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MARINE BIOLOGY

Psychrotrophic Hydrocarbon-Oxidizing Bacteria Isolated from Bottom Sediments of Peter the Great Bay, Sea of Japan

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Abstract—Five strains of psychrotrophic hydrocarbon-oxidizing bacteria were isolated from the bottom sediments of Peter the Great Bay of the Sea of Japan. They were classified into the following species: *Rhodococcus erythropolis*, *Rhodococcus* sp., *Sphingomonas* sp., *Pseudomonas* sp., and *Alcanivorax* sp. All studied bacteria showed high oxidizing ability in relation to the decomposition of *n*-alkanes (C9–C27), phytane, pristane, and polycyclic aromatic hydrocarbons at 5 and 22°C. At the same time, the degradation of hydrocarbons was more intense at 5°C. Despite the different taxonomic affiliations of the obtained microorganisms, all strains primarily utilized short- (C9–C13) and long-chain (C21–27) alkanes, as well as polycyclic aromatic hydrocarbons. The highest hydrocarbon-oxidizing activity was shown by the strain *Rhodococcus erythropolis* AP_291. The latter utilized more than 50% of all hydrocarbons in the model mixture during the first week of the experiment at 5°C.

Keywords: psychrotrophs, hydrocarbon-oxidizing bacteria, oil, hydrocarbons, Sea of Japan, alkanes, polycyclic hydrocarbons

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The anthropogenic load growing worldwide makes the search for hydrocarbon-degrading microorganisms, as well as the development of preparations based on these microorganisms to eliminate oil pollution of the marine environment, an urgent scientific task [16, 46]. Such biological products have proven to be effective and environmentally friendly preparations for the purification and remediation of marine ecosystems [36]. The search for psychrophilic and psychrotolerant hydrocarbon-oxidizing microorganisms (HCOMs) that are able to efficiently degrade oil components at low temperatures is of particular interest.

Recent studies have indicated that the use of indigenous microorganisms adapted to the degradation of hydrocarbons (HC) under certain environmental conditions is promising for bioremediation in each specific region. At the same time, these microorganisms are not genetically modified and have no toxic effect on local flora and fauna [40].

Bacteria of the genera *Mycobacterium*, *Brevibacterium*, *Nocardia*, *Corynebacterium*, *Rhodococcus*, and *Arthrobacter*, which are usually isolated from the areas heavily polluted with oil, are among the most common HCOMs in the marine environment [1, 7, 8]. The ability to oxidize HC is often found in *Acinetobacter*, *Achromobacter*, *Bacterium*, *Bacillus*, *Micrococcus*, and *Pseudomonas*. Hydrocarbon-oxidizing (HCO) members of the genera *Pseudoalteromonas, Psychrobacter, Marinobacter, Marinomonas, Oleispira*, and *Shewanella* have been detected in sea water and ice [32].

Information about HCOMs of the Far Eastern seas is scarce in the scientific literature. The study of the microbiota of the Bering Sea has reported the ability of bacteria of the genera *Bacillus*, *Pseudomonas*, *Micrococcus*, *Pseudobacterium*, *Achromobacter*, *Bacterium*, and *Brevibacterium* to degrade HC [6]. Among the oil oxidizers of the Sea of Okhotsk, *Cobetia*, *Pseudoalteromonas*, *Oceanisphaera*, *Shewanella*, *Pseudomonas*, *Marinomonas*, and *Thalassospira* have been revealed [14].

The ecosystem of the Sea of Japan is characterized by considerable anthropogenic impact associated with an increase in the population, urbanization of coasts, active shipping, industrialization, and tourism development. On the shores of Peter the Great Bay, which

is the largest bay on the Russian coast of the Sea of Japan, the ports of Vladivostok, Nakhodka, Bolshoy Kamen, and an urban-type settlement Posyet, as well as a specialized oil-loading port of Kozmino, are situated. In most coastal areas of Peter the Great Bay, a high content of petroleum HC is recorded [4]. The problem of oil pollution is exacerbated by the effect of low temperatures in the waters of the Sea of Japan for most of the year, retarding the processes of HC oxidation. For instance, the average annual temperature of the surface waters of the Sea of Japan is 12°C [2], while the temperature of bottom shelf waters rarely rises above 0°C [5].

Low temperatures have adverse effects on the rate of oil biodegradation due to a decreased solubility of its compounds. These conditions of the existence of microorganisms result in a decrease in the activity of biochemical reactions, an increase in the viscosity of the medium, as well as changes in membrane fluidity and protein conformation. In response to low temperatures, psychrophilic and psychrotrophic microorganisms have developed the following adaptations: an increase in the content of polyunsaturated, short-chain, and branched fatty acids in membrane lipids; biosynthesis of cryoprotectants and antifreeze proteins; production of heat and cold shock proteins. An important adaptation of psychrophilic hydrocarbon-oxidizing bacteria is the synthesis of cold-active enzymes, as well as bioemulsifiers to reduce the viscosity and surface tension of the medium [33].

Despite the chronic pollution of the Sea of Japan with oil products, the HCO microbiota of this region and its participation in the self-purification of the ecosystem has not been sufficiently studied. Some bacteria from the surface coastal waters of Japan are known to oxidize HC. These are *Alcanivorax* [21, 25, 35, 41], *Pseudomonas aeruginosa* [15], *Sphingomonas subarctica* [25], and *Cycloclasticus pugetii* [35]. However, the modern scientific literature lacks information about the taxonomic diversity and metabolic potential of psychrophilic and psychrotrophic HCOMs in the Sea of Japan. Therefore, the goal of this research was to obtain cultures of psychrotrophic hydrocarbon-oxidizing bacteria from the bottom sediments of Peter the Great Bay of the Sea of Japan, to determine their taxonomic position, and to evaluate their ability to oxidize oil hydrocarbons.

MATERIALS AND METHODS

Study area and sampling. Samples of the upper oxidized layer of the bottom sediments of Peter the Great Bay (the Sea of Japan) obtained in 2018–2019 during coastal expeditions were used as material for this study.

Sediment samples were obtained according to the regulatory documents and methodological recommendations and requirements for sampling the bottom sediments of water bodies for pollution analysis [10]. From the moment of sampling to the beginning of the study, the samples were stored in a freezer at –30°C.

Obtaining cultures of hydrocarbon-oxidizing bacteria. To obtain cultures of hydrocarbon-oxidizing bacteria (HCOB), marine fuel oil was used as a source of HC. Marine fuel is among the oil products that are most characteristic of anthropogenic pollution of the marine environment. To obtain the enrichment cultures of HCOB, bottom sediments were placed in the modified Voroshilova–Dianova mineral medium containing sterile marine fuel oil (2.5%) as the only carbon and energy source [9]. The composition of the mineral medium was as follows (g/L of distilled water): NaCl, 10.0; NH_4NO_3, 1.0; K_2HPO_4, 1.0; KH_2PO_4, 1.0; $MgSO_4$, 0.2; $CaCl_2$, 0.02; $FeCl_2$, two drops of the saturated solution. Incubation was carried out for 30 days at 5°C. To obtain pure cultures of HCOB, the cultivation was carried out on a similar agar medium under the same conditions. To assess the ability of the obtained isolates of HCOB to utilize HC at higher temperatures, the incubation was carried out on the solid mineral medium containing fuel oil for 10 days at 22°C.

Molecular genetic identification of hydrocarbon-oxidizing bacteria and phylogenetic analysis. The genomic DNA was extracted from bacterial cultures using the commercial Genomic DNA purification kit (Thermo Fisher Scientific, United States) according to the manufacturer's recommendation. The 16S rRNA gene fragment was amplified using the following pair of primers: 27F (5'–AGAGTTTGATCATGGCTCAG–3') and 1350R (5'–GACGGGCGGTGTGTACAAG–3') [30]. The PCR reaction products were separated in agarose gel (1%) containing ethidium bromide in an electrophoresis chamber. Amplification products of the required length were cut out from the gel and extracted by freezing at –80°C (30 min), followed by centrifugation at 13 400 rpm for 20 min. The obtained PCR products were sequenced by the Sanger method using the Big Dye Terminator v.3.1 Cycle Sequencing Kit (Thermo Fisher Scientific, United States) and an ABI 3500 Genetic Analyzer (Applied Biosystems, United States) at the Biotechnology and Genetic Engineering Center for Collective Use (Federal Scientific Center for the East Asia Terrestrial Biodiversity, Far Eastern Branch, Russian Academy of Sciences).

Phylogenetic analysis was performed on 16S rRNA gene sequences from the international data bank (GenBank) using BLAST software [12] (http://www.ncbi.nlm.nih.gov/blast). Sequences were edited with the BioEdit editor and aligned using the CLUSTAL W algorithm (http://www.genebee.msu.su/clustal). The phylogenetic tree was constructed using the neighbor-joining method based on the Kimura two-parameter model with the MEGA X software [29]. The confidence value for branching order was determined by bootstrap analysis of 100 alternative trees. Nucleotide sequences of the bacterial 16S rRNA gene fragments were deposited in the GenBank database.

Evaluation of the ability of bacterial strains to oxidize oil hydrocarbons. The model mixture of HC was prepared based on the commercially available summer diesel fuel (grade C, type III (DT-L-K5), GOST R 52368–2005 (EN 590:2009)). Additionally, chemically pure naphthalene, anthracene, and phenanthrene (8 mmol) were dissolved in 1 L of the sterile diesel fuel. Before the experiment, the model mixture of HC was repeatedly autoclaved.

Physiological saline (50 μL) supplemented with a 24-h culture of the studied bacteria (10^5 cells/mL) was added to sterile penicillin vials containing the modified liquid Voroshilova–Dianova medium (4650 μL) and the model mixture of HC (300 μL). The vials were sealed with rubber stoppers and aluminum caps. Incubation was carried out for 70 days at 5°C and 28 days at 22°C. Sterile saline (instead of the bacterial suspension) was used as a control. All experiments were carried out in triplicate.

For all samples, the extraction was performed with trichloromethane (chemically pure, distilled) on days 7, 14, and 28, as well as on day 70, but only for the samples incubated at 5°C. Aliquots (1 mL) were collected from the extracts diluted six times with trichloromethane before the chromatography-mass spectrometry analysis.

Chromatography-mass spectrometry analysis was performed using a Shimadzu GCMS 2010 Ultra chromatography-mass spectrometer. The components were separated on an Ultra ALLOY-5 MS/HT capillary column (Frontier Lab., Japan; length, 30 m; film thickness, 0.25 μm; diameter, 0.25 mm; phase, 5% diphenyl/ 95% dimethyl polysiloxane). Helium (grade 6.0) was used as a carrier gas. Using an autosampler, a sample dissolved in chloroform (1 μL) was introduced into an injector heated to 230°C (split mode; flow splitting, 1 : 40). The linear velocity of the carrier gas was 40 cm/s, and the initial gas pressure was 66 kPa. The samples were chromatographed in the following mode: isothermally for 7 min at 40°C, with a temperature gradient from 40 to 270°C and a heating rate of 4°C/min; after that, isothermally for 20 min at 270°C. The temperature of the ion source was 200°C; the temperature of the mass spectrometer interface was 270°C. The scan range of the mass spectrometer was from 35 to 600 amu, with electron impact ionization at 70 eV. Mass fragmentograms were recorded using the total ion current and in the mode of selected ion monitoring for PAHs (m/z = 128 for naphthalene; m/z = 178 for anthracene and phenanthrene). Interpretation of organic compounds was based on the comparison of the obtained mass spectra with the mass spectra of compounds from the NIST 08 library. The HC content was expressed as a percentage and calculated by the method of internal normalization of mass fragmentograms by total ion current (TIC) for aliphatic alkanes and in the mode of selected ion monitoring (SIM) for PAHs (naphthalene, anthracene, and phenanthrene).

The level of HC utilization by bacteria was expressed as a percentage and estimated from the change in the concentration of the components of the model HC mixture during incubation with respect to the initial values. The microbial abundance was determined in dynamics by staining cells with the DAPI (4,6-diamino-2-phenylindole) fluorochrome dye [39]. Cell counts were performed with a Zeiss Axio Imager M1 epifluorescence microscope using the Image Test software.

RESULTS

Obtaining a collection of HCOB from bottom sediments of the Sea of Japan. Five strains of HCOB capable of growth at 5°C on the medium containing fuel oil as the sole source of carbon were isolated from bottom sediments of Peter the Great Bay, Sea of Japan, and were taxonomically characterized. For the studied bacterial strains, a phylogenetic analysis was carried out by searching for homologous sequences in the international data bank (GenBank), and a phylogenetic tree was constructed (Fig. 1).

Two strains were isolated from the station with the following coordinates: 42°30′ N, 132°41′ E (depth, 174 m). These were *Sphingomonas* sp. AP_231 (MW784855) and *Rhodococcus* sp. AP_244 (MW784849). The nucleotide sequence of the 16S rRNA gene of the strain *Sphingomonas* sp. AP_231 showed a high percentage of similarity (98.20%) to *Sphingomonas paucimobilis* 20ABA1 (MH482346) and *Sphingomonas* sp. D36 (MN540917). *Rhodococcus erythropolis* IEGM 1188 (MG637011) proved the closest homolog of the isolate *Rhodococcus* sp. AP_244 (98.79%).

Rhodococcus erythropolis AP_291 (MW784848) was another *Rhodococcus* member isolated from the bottom sediments of the station with the following coordinates: 42°39′ N, 133°02′ E (depth, 90 m). This sampling site was located near the oil-loading seaport of Kozmino. The analysis of the nucleotide sequence of the 16S rRNA gene of this strain showed the highest similarity (99.53%) to *Rhodococcus erythropolis* BD7100 (LC030232).

The strain of *Pseudomonas* sp. AP_27 (MW784854) was also isolated from bottom sediments in the area of the sea oil port of Kozmino (42°34′ N, 133°02′ E; depth, 110 m). The studied strain had an equally high percentage of 16S rRNA gene homology (99.77%) with three different *Pseudomonas* species: *Pseudomonas extremaustralis* cqsV14 (MN826583), *Pseudomonas marginalis* PMK1 (MT583077), and *Pseudomonas rhodesiae* D12 (MT374265).

The strain *Alcanivorax* sp. AP_54 (MW784853) was isolated from the station (42°41′ N, 132°27′ E; depth, 82 m). The isolate had the same percentage of

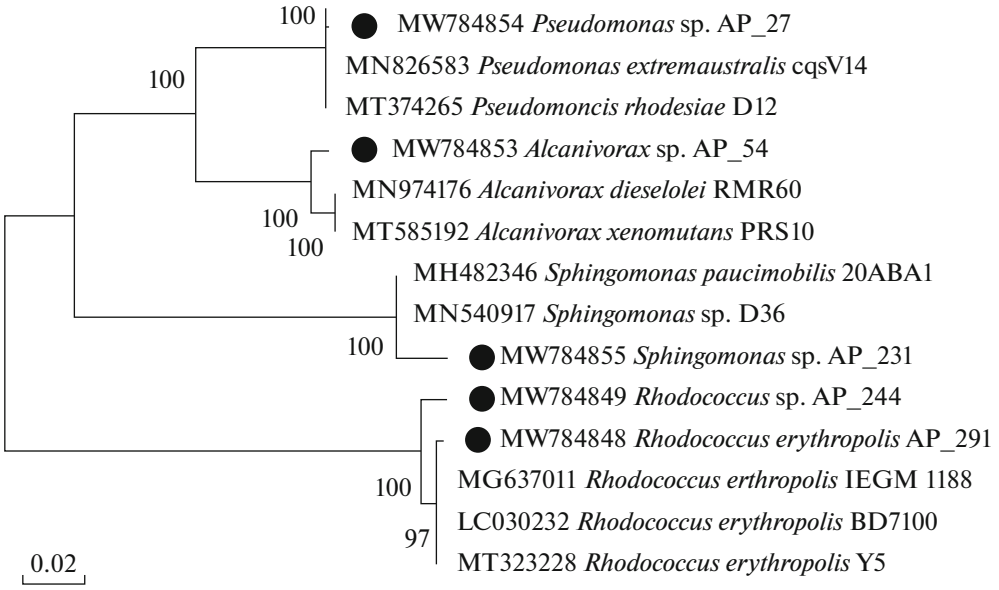

Fig. 1. Phylogenetic tree constructed based on the analysis of sequences of the 16S rRNA gene fragments of psychrotrophic HCOB from the bottom sediments of the Sea of Japan. The dendrogram was constructed using the neighbor-joining (NJ) algorithm. The sequences obtained in this study are marked with a black circle. The scale corresponds to two nucleotide substitutions per every 100 bp. Bootstrap support values above 50% are shown.

the 16S rRNA gene similarity (98.40%) to *Alcanivorax dieselolei* RMR60 (MN974176) and *Alcanivorax xenomutans* Y50-2 (MT585192).

Evaluation of the ability of bacterial strains to oxidize oil hydrocarbons. For the five isolated bacterial strains, the dynamics of the utilization of the model mixture HC were assessed for 70 days at 5°C. By the 7th day of the experiment, a noticeable decrease in the HC concentration was observed in all samples (Table 1). The highest values of the HCO activity were recorded for the strain *Rhodococcus erythropolis* AP_291 (Fig. 2). Within a week, the bacterial strain cells decomposed more than 50% of all HC from the mixture. Alkanes C9-C14 and C18-C27, as well as all PAHs, were oxidized best of all. Bacteria showed a lower level of utilization of HC with a carbon chain length of 15–17 atoms and pristane.

Relatively high enzymatic activity was demonstrated by the strain *Pseudomonas* sp. AP_27. As in the case of the strain *Rhodococcus erythropolis* AP_291, bacteria of the genus *Pseudomonas* oxidized short- and long-chain alkanes, as well as naphthalene and anthracene, more efficiently. A poorer utilization was shown for C15-C19 alkanes (25% or less), pristane (21%), phytane (23%), and phenanthrene (10%). It is noteworthy that both strains were isolated from neighboring stations located near the oil-loading port of Kozmino.

Despite the lower values of HC utilization in the first week of the experiment, all other strains showed similar trends in the preference for oxidizable substrates. Among alkanes, HC with a carbon chain length of 9–13 and 21–27 atoms were decomposed first. In the case of PAHs, all strains decomposed naphthalene (up to 82%) and anthracene (up to 67%) best of all. Phenanthrene turned out to be a less preferred HC, especially for the strains *Rhodococcus* sp. AP_244 and *Alcanivorax* sp. AP_54 (utilization levels of 12 and 18%, respectively). The C14–C20 alkanes, including phytane and pristane, were almost not used by bacteria in the first week of the experiment (the level of utilization was 0–25%).

On day 14 of the experiment with the strains *Rhodococcus* sp. AP_244 and *Alcanivorax* sp. AP_54, chromatography-mass spectrometry analysis of the samples showed an abrupt increase in the consumption of all components of the model mixture, including C14-C20 and phenanthrene. For other strains, the level of utilization of all HC increased more smoothly compared to the previous week. Noteworthy, all strains, except for *Sphingomonas* sp. AP_231, oxidized most of the studied hydrocarbons by the 28th day of observation. On day 70, no significant difference in the level of HC utilization, compared to the data obtained on day 28, was found. At the same time, the strain *Sphingomonas* sp. AP_231 continued the active oxidation of HC from the mixture for 70 days of the experiment. Thus, by the end of the experiment, a decrease in alkanes and PAHs was 43–100 and 67–91%, respectively, for all studied microbial strains.

The dynamics of the microbial cell abundance during the experiment correlated with the level of utilization of the ambient HC. On day 7, cell numbers of the most active strains *Rhodococcus erythropolis* AP_291 and *Pseudomonas* sp. AP_27 increased from 10^3 to

Table 1. Utilization level of HC (%) at 5°C relative to the initial HC concentration in the model mixture

Hydrocarbon	*Rhodococcus erythropolis* AP_291				*Rhodococcus* sp. AP_244				*Alcanivorax* sp. AP_54				*Pseudomonas* sp. AP_27				*Sphingomonas* sp. AP_231			
	7 days	14 days	28 days	70 days	7 days	14 days	28 days	70 days	7 days	14 days	28 days	70 days	7 days	14 days	28 days	70 days	7 days	14 days	28 days	70 days
Nonane (C9)	73 ± 4	76 ± 3	82 ± 4	100	51 ± 3	75 ± 2	77 ± 2	100	50 ± 2	71 ± 2	79 ± 3	100	64 ± 2	65 ± 2	76 ± 3	100	55 ± 4	60 ± 2	68 ± 2	100
Decane (C10)	66 ± 3	72 ± 2	73 ± 2	73 ± 3	54 ± 4	70 ± 1	63 ± 5	72 ± 1	47 ± 3	64 ± 2	67 ± 2	67 ± 2	60 ± 2	62 ± 3	63 ± 3	60 ± 3	51 ± 4	54 ± 2	57 ± 2	68 ± 2
Undecane (C11)	70 ± 2	73 ± 1	73 ± 0	71 ± 3	44 ± 2	62 ± 3	64 ± 3	72 ± 3	33 ± 5	70 ± 2	68 ± 3	67 ± 4	53 ± 2	58 ± 4	63 ± 2	62 ± 1	50 ± 2	56 ± 2	51 ± 2	67 ± 4
Dodecane (C12)	59 ± 1	59 ± 2	65 ± 2	63 ± 2	35 ± 4	52 ± 2	57 ± 3	64 ± 3	25 ± 5	58 ± 2	60 ± 3	68 ± 2	43 ± 2	49 ± 2	56 ± 4	52 ± 2	42 ± 2	43 ± 1	45 ± 3	61 ± 2
Tridecane (C13)	61 ± 2	62 ± 2	63 ± 3	61 ± 2	29 ± 4	62 ± 2	56 ± 4	63 ± 2	17 ± 4	59 ± 1	59 ± 1	58 ± 2	34 ± 3	41 ± 2	53 ± 4	49 ± 2	38 ± 3	43 ± 2	42 ± 2	54 ± 4
Tetradecane (C14)	59 ± 1	58 ± 1	61 ± 2	60 ± 3	21 ± 3	58 ± 2	53 ± 5	61 ± 2	11 ± 3	57 ± 1	57 ± 0	54 ± 2	29 ± 2	29 ± 2	50 ± 4	49 ± 2	24 ± 2	39 ± 0	39 ± 2	52 ± 2
Pentadecane (C15)	49 ± 3	53 ± 2	59 ± 3	60 ± 4	12 ± 1	48 ± 4	51 ± 2	52 ± 1	6 ± 2	47 ± 3	56 ± 2	54 ± 2	22 ± 3	37 ± 3	48 ± 0	48 ± 2	21 ± 2	28 ± 3	37 ± 2	50 ± 3
Hexadecane (C16)	38 ± 2	46 ± 2	53 ± 3	57 ± 2	9 ± 3	34 ± 3	46 ± 4	45 ± 4	11 ± 2	38 ± 3	56 ± 2	53 ± 2	21 ± 3	36 ± 3	48 ± 5	42 ± 2	26 ± 4	25 ± 4	40 ± 3	48 ± 3
Heptadecane (C17)	48 ± 2	51 ± 2	62 ± 3	68 ± 2	0	53 ± 1	53 ± 0	55 ± 1	13 ± 2	58 ± 2	60 ± 2	63 ± 2	23 ± 3	39 ± 3	46 ± 5	55 ± 2	21 ± 3	31 ± 2	39 ± 2	60 ± 4
Pristane	36 ± 2	48 ± 3	55 ± 1	59 ± 0	0	27 ± 4	46 ± 2	49 ± 2	3 ± 1	41 ± 3	63 ± 2	65 ± 2	21 ± 3	32 ± 1	41 ± 1	43 ± 1	20 ± 4	32 ± 3	36 ± 2	53 ± 5
Octadecane (C18)	56 ± 2	55 ± 2	55 ± 0	64 ± 1	0	30 ± 2	47 ± 3	51 ± 3	11 ± 2	47 ± 3	53 ± 2	50 ± 2	21 ± 4	38 ± 3	43 ± 2	49 ± 2	22 ± 1	23 ± 1	32 ± 1	59 ± 3
Phytane	56 ± 1	58 ± 1	59 ± 1	61 ± 4	3 ± 1	36 ± 4	45 ± 2	50 ± 2	14 ± 2	51 ± 2	55 ± 2	57 ± 2	23 ± 1	38 ± 3	43 ± 2	48 ± 2	23 ± 2	26 ± 2	36 ± 3	55 ± 2
Nonadecane (C19)	54 ± 2	59 ± 2	57 ± 2	64 ± 1	0	34 ± 3	48 ± 4	54 ± 2	16 ± 2	45 ± 4	53 ± 2	54 ± 1	25 ± 3	31 ± 2	45 ± 2	51 ± 1	19 ± 1	29 ± 1	34 ± 2	62 ± 3
Eicosane (C20)	56 ± 2	60 ± 2	59 ± 2	67 ± 3	5 ± 1	24 ± 2	50 ± 2	60 ± 3	24 ± 4	54 ± 3	59 ± 2	60 ± 2	35 ± 3	39 ± 2	48 ± 1	58 ± 2	25 ± 2	34 ± 2	39 ± 2	67 ± 4
Heneicosane (C21)	55 ± 2	62 ± 2	64 ± 3	72 ± 1	19 ± 2	31 ± 2	56 ± 2	68 ± 3	34 ± 1	56 ± 2	63 ± 1	57 ± 5	41 ± 2	54 ± 2	57 ± 2	63 ± 2	33 ± 1	34 ± 2	48 ± 2	70 ± 4
Docosane (C22)	57 ± 1	60 ± 2	69 ± 2	75 ± 3	29 ± 3	44 ± 1	64 ± 2	71 ± 3	42 ± 1	51 ± 2	67 ± 3	64 ± 4	50 ± 1	52 ± 1	59 ± 1	70 ± 2	40 ± 2	45 ± 2	55 ± 2	76 ± 2
Tricosane (C23)	60 ± 2	64 ± 2	71 ± 1	80 ± 2	31 ± 3	46 ± 3	65 ± 1	70 ± 1	43 ± 2	52 ± 2	68 ± 2	67 ± 2	55 ± 1	56 ± 1	63 ± 2	70 ± 3	41 ± 2	54 ± 2	55 ± 3	78 ± 2
Tetracosane (C24)	60 ± 0	62 ± 2	77 ± 2	83 ± 2	43 ± 2	48 ± 1	75 ± 3	80 ± 1	55 ± 1	64 ± 2	76 ± 2	75 ± 2	67 ± 2	69 ± 2	72 ± 2	79 ± 3	44 ± 3	47 ± 4	65 ± 2	85 ± 3
Pentacosane (C25)	61 ± 2	65 ± 2	74 ± 1	81 ± 3	43 ± 2	53 ± 2	73 ± 3	77 ± 3	54 ± 2	67 ± 2	73 ± 2	70 ± 3	65 ± 1	65 ± 1	68 ± 2	80 ± 4	38 ± 2	54 ± 2	59 ± 3	80 ± 3
Hexacosane (C26)	67 ± 0	67 ± 0	75 ± 2	82 ± 3	39 ± 2	59 ± 2	74 ± 2	75 ± 2	56 ± 3	66 ± 3	76 ± 2	75 ± 2	68 ± 2	69 ± 2	71 ± 2	82 ± 3	36 ± 2	45 ± 2	58 ± 1	76 ± 3
Heptacosane (C27)	65 ± 2	65 ± 1	71 ± 3	78 ± 2	38 ± 4	59 ± 2	69 ± 3	75 ± 2	56 ± 2	64 ± 1	74 ± 0	71 ± 0	67 ± 2	67 ± 2	65 ± 2	81 ± 2	32 ± 2	47 ± 2	58 ± 2	76 ± 1
Naphthalene	86 ± 2	90 ± 2	91 ± 0	91 ± 3	70 ± 3	83 ± 3	91 ± 2	89 ± 1	64 ± 2	86 ± 3	81 ± 2	82 ± 1	76 ± 3	81 ± 1	88 ± 2	83 ± 1	82 ± 2	85 ± 3	79 ± 2	88 ± 4
Anthracene	58 ± 2	61 ± 2	80 ± 2	80 ± 2	35 ± 3	71 ± 2	73 ± 1	72 ± 3	44 ± 2	66 ± 3	73 ± 0	67 ± 5	37 ± 3	56 ± 2	71 ± 2	68 ± 3	67 ± 2	71 ± 3	68 ± 2	77 ± 2
Phenanthrene	54 ± 2	57 ± 0	74 ± 4	85 ± 1	12 ± 2	66 ± 2	71 ± 2	78 ± 3	18 ± 3	55 ± 3	78 ± 2	72 ± 5	10 ± 2	45 ± 3	66 ± 3	77 ± 2	57 ± 3	73 ± 2	64 ± 2	74 ± 1

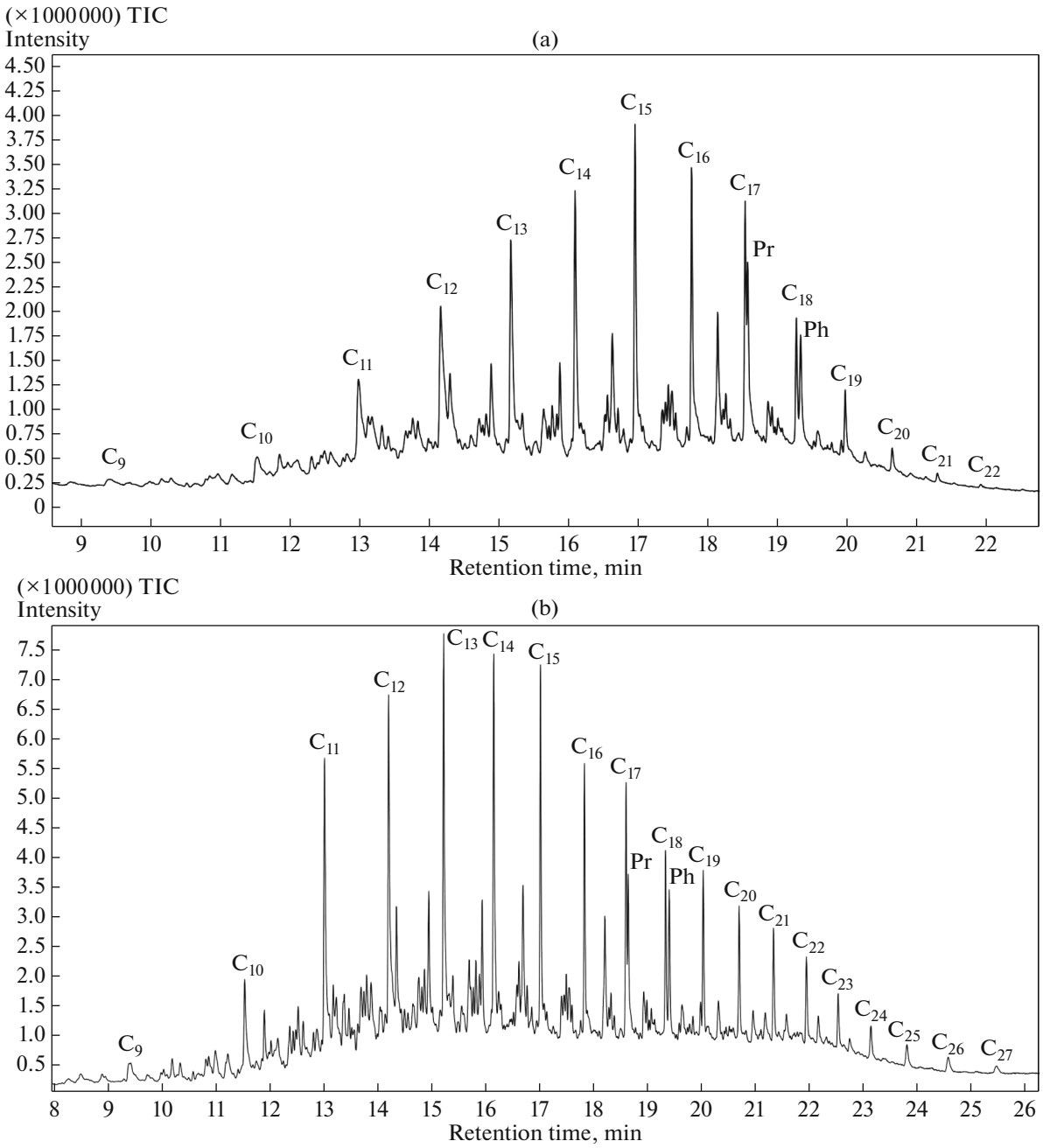

Fig. 2. Mass fragmentograms of the composition of the model HC mixture on day 7 of the experiment at 5°C: (a) strain *Rhodococcus erythropolis* AP_291; (b) control. Symbols: C9–C27, *n*-alkanes with the corresponding carbon chain length; Pr, pristane; Ph, phytane.

10^5 cells/mL. The abundance of other strains increased to 10^4 cells/mL. On day 14, cell numbers of *Rhodococcus* sp. AP_244, *Sphingomonas* sp. AP_231, and *Alcanivorax* sp. AP_54 increased from 10^4 to 10^5 cells/mL, which correlated with an abrupt increase in the consumption of HC on days 7–14. The number of *Rhodococcus erythropolis* AP_291 and *Pseudomonas* sp. AP_27 cells remained unchanged for a week. On day 28, the abundance decreased from 10^5 to 10^4 cells/mL in all samples and did not change until the end of the experiment.

At the next stage of this research, HCOB from the obtained collection were incubated on a solid mineral medium containing fuel oil for 10 days at 22°C to assess their ability to utilize hydrocarbons at increased temperatures. All studied microorganisms were shown to grow under these conditions. Therefore, the dynamics of the consumption of individual HC by

bacteria using chromatography-mass spectrometry were also assessed at 22°C (Table 2).

The results indicated that the level of the HC utilization by the strain *Rhodococcus erythropolis* AP_291, which showed the highest HCO activity at 5°C, was the lowest. With an increase in temperature, the oxidation of HC by this strain slowed down significantly. The other four bacterial strains decomposed HC with the same or less intensity than at 5°C.

For all studied bacterial cultures, the same selectivity in the choice of oxidizable substrates, as at low temperatures, was shown. The highest level of degradation by bacteria was noted for short- and long-chain alkanes, as well as PAHs. The poorest decomposition was shown for hydrocarbons with 15 to 19 carbon atoms. Among PAHs, naphthalene and anthracene were primarily oxidized by microorganisms; after that, phenanthrene was used.

In the first week of the experiment, the average oxidation level of HC of the model mixture by all studied bacteria was ~40%. However, on days 7–14, HC were almost not consumed. A steep increase in the HC utilization rate was noted by day 28. The dynamics of the cell numbers during the experiment agreed with the level of the HC utilization. For instance, the abundance of all strains increased from 10^3 to 10^4 cells/mL on day 7 of the experiment and remained unchanged until day 28 on which cell numbers increased to 10^5 cells/mL in all samples.

DISCUSSION

Taxonomic characterization of HCOB from bottom sediments of the Sea of Japan. *Sphingomonas* sp. AP_231 (MW784855) are Gram-negative aerobic rods assigned to the class Alphaproteobacteria. According to the literature data, members of the genus *Sphingomonas* isolated from polluted waters and soils often show the ability to degrade various PAHs, such as naphthalene, phenanthrene, anthracene, fluorene, pyrene, benzo(a)pyrene, and others [38].

Rhodococcus erythropolis AP_291 (MW784848) and *Rhodococcus* sp. AP_244 (MW784849) are Gram-positive aerobic rods belonging to the phylum Actinobacteria. Members of the genus *Rhodococcus* are typical soil microorganisms, which are often found in other ecological niches of different climatic zones. In the literature, a large amount of information concerns the possibility of their use for bioremediation, since they are able to utilize linear and cyclic alkanes, as well as a wide range of PAHs [27]. Moreover, data on psychrotrophic HCO strains of *Rhodococcus erythropolis* have been obtained [17, 37].

The strain *Pseudomonas* sp. AP_27 (MW784854) is represented by Gram-negative aerobic rods belonging to the class Gammaproteobacteria. Bacteria of the genus *Pseudomonas* are ubiquitous and typical for various ecosystems. They possess a wide range of enzyme activity, including the ability to oxidize petroleum HC. The involvement of bacteria of this genus isolated from the waters of the Sea of Okhotsk [14] and the Sea of Japan [15] in the HC degradation has been previously reported.

Cells of the strain *Alcanivorax* sp. AP_54 (MW784853) are Gram-negative aerobic rods assigned to the class Gammaproteobacteria. The genus *Alcanivorax* includes microorganisms that are characteristic of marine biocenoses. These microorganisms have a common ability to degrade HC and synthesize surfactants. There is a large amount of data on the HCO activity of these bacteria in various seas, including the Sea of Japan [21, 25, 35, 41].

Evaluation of the ability of bacterial strains to oxidize oil hydrocarbons. According to the literature data, a few bacterial species can grow on media with *n*-alkanes containing up to 8 carbon atoms. The use of hydrocarbons with 9 to 18 carbon atoms as a substrate is quite widespread among microorganisms. Under the action of enzymes, aliphatic hydrocarbons are oxidized at the terminal methyl group to the corresponding alcohols, which are further oxidized to fatty acids by NAD-dependent dehydrogenases [3].

Numerous bacteria are able to grow using a wide range of *n*-alkanes; therefore, they can contain several variants of alkane hydroxylase enzyme systems [42]. The *n*-alkane degradation genes have been reported to be selectively expressed depending on the HC composition, phase of bacterial culture growth, and incubation conditions [11, 34, 43]. For instance, the addition of yeast extract to the medium stimulated growth and accelerated the oxidation of linear and branched alkanes in the psychrotrophic strain *Rhodococcus* sp. Q15 at 5°C on a medium containing diesel [45]. The authors suggested that the growth factors and vitamins composing the yeast extract contributed to a more rapid increase in bacterial biomass, which resulted in accelerated HC degradation.

One of the studies has reported that a consortium of HCOMs isolated from the samples that contained a mixture of sea ice and seawater (Spitsbergen Island) can completely degrade *n*-alkanes (C8-C34) and isoprenoids (pristane and phytane) within 28 days of incubation at 4°C [18]. First, these microorganisms degraded short-chain alkanes (C8-C14), the utilization of which occurred during the first two weeks of cultivation. After that, HCOMs switched to the oxidation of long-chain hydrocarbons (C15-C34), including pristane and phytane. The molecular genetic analysis allowed the identification of six genera in the bacterial consortium: *Marinobacter*, *Pseudomonas*, *Pseudoalteromonas*, *Psychrobacter*, *Shewanella*, and *Agreia* [18].

Bacteria from the bottom sediments of Peter the Great Bay also oxidized most of the HC of the model mixture within 28 days of the experiment; however, they exhibited a slightly different substrate specificity. In the first week of research, the microorganisms pre-

Table 2. The level of HC utilization (%) at 22°C in relation to the initial concentrations of HC in the model mixture

Hydrocarbon	*Rhodococcus erythropolis* AP_291			*Rhodococcus* sp. AP_244			*Alcanivorax* sp. AP_54			*Pseudomonas* sp. AP_27			*Sphingomonas* sp. AP_231		
	7 days	14 days	28 days	7 days	14 days	28 days	7 days	14 days	28 days	7 days	14 days	28 days	7 days	14 days	28 days
Nonane (C9)	48 ± 3	54 ± 2	76 ± 3	57 ± 2	61 ± 2	83 ± 3	56 ± 1	54 ± 3	77 ± 2	65 ± 0	62 ± 4	68 ± 3	59 ± 3	58 ± 3	71 ± 2
Decane (C10)	48 ± 2	47 ± 2	67 ± 3	60 ± 2	59 ± 2	65 ± 2	56 ± 1	48 ± 5	64 ± 3	55 ± 2	58 ± 3	56 ± 3	57 ± 1	52 ± 5	60 ± 4
Undecane (C11)	36 ± 2	37 ± 2	62 ± 3	49 ± 2	45 ± 3	66 ± 3	44 ± 3	46 ± 1	62 ± 3	57 ± 2	59 ± 2	56 ± 4	47 ± 2	45 ± 4	59 ± 2
Dodecane (C12)	28 ± 1	28 ± 2	54 ± 4	43 ± 3	46 ± 2	58 ± 4	37 ± 2	39 ± 2	54 ± 3	48 ± 2	52 ± 2	47 ± 5	40 ± 2	38 ± 4	52 ± 4
Tridecane (C13)	27 ± 2	21 ± 5	48 ± 3	33 ± 0	33 ± 0	55 ± 2	28 ± 2	26 ± 4	49 ± 2	40 ± 2	41 ± 2	45 ± 2	32 ± 2	35 ± 2	50 ± 3
Tetradecane (C14)	25 ± 2	26 ± 1	43 ± 1	25 ± 2	30 ± 3	53 ± 2	22 ± 0	25 ± 3	47 ± 2	35 ± 2	37 ± 4	40 ± 2	26 ± 1	34 ± 1	46 ± 0
Pentadecane (C15)	19 ± 2	17 ± 3	39 ± 2	16 ± 1	26 ± 3	49 ± 2	15 ± 1	21 ± 2	44 ± 2	28 ± 2	23± 5	36 ± 2	18 ± 2	31 ± 1	43 ± 1
Hexadecane (C16)	22 ± 0	22 ± 2	38 ± 2	17 ± 1	19 ± 2	49 ± 2	19 ± 2	25 ± 1	45 ± 2	28 ± 2	23 ± 2	26 ± 3	18 ± 1	34 ± 0	44 ± 2
Heptadecane (C17)	19 ± 1	22 ± 2	38 ± 2	17 ± 2	21 ± 2	48 ± 2	23 ± 1	24 ± 2	47 ± 3	20 ± 2	22 ± 3	42 ± 4	18 ± 3	32 ± 4	44 ± 4
Pristane	8 ± 3	12 ± 2	36 ± 2	12 ± 4	19 ± 2	42 ± 2	22 ± 4	26 ± 3	39 ± 3	23 ± 4	24 ± 4	23 ± 3	18 ± 4	23 ± 3	41 ± 5
Octadecane (C18)	15 ± 2	10 ± 4	30 ± 2	15 ± 3	19 ± 4	46 ± 2	18 ± 2	17 ± 2	42 ± 3	19 ± 3	24 ± 1	30 ± 2	23 ± 5	25 ± 2	39 ± 2
Phytane	14 ± 2	19 ± 3	33 ± 2	18 ± 2	23 ± 2	43 ± 1	26 ± 2	28 ± 2	46 ± 3	20 ± 3	26 ± 1	28 ± 2	19 ± 2	27 ± 1	42 ± 3
Nonadecane (C19)	17 ± 2	14 ± 4	34 ± 2	22 ± 2	18 ± 2	46 ± 2	22 ± 3	27 ± 2	43 ± 2	19 ± 1	23 ± 0	28 ± 0	28 ± 0	28 ± 2	40 ± 2
Eicosane (C20)	15 ± 2	23 ± 3	38 ± 2	31 ± 2	31 ± 2	49 ± 3	28 ± 2	31 ± 4	48 ± 2	24 ± 2	27 ± 2	33 ± 1	36 ± 1	29 ± 5	42 ± 2
Heneicosane (C21)	25 ± 2	32 ± 2	46 ± 1	39 ± 2	34 ± 3	55 ± 2	40 ± 1	41 ± 1	54 ± 0	32 ± 1	28 ± 5	39 ± 2	49 ± 2	49 ± 1	49 ± 3
Docosane (C22)	34 ± 2	43 ± 1	51 ± 2	48 ± 3	50 ± 3	60 ± 1	47 ± 3	48 ± 4	59 ± 2	39 ± 2	38 ± 3	44 ± 2	56 ± 0	56 ± 0	56 ± 2
Tricosane (C23)	39 ± 2	46 ± 2	51 ± 3	52 ± 2	45 ± 1	64 ± 3	50 ± 2	53 ± 3	61 ± 1	42 ± 3	43 ± 2	49 ± 0	50 ± 2	48 ± 4	54 ± 2
Tetracosane (C24)	45 ± 2	55 ± 2	63 ± 3	58 ± 3	61 ± 2	71 ± 2	61 ± 4	58 ± 5	68 ± 1	46 ± 2	55 ± 2	61 ± 0	52 ± 3	52 ± 2	65 ± 2
Pentacosane (C25)	52 ± 1	53 ± 1	54 ± 2	57 ± 2	58 ± 2	71 ± 1	57 ± 1	52 ± 2	67 ± 2	47 ± 1	47 ± 2	68 ± 2	60 ± 2	59 ± 2	63 ± 2
Hexacosane (C26)	50 ± 2	55 ± 3	54 ± 2	61 ± 0	61 ± 0	70 ± 2	60 ± 1	55 ± 1	65 ± 2	48 ± 1	52 ± 2	65 ± 3	61 ± 4	62 ± 2	62 ± 2
Heptacosane (C27)	46 ± 2	43 ± 4	49 ± 1	49 ± 1	58 ± 1	65 ± 1	54 ± 2	56 ± 3	63 ± 1	41 ± 3	37 ± 4	66 ± 2	55 ± 1	55 ± 0	62 ± 1
Naphthalene	50 ± 1	69 ± 2	89 ± 3	66 ± 2	65 ± 4	83 ± 2	74 ± 2	70 ± 4	85 ± 2	76 ± 2	73 ± 5	84 ± 2	75 ± 1	71 ± 4	83 ± 2
Anthracene	45 ± 0	46 ± 1	59 ± 1	45 ± 2	57 ± 1	76 ± 1	40 ± 2	47 ± 3	62 ± 2	49 ± 2	53 ± 0	60 ± 2	41 ± 1	47 ± 2	69 ± 1
Phenanthrene	18 ± 6	4 ± 1	52 ± 2	15 ± 2	29 ± 2	72 ± 3	25 ± 1	29 ± 2	67 ± 2	32 ± 2	31 ± 3	56 ± 2	26 ± 3	40 ± 4	62 ± 1

ferred to decompose short- (C9–C13) and long-chain (C21–27) alkanes. And by the second week of the experiment alone, active degradation of medium-chain hydrocarbons (C14–C20), including pristane and phytane, was noted. This may be because different alkane hydroxylases responsible for the decomposition of alkanes depending on the length of their carbon chain can be synthesized at different rates [43].

Independently of their taxonomic affiliation, all bacteria of the studied region oxidized alkanes with different carbon chain lengths according to the same scenario. Probably, the corresponding HC catabolism genes are transferred from one microorganism to another by horizontal transfer via conjugation. Plasmid localization of the genes responsible for the HC oxidation provides their distribution within one bacterial population, as well as among members of other bacterial species, increasing the metabolic potential of microorganisms under unstable environmental conditions. Possibly, due to this, microbial communities possessing certain catabolic properties can be formed in some marine areas.

The ability to simultaneously oxidize *n*-alkanes and PAHs from the first day of the experiment was an interesting feature of the bacterial strains obtained in this study. Based on the chemical structure, the bioavailability of hydrocarbons for microorganisms decreases in the following order: *n*-alkanes > branched alkanes > low molecular weight aromatic compounds > cycloalkanes > PAHs [44]. For different microorganisms, aromatic HC are less preferred substrates than aliphatic hydrocarbons, since their utilization requires more complex enzymatic reactions [3]. Therefore, as a rule, linear HC are degraded first, and then all the others. In the modern scientific literature, few studies have reported the proven ability of bacteria to simultaneously oxidize *n*-alkanes and PAHs. Such phenomena of co-metabolism, when HC of different classes are simultaneously degraded, have been shown in the strains *Rhodococcus* sp. 1B [13] and *Mycobacterium vanbaalenii* PYR-1 [27].

All strains obtained by us are bacteria that can often oxidize various PAHs, according to the literature data [20]. Moreover, the PAH degradation genes of Gram-positive and Gram-negative bacteria may differ in their structural organization. For instance, in contrast to *Pseudomonas* and other Gram-negative bacteria, the naphthalene degradation genes (*nah*) of which are clustered into one operon, the Gram-positive *Rhodococcus* members usually have three separate structural genes for naphthalene degradation (*narAa*, *narAb*, and *narB*) [28, 31]. Moreover, the transcription of the naphthalene degradation genes in *Rhodococcus* is induced by the presence of naphthalene in the medium, while the *nah* genes of *Pseudomonas* are transcribed in response to the accumulation of salicylates [28, 31].

Most aromatic compounds are converted by bacteria into protocatechuic acid or catechol, which are further decomposed by *ortho*- or *meta*-cleavage [3]. In contrast to *nar* and *nah*, the genes encoding the synthesis of the key enzyme of catechol *meta*-cleavage (catechol 2,3-dioxygenase) in Gram-negative and Gram-positive bacteria are often highly homologous and easily transferred by conjugation [22].

Intensive consumption of PAHs by the microorganisms obtained in this research may be associated with their adaptation to high concentrations of PAHs in the pollutants of the study area. Similar results have been previously reported for the bacterial strains isolated from the coastal waters of the south of Island Sakhalin, which are subject to significant anthropogenic pollution [14]. In the cited paper, bacteria of the genera *Cobetia*, *Pseudoalteromonas*, *Oceanisphaera*, *Shewanella*, *Pseudomonas*, *Marinomonas*, and *Thalassospira* first decomposed aromatic compounds and then aliphatic HC.

The microorganisms studied by us showed similar substrate specificity at low and increased temperatures of incubation. At the same time, the HC oxidation occurred more intense in a refrigerator than at 22°C for all strains, except for *Sphingomonas* sp. AP_231. The latter decomposed HC of the model mixture with the same efficiency, regardless of the selected temperature. The results obtained can be associated with the permanently low temperatures in the habitats of the studied strains and the emergence of the corresponding adaptation to these temperatures in microorganisms. Probably, the optimum for the HCO enzyme functioning in these bacteria is at low temperatures, and a change in the incubation conditions decreases the enzymatic activity. It is also possible that these microorganisms can produce isoenzymes that are responsible for the degradation of the same HC at different temperatures. The switch from the synthesis of one enzyme type to another can take a long time. Possibly, for this reason, no consumption of HC and no increase in the microbial abundance was noted on days 7–14 of the experiment at 22°C.

At both temperatures, the dynamics of the number of microbial cells in the experiments was comparable with the level of the ambient HC utilization. However, the number of cells of all strains increased by no more than two orders of magnitude (compared to the initial values) by the end of the exponential phase, despite the intense oxidation of HC. This might be due to the effect of intraspecific competition for a carbon source under the limiting conditions and to nitrogen deficiency. In the absence of nitrogen, many microorganisms are known to reserve carbon as intracellular inclusions. For instance, bacterial strains of the genus *Alcanivorax* accumulate triacylglycerols and wax esters in the presence of excess *n*-alkanes in the medium [24]. *Pseudomonas putida* GPo1 can form intracellular inclusions of poly(β-hydroxyoctanoate) when growing with *n*-octane

[19], while *Acinetobacter* sp. M-1 forms wax esters on a medium containing hexadecane [23].

Thus, in this study, we obtained the first collection of psychrotrophic HCOB from the Sea of Japan that are capable of efficient oxidation of a wide range of HC. Understanding the exact mechanisms for the HC degradation by bacteria under different conditions requires additional studies of the properties of the HCO enzymes and the genes involved in their synthesis. These bacterial strains can be subsequently used to develop biological preparations for purification of the sea areas of the Far East region from accidental spills of oil and oil products.

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