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EXPERIMENTAL ARTICLES

Oil-Oxidizing Activity of Bacteria Isolated from South Sakhalin Coastal Waters

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Abstract—Ability of bacteria isolated from the southern coastal waters of the Sakhalin Island to degrade various hydrocarbons was studied. The population of marine microorganisms grown on oil was heterogeneous in terms of hydrocarbon degradation. The rate of bacterial degradation of oil hydrocarbons was shown to correlate with their growth rate on the model medium. The degradation rates were higher for aromatic hydrocarbons than for alkanes. Based on our data, the studied bacteria were conditionally assigned to three groups: active, intermediately active, and passive degraders. Ability to oxidize oil was previously not reported for members of the genus *Cobetia*.

Keywords: bioremediation, petroleum hydrocarbons, marine bacteria, destructors
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One of the most promising ways of elimination of marine pollution is bioremediation, which is a combination of methods to clean the environment using biochemical activity of various living objects, including marine organisms and microorganisms of oil reservoirs (Tan et al., 1999; Wackett, 2008). Bacteria are highly significant as degraders of oil hydrocarbons in the sea, since higher organisms cannot carry out their full degradation. In coastal regions, which are permanently contaminated by oil and oil products, specific communities of heterotrophic microorganisms are formed, with the ability to oxidize a broad range of hydrocarbons and products of their transformation (Mironov, 2002).

To improve the efficiency of bioremediation, it is needed to isolate the strains of microorganisms, which are adapted to the degradation of oil hydrocarbons (OH) in certain environmental conditions (Bryanskaya et al., 2014), and to analyze their biochemical activity. To this end, it is necessary to pay special attention to the study of the diversity and biological properties of microorganisms oxidizing OH in each particular region. This is especially true for the Northern water areas with low temperatures, where light oil fractions form a film on the water surface as they evaporate, and where their degradation by microorganisms is slower, while the heavy components settle to the bottom becoming a source of secondary pollution (Koronelli et al., 1989).

Coastal waters of Sakhalin Island give the unique opportunity to study the activity of oil-oxidizing bacteria in the Northern Seas as there exist all possible sources of oil pollution of both anthropogenic and natural origin.

The goal of the present work was to study the ability of marine bacteria, isolated from the South Sakhalin coastal waters, to decompose oil hydrocarbons in order to use the active strains in the technology of biodegradation of oil pollution of water in this region.

MATERIALS AND METHODS

Subjects of the study. Samples of the coastal waters from seven stations in the southern end of Sakhalin Island were studied: four from Aniva Bay (Prigorodnoe settlement, Korsakov port, Lososei Bay, Zolotorybnoe settlement), two from the Strait of Tartary (Polyakov Bay, Kholmsk port), and one from Mordvinov Gulf (Okhotskoe port). For the isolation of bacteria biodegrading OH, water samples were plated on Voroshilova–Dianova agar medium (Voroshilova and Dianova, 1952) supplemented with Sakhalin crude oil (Chaivo oil field) at a concentration of 1% and incubated at room temperature. The aforementioned oil is an oil of medium density (0.876 g/cm^3), low sulfur (0.28%), low tar (the content of silica gel resins up to 5.33%, of asphaltenes, 0.75%), paraffinaceous (3.96%) with high yield of gasoline fractions (up to

54%) (Golovko et al., 2004). The composition of the mineral medium was as follows (g/L distilled water): NH_4NO_3, 1; K_2HPO_4, 1; KH_2PO_4, 1; $MgSO_4$, 0.2; $CaCl_2$, 0.02; $FeCl_2$, 2 drops or 0.1 mL of the saturated solution; and agar, 15. Bacterial cultures grown on the solid medium were used for further experiments.

To determine the resistance of microorganisms to OH, bacterial suspension of each strain (0.1 mL of inoculum contained 100 cells) was plated on Voroshilova–Dianova agar medium with different oil content (1, 2, and 2.5%). The incubation was carried out at room temperature for 7 days. CFU of each bacterial strain were then determined for media with different concentrations of oil. The experiments were repeated three times.

Preparation of the model mixture of oil hydrocarbons for bacterial incubation. The chosen composition was based on the presence of these OH in the marine environment as real pollutants (Repina, 2009) and on the possibility of quantifying each component of the mixture. As a result, the mixture of saturated and aromatic hydrocarbons had the following composition (g/L): tridecan, 113.5; benzene, 131.7; hexadecan, 116.8; heptane, 220.8; naphthalene, 240.0; and toluene, 65.0.

The model OH mixture (20 g) was added into a sterile flask containing 180 mL of liquid Voroshilova–Dianova medium. After that, the cells of bacteria washed in physiological saline were introduced into the flasks (the final concentration in the media was 10^7 cells/mL) and were incubated in batch culture for 30 days at 20°C. Dynamics changes in the optical density of the cultures were registered spectrophotometrically at the wavelength of 540 nm (Schlegel, 1985) to plot the growth curve.

Quantitative assessment of oil hydrocarbons. The method of vapor-phase analysis (RD 52.24.473–2012) was used for sample preparation. Determination of OH was conducted on a GC 6890 Plus gas chromatograph with a 5973N mass selective detector (Agilent Technologies, United States). An HP5-MC quartz capillary column (30 m × 0.25 mm) was used with gelium as a carrier gas, flow rate of 1 mL/min, the range of mass scanning 15 to 250 *m/z*, injector temperature 260°C, without dividing the flow; the temperature program for the analysis was as follows: 40°C (3 min)–10°C (1 min)–260°C (10 min). Identification of chromatographic peaks was performed by retention time and mass spectrum (electronic library of mass spectra NIST 98). The amount of OH was assessed from the peak areas using calibration graphs.

The degree of OH degradation by bacteria in the dynamics of their growth was expressed in percent and calculated as a ratio of OH in the test samples to OH in the control samples without bacteria.

Molecular genetic identification of oil-degrading bacteria. Extraction of genomic DNA was performed from daily bacterial cultures by enzymatic lysis (Belkova, 2004). A fragment of the 16S rRNA gene was amplified using a pair of primers 27F (5'-AGAGTTTGATCATGGCTCAG-3') and 1350R (5'-GACGGGCGGTGTGTACAAG-3'), nucleotide sequences were determined with a Beckman Coulter CEQ 8800 Genetic Analysis System automated sequencer. The sequences were deposited in the international database with the following numbers: AM423068–AM423077, LN877941–LN877946. Comparative (using the online services FASTA and BLAST) and phylogenetic analyses (Mega 6.06, neighbor joining method, model Kimura 2-parameter) were made for strain identification at the species level.

RESULTS AND DISCUSSION

Investigation of oil-degrading abilities of microorganisms isolated from the coastal waters of Sakhalin Island. As a result of cultivation on selective solid medium with oil, 45 strains of organotrophic bacteria were identified. The study of their oil-degrading ability was conducted on the model mixture of the OH, which included saturated and aromatic hydrocarbons. Apart from the processes of biodegradation, a substantial decrease in the content of OH in seawater is associated with photooxidation and evaporation of light hydrocarbons. Thus, in order to get objective results, the content of each OH model compound was determined in the control mineral medium not containing microorganisms. Since our data showed a regular decrease of the concentration of all components in the control, further calculations were based on these results.

All selected isolates were divided into three groups (Table 1) according to their ability to decompose OH. The first group included the so-called active degraders, i.e., organisms that rapidly and almost completely utilized all OH from the mixture (7 strains); microorganisms of the second group (14 strains of intermediately active degraders) absorbed OH but worse than the strains of the first group. Bacteria of the third group (24 strains) practically did not digest OH and were named passive degraders.

It was shown that bacteria of the first group degraded the aromatic and saturated hydrocarbons almost completely already by the ninth day and within one month of growth, respectively. Bacteria of the second group also fully oxidized aromatic compounds but only by the 30th day of observations. It is significant that decomposition of aromatic hydrocarbons by microorganisms is a more complex process than the oxidation of aliphatic compounds. It requires, firstly, synthesis of the enzymes that convert aromatic hydrocarbons into catechol or protocatechuic acid and then a number of enzymes to break down these substrates to obtain compounds of the central metabolic pathways (Gottschalk, 1979). Our data indicated that the studied strains selectively oxidized certain OH. This may

Table 1. Changes in the content of oil hydrocarbons in selective medium in the presence of bacteria assigned to different groups of degraders

Mixture component	Content of OH compared to the control, %		
	9 days	14 days	30 days
Active degraders			
Tridecane	0	0	0
Benzene	0	0	0
Hexadecane	13 ± 3	0	0
Heptane	73 ± 13	55 ± 8	30 ± 7
Naphthalene	5 ± 1	0	0
Toluene	0	0	0
Intermediately active degraders			
Tridecane	30 ± 2	17 ± 2	12 ± 2
Benzene	20 ± 2	12 ± 2	0
Hexadecane	57 ± 1	32 ± 1	18 ± 5
Heptane	93 ± 18	53 ± 9	45 ± 5
Naphthalene	25 ± 3	10 ± 2	0
Toluene	10 ± 2	10 ± 2	0
Passive degraders			
Tridecane	78 ± 16	65 ± 14	50 ± 10
Benzene	73 ± 16	73 ± 15	60 ± 11
Hexadecane	90 ± 18	73 ± 14	60 ± 13
Heptane	95 ± 19	70 ± 13	47 ± 8
Naphthalene	67 ± 13	50 ± 9	33 ± 7
Toluene	85 ± 16	60 ± 13	55 ± 10

be caused either by the predominance of aromatic hydrocarbons among contaminants of the waters from which microorganisms were isolated, or by the adaptation of bacteria to a competitive existence in the oil-degrading microbial community due to assimilation of more complex organic substances (Bryanskaya et al., 2014).

Both for active and intermediately active degraders heptane (the shortest carbon chain length among the components of the model OH mixture) were most difficult to split, which is consistent with the literature (Gottschalk, 1979). It is known that the use of hydrocarbons containing up to eight carbon atoms by microorganisms requires effective systems of intracellular transport and it is not as widespread as the use of *n*-alkanes with 10 to 18 carbon atoms.

Despite growing on selective mineral medium that contained only 1% of oil, bacteria of the third group showed low activity in decomposition of the model OH mixture.

It is important to note that the growth curves of the above mentioned groups of microorganisms remarkably differed from each other (Fig. 1). Thus, active degraders adapted quickly to the environmental conditions and entered the exponential growth phase after 24 h (Fig. 1a). Bacteria of the group of intermediate degraders adapted slowly to the model OH mixtures and increased their number later (Fig. 1b). As for the growth curves of the passive degraders, they were characterized by a long lag phase (up to 10 days), showing the adaptation of microorganisms to adverse conditions, and low bacterial numbers during other growth phases (Fig. 1c). These features suggest that microorganisms of the third group are not oil oxidizers; they are able only to maintain viability at certain concentrations of OH in a selective medium.

To confirm these conclusions, all 45 studied strains were plated on Voroshilova–Dianova medium containing 1, 2, and 2.5% crude oil. After incubation, the colonies of each strain on media with different concentrations of oil were counted (Table 2). The results showed that the increase in oil concentration up to 2.5% had no significant effect on the number of bacteria of the first and the second groups; however, it inhibited growth of the microorganisms of the third group.

Table 2. Number oil-oxidizing bacteria (CFU) on agar medium with crude oil

Oil concentration	Active degraders	Intermediately active degraders	Passive degraders
1%	89 ± 11	60 ± 2	22 ± 2
2%	78 ± 9	53 ± 12	3 ± 2
2.5%	75 ± 8	33 ± 8	0

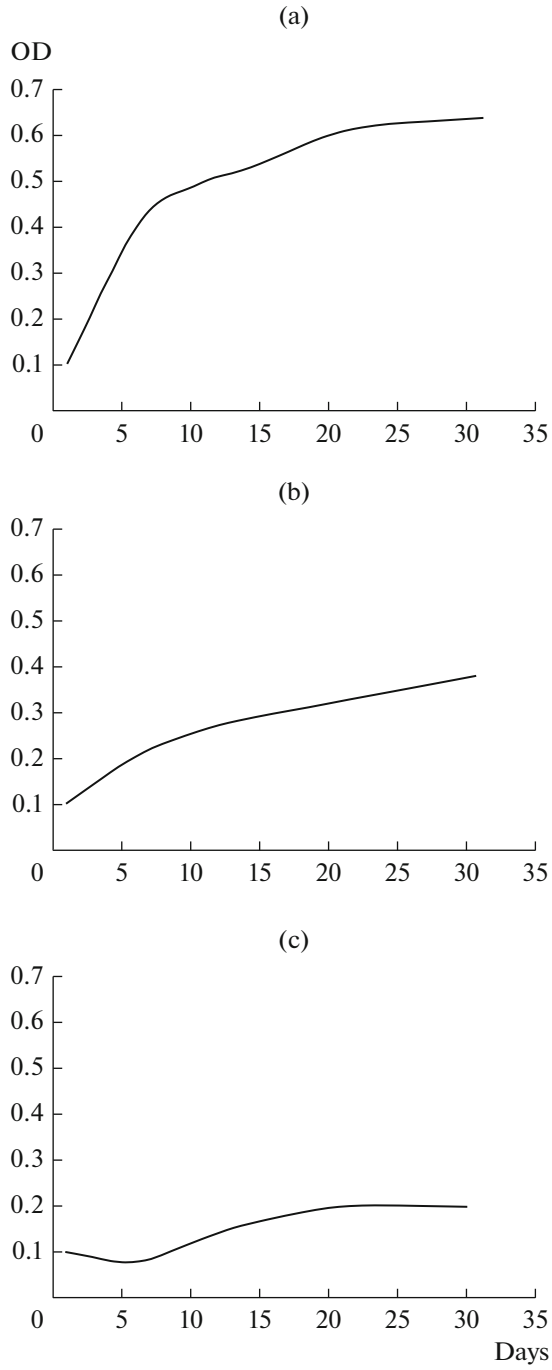

Fig. 1. Growth curves of marine bacteria isolated on the medium with OH: active destructors (a); intermediately active destructors (b); and passive destructors (c).

Taxonomic position of isolated oil-oxidizing microorganisms. Molecular genetic identification based on the ribosomal phylogeny was conducted for all active degraders (seven strains) and for nine of the most active strains from the second physiological group. We identified the representatives of the following genera: *Cobetia*, *Pseudoalteromonas, Oceanisphaera, Shewanella, Pseudomonas, Marinomonas*, and *Thalassospira*. Strain MS-715 belonging to *Alphaproteobacteria* was identified as *Thalassospira* sp. because comparative analysis showed high homology (99.3%) with the sequence of an uncultured alphaproteobacterium (AB024595), and 98.0% homology to its closest cultured relative, *Thalassospira lucentensis* (AM294944). Phylogenetic analysis of the sequences of other isolates, which belonged to different families of gammaproteobacteria, made it possible to clarify their taxonomic position (Fig. 2). Species identification was carried out for strains *Shewanella arctica* MS-96, *Pseudomonas composti* MS-8, and *Pseudoalteromonas marina* MS-21 and MS-031; genus was determined for *Cobetia* sp. MS-85, MS-64, and MS-65; *Pseudoalteromonas* sp. MS-87, MS-517, and MS-20; *Pseudomonas* sp. MS-812, MS-59, and MS-511; *Oceanisphaera* sp. MS-83 and *Marinomonas* sp. MS-810. Moreover, it was shown that nucleotide sequences of the analyzed strains clustered together with the sequences of the relevant taxa, which were isolated from marine ecosystems: water, sediments, and associations with marine organisms, mainly algae (Ivanova et al., 2000, 2002; Sawabe et al., 2000). Some strains were found to be oil-degrading (Wang et al., 2014).

Our results suggested that species of OH-oxidizing microorganisms from the Sakhalin collection were different from the species isolated from other areas and described in the literature (Table 3). We did not discover active oil degraders, i.e., *Mycobacterium*, *Brevibacterium*, *Corynebacterium*, bacteria with a lipophilic surface, which were mainly allocated in areas of high oil contamination, where the content of the OH was more than 1 g/L (oil films on the water surface). The reason for this finding was that our samples were collected from the places where there was no oil film on the water surface, with the exception of the ports. The collection therefore consisted of the strains involved in the second stage of OH degradation (Gusev and Koronelli, 1981). It should be noted that OH oxidation by members of the genus *Cobetia* was reported for the first time, thus expanding the list of known oil biodegraders.

Thus, the microorganisms isolated from the coastal zone of the southern Sakhalin Island had different destructive activity against different classes of oil hydrocarbons. The greatest effect was detected in the strains assigned by us to the group of active degraders; their possibile application in the liquidation of oil contamination should be considered. The studied strains had specificity in the degradation of OH. The isolates degraded aromatic hydrocarbons more intensively, in spite of their more complex structure impeding their disposal and requiring the certain enzyme systems (Gottschalk, 1979). The research revealed the peculiarities of the oil-oxidizing bacteria in the coastal zone of the southern Sakhalin Island, which, on the one hand, is of great theoretical interest, and, on the

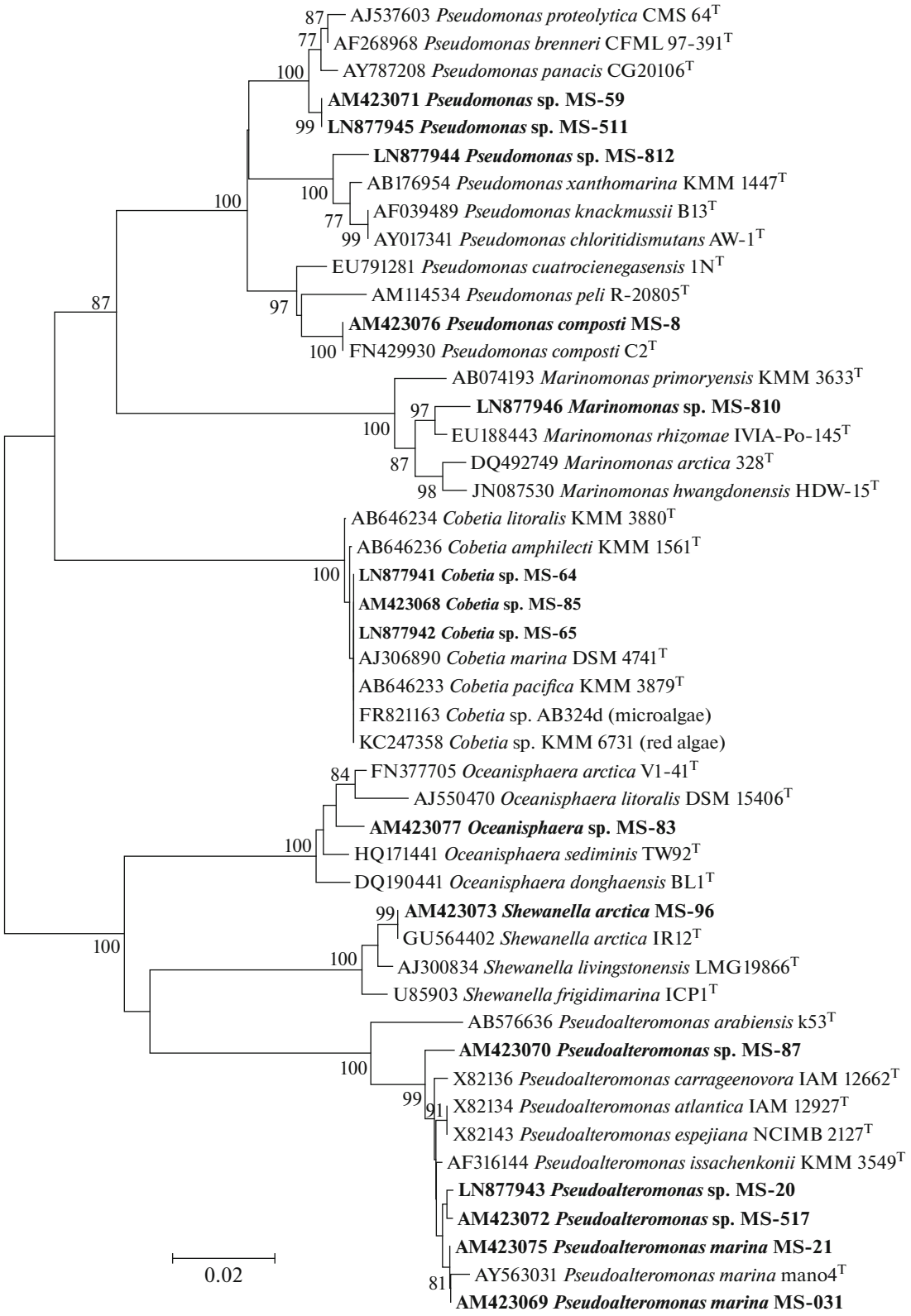

Fig. 2. Phylogenetic tree of gene fragments of 16S rRNA for hydrocarbon-degrading strains and type strains of the genera constructed using the neighbor joining method. Sequences obtained in this study are in boldface. The scale corresponds to 2 nucleotide substitutions per each 100 bp, bootstrap values are above 75%.

Table 3. Oil-oxidizing microorganisms isolated from different water basins (literature data)

Place of discovery	Genus	Authors
Baltic Sea	*Pseudomonas, Rhodococcus, Mycobacterium, Arthrobacter, Brevibacterium*	Koronelli et al., 1987
Barents Sea	*Bacillus, Bacterium, Pseudomonas, Pseudobacterium, Micrococcus*	Trunova, 1979
Black Sea	*Pseudomonas, Azotobacter, Erithrobacter, Marinococcus, Mezophilobacter, Alteromonas,, Bacillus, Micrococcus, Vibrio, Microbacterium, Arthrobacter*	Rubtsova and Egorov, 2004
Caspian Sea	*Pseudomonas, Bacillus, Micrococcus, Acinetobacter, Mycobacterim, Arthrobacter, Aeromonas, Vibrio, Flavobacterium, Alcaligenes*	Alekperova, 2009
Northern part of the Pacific Ocean	*Mycobacterium*, *Arthobacter*	Il'insky, 2000
Persian Gulf	*Haloferax, Halobacterium, Halococcus*	Al-Mailem et al., 2010
Yellow Sea	*Alcanivorax, Marinobacter, Novosphingobium, Rhodococcus, Pseudoalteromonas Algoriphagus, Aestuariibacter, Celeribacter, Fabibacter, Zobellia, Tenacibaculum, Citreicella, Roseivirga, Winogradskyella, Thioclava, Polaribacte, Pelagibaca*	Wang et al., 2014
Indian Ocean	*Nitratireductor indicus* sp. nov., *Altererythrobacter marinus* sp. nov.	Lai et al., 2009, 2011
Arctic waters	*Rhodococcus, Mycobacterium, Colwellia, Marinomonas, Glaciecola*	Koronelli et al., 1987; Brakstad et al., 2008
Coast of Alaska	*Alcanivorax*	Harayama et al., 1999
Mediterranean Sea	*Desulfosarcina, Desulfococcus, Oleispira, Percisivirga, Roseobacter, Phaeobacter*	Jaekel et al, 2015; Sauret et al., 2015
Gulf of Mexico	*Pseudomonas*	Koronelli et al., 1994
The area of Cuba	*Rhodococcus, Arthobacter*	
Antarctica	*Rhodococcus*	
Bering Sea	*Bacillus, Pseudomonas, Micrococcus, Pseudobacterium, Achromobacter, Bacterium, Brevibacterium*	Israel and Tsyban, 1989
Kara Sea and Laptev Sea	*Mycobacterium*	Gusev et al., 1977
California coastal waters; Bays of New Jersey; Alaska coast	*Corynebacterium, Arthrobacter, Brevibacterium, Marinomonas, Mycobacterium, Shewanella*	Atlas and Bartha, 1972
Sea of Okhotsk	*Pseudoalteromonas, Oceanisphaera*	Struppul et al., 2009; Bogatyrenko et al., 2013
Japan coastal waters	*Thalassospira*	Tsubouchi et al., 2014

other hand, has practical significance in a creation of strains collection for bioremediation technologies.

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