

Plating of suspensions of the oil-slime revealed high numbers of aerobic heterotrophic microorganisms and specific oil-slime degraders (up to 10^8 and 10^7 CFU/g dry slime, respectively). The diversity of colony morphotypes revealed on NA did not exceed 10–12, with 90% of the colonies belonging to three morphotypes (table). The colonies obtained on the medium with oil-slime belonged to two morphotypes. According to [8], a similar procedure used to study microbial diversity in soil yielded tens of morphotypes. Thus, cultural techniques were used to obtain the first impression of the oil-slime microbial community. It differs from the well-studied soil communities in high abundance of specific degraders, as well as in their limited diversity and high share among the culture microorganisms.

Members of the dominant morphotypes were isolated in pure cultures and identified based on their 16S rRNA gene sequences. The isolates were shown to belong to the genera *Pseudomonas, Stenotrophomonas*, and *Enterobacter* within the class *Gammaproteobacteria* (table, Fig. 1). Gammaproteobacteria were previously shown to predominate in the microbial community of oil industry slimes and oil-contaminated soils [9, 10].

T-RFLP analysis of the total DNA confirmed the limited microbial diversity. Three phylotypes predominated among the 20 revealed (Fig. 2). T-RFLP analysis of marine sediments yielded similar results [11]. In the case of hydrocarbon contamination, microbial diversity did not exceed 20 phylotypes, while uncontaminated samples contained over 100 taxonomic units [11].

Our results demonstrate the diversity of bacteria in hydrocarbon slimes, which, according to their chemical and biological properties, are ecological niches with an extreme anthropogenic load. Such limited diversity has been shown for naturally extreme environments, such as saline lakes and hot springs, where one class of microorganisms predominated [12, 13]. Thus, minimization of the species diversity with predominance of the few forms most resistant to stress factors (associated, for example, with heavy hydrocarbon contamination) is the universal strategy of adaptation of microbial communities to extreme environments.

ACKNOWLEDGMENTS

The work was supported by the Federal Target Program "Research and Development in Priority Directions of the Russian Scientific and Technological Complex in 2007–2012" (2011-1.5-515-034), state contract no. 16.515.11.5043.

REFERENCES

1. Nikitina, E.I., Yakusheva, O.I., Zaripov, S.A., Galiev, R.A., Garusov, A.V., and Naumova, R.P., Distribution and physiological state of microorganisms in petrochemical oily sludge, *Microbiology*, 2003, vol. 72, no. 5, pp. 621–627.
2. Galiev, R.A., Ziganshin, A.M., Yakusheva, O.I., Nikitina, E.V., Zaripov, S.A., and Naumov, A.V., Ecologically hazardous petrochemical sludges as a nutrient source for microorganisms, *Environ. Radioecol. Appl. Ecol.*, 2003, no. 4, pp. 13–21.
3. Naumova, R.P., Grigoryeva, T.V., Rizvanov, A.A., Gorshkov, V.Y., Kudrjashova, N.V., and Laikov, A.V., Dia-

zotrophs originated from petrochemical sludge as a potential resource for waste remediation, *World Appl. Sci. J.*, 2009, vol. 6, pp. 154–157.

4. Maloy, S.R., *Experimental Techniques in Bacterial Genetics*, Sudbury: Jones and Bartlett learning, 1989.
5. Weisburg, W.J., Barns, S.M., Pelletier, D.A., and Lane, D.J., 16S ribosomal dna amplification for phylogenetic study, *J. Bacteriol.*, 1991, vol. 173, no. 2, pp. 697–703.
6. Li, F., Hullar, M.A.G., and Lampe, J.V., Optimization of terminal restriction fragment polymorphism (TRFLP) analysis of human gut microbiota, *J. Microbiol. Meth.*, 2007, vol. 68, pp. 303–311.
7. Hall, T.A., BioEdit: a user-friendly biological sequence alignment editor and analysis program for Windows 95/98/NT, *Nucleic Acids Symp. Ser.*, 1999, vol. 41, pp. 95–98.
8. Dobrovol'skaya, T.G., Golovchenko, A.V., Pankratov, T.A., Lysak, L.V., and Zvyagintsev, D.G., Assessment of the bacterial diversity in soils: evolution of approaches and methods, *Euras. Soil Sci.*, 2009, no. 10, pp. 1138–1147.
9. Barragan, V.A., Aveiga, I., and Trueba, G., Microbial community composition in petroleum-contaminated and uncontaminated soil from Francisco de Orellana, in the northern Ecuadorian Amazon, *Int. Microbiol.*, 2008, vol. 11, no. 2, pp. 121–126.
10. Katsivela, E., Moore, E.R.B., Maroukli, D., Strompl, C., Pieper, D., and Kalogerakis, N., Bacterial community dynamics during in-situ bioremediation of petroleum waste sludge in landfarming sites, *Biodegradation*, 2005, vol. 16, no. 2, pp. 169–180.
11. Danovaro, R., Luna, G.M., Dell'Anno, A., and Pietrangeli, D., Comparison of two fingerprinting techniques, terminal restriction fragment length polymorphism and automated ribosomal intergenic spacer analysis, for determination of bacterial diversity in aquatic environments, *Appl. Environ. Microbiol.*, 2006, vol. 72, pp. 5982–5989.
12. Huang, Q., Dong, C.Z., Dong, R.M., Jiang, H., Wang, S., Wang, G., Fang, B., Ding, X., Niu, L., Li, X., Zhang, C., and Dong, H., Archaeal and bacterial diversity in hot springs on the Tibetan Plateau, China, *Extremophiles*, 2011, vol. 15, no. 5, pp. 549–563.
13. Mesbah, N.M., Abou-El-Ela, S.H., and Wiegel, J., Novel and unexpected prokaryotic diversity in water and sediments of the alkaline, hypersaline lakes of the Wadi An Natrun, Egypt, *Microb. Ecol.*, 2007, vol. 54, pp. 598–617.

Translated by P. Sigalevich