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ECOLOGY

# Study of Hydrocarbon Oxidizing Microorganisms from Deep Groundwater of the Puchezh–Katunki Impact Structure

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**Abstract**—The presence of viable hydrocarbon-oxidizing microorganisms has been shown in the underground waters exposed by the Vorotilovskaya deep well (the Puchezh–Katunki astrobleme, 75 km north of Nizhny Novgorod, 1900- and 3200-m deep) using the method of chromatography–mass spectrometry of specific biomarkers of the microbial cell wall and the classical methods of bacteriology. Several microbial species have been isolated in pure culture and identified. Two bacillary species, *Bacillus pumilus* KTB-2 and *Bacillus subtilis* KTB-4, were maintained in pure cultures at reinoculations. The effects of mineralization and aeration of the medium on the growth characteristics of *Bacillus pumilus* KTB-2 in batch culture have been studied.

***Keywords***: deep underground waters, hydrocarbon-oxidizing microorganisms, *Bacillus pumilus*, high mineralization of the medium, kinetic parameters of growth of microorganisms.

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Environmental pollution with oil hydrocarbons is one of the most pressing problems today. One of the first elements in the complex series of processes leading to the degradation of oil hydrocarbons in natural ecosystems is the action of hydrocarbon-oxidizing microorganisms. Wide spread occurrence of this group of microorganisms has been shown for aboveground ecosystems and the methods of bioremediation of polluted territories are actively developed using indigenous or specially selected strains or bacterial communities, as well as their microbiological preparations. The hydrocarbon-oxidizing microorganisms of the underground environment are studied mainly in the zones of occurrence of oil-bearing strata. These studies are important for enhanced oil recovery. Among underground waters, mainly the brine waters of oil fields with low or moderate mineralization (up to 40 g/L) are studied [1, 2]. In view of the above, it would be interesting to discover carbohydrate-oxidizing microorganisms in ecologically specific biotopes, such as highly mineralized underground waters. In prospect, such studies provide opportunities for obtaining new hydrocarbon-oxidizing strains–destructors for bioremediation of the territories with combined pollution, since the development of oil fields is very often accompanied by not only hydrocarbon pollution but also salinization.

The objectives of research were to detect hydrocarbon-oxidizing microorganisms in highly mineralized underground waters, to isolate pure cultures capable of utilizing hydrocarbons as a sole carbon and energy source, and to study their properties.

## MATERIALS AND METHODS

The research object was the underground water exposed by the Vorotilovskaya deep well (VDW) (the Puchezh–Katunki impact structure, 75 km north of Nizhny Novgorod, drilling depth 5374 m). The bromine/calcium chloride waters with total mineralization up to 160–190 g/L are of the nitrogen class with helium and hydrogen. Dissolved organic matter is presented by gaseous and liquid n-alkanes, as well as by natural and technogenic aromatic hydrocarbons that formed as a result of water pollution by oil additives and the peat–humate components of drilling fluid [3]. While VDW was being drilled, the permeable beds were filled with drilling fluid filtrate that not only had a certain effect on chemical composition of the waters but also, as we had shown previously, substantially influenced the functional diversity and composition of microbial communities of the groundwater [4]. In the present work, we have studied the waters from the zones of maximum water production (depths of 1900 and 3200 m) since their microbial coenosis, according to the results of the previous studies, is most similar to the indigenous one [5]. Water sampling and physicochemical investigation were performed by the Federal State Unitary Enterprise "Research and Production Center for Superdeep Drilling and Comprehensive

**Table 1.** Main physicochemical parameters of VDW groundwater

| Depth, m | $t$, °C | pH | Mineral-ization, g/L | Major ion composition, g/L* | | | | | | ΣHCG, vol % | DOM, mg/L |
|---|---|---|---|---|---|---|---|---|---|---|---|
| | | | | $Ca^{2+}$ | $Mg^{2+}$ | $Na^+ + K^+$ | $HCO_3^-$ | $Cl^-$ | $SO_4^{2-}$ | | |
| 1900 | 32 | $\frac{7.8}{8.0}$ | $\frac{129}{188}$ | $\frac{42.57}{61.32}$ | $\frac{0.06}{0.12}$ | $\frac{4.21}{6.86}$ | $\frac{0.17}{0.11}$ | $\frac{80.99}{118.79}$ | $\frac{1.22}{0.78}$ | $\frac{0.07}{0.03}$ | $\frac{11.4}{–}$ |
| 3200 | 60 | $\frac{7.8}{6.7}$ | $\frac{115}{187}$ | $\frac{36.89}{59.32}$ | $\frac{0.10}{0.12}$ | $\frac{5.01}{8.67}$ | $\frac{0.22}{0.10}$ | $\frac{72.20}{117.97}$ | $\frac{1.39}{0.90}$ | $\frac{0.04}{0.03}$ | $\frac{9.7}{–}$ |

Note: *, the level of 1995 in the numerator and the level of 2000 in the denominator; –, no data; HCG, hydrocarbon gases; DOM, dissolved organic matter.

Study of the Earth Interior” (Nedra, Yaroslavl) (Table 1).

Compositional and structural reconstruction of the microbial community of the waters and culture identification were performed using specific chemical markers (fatty acids, aldehydes, and oxyacids) by the method of gas chromatography/mass spectrometry (GC-MS) using an AT5973 D chromatograph–mass spectrometer (Agilent Technologies, United States) according to the procedure [6, 7]. Lipid markers were analyzed by Dr. G.A. Osipov (D.Sc. (Biology), Moscow). Different bacterial groups were accounted by the methods of classical bacteriology by inoculating water samples into selective nutrient media prepared from groundwater from the respective depth. The following two variants of the media were used: B-1 (mineralization level of 2000) and B-2 (mineralization level of 1995, approximately 1.5 times lower). The cultivation temperature was close to abyssal temperatures (Table 1). As we have already shown previously, the combination of temperature factor with the higher or lower mineralization of nutrient media makes it possible to shut off allochthonic bacteria and to isolate indigenous bacteria [8]. Hydrocarbon-oxidizing bacteria (HOB) were quantitatively accounted for by the tenfold dilution technique in liquid Raymond’s medium with the addition of oil or hexadecane as a carbon and energy source, as well as on the K medium for the bacteria utilizing gaseous (propane/butane) hydrocarbons. Physiological and biochemical properties were studied in the standard differential–diagnostic media [9, 10]. The effect of mineralization on pure culture growth was studied using a liquid K medium prepared from distilled water with addition of calcium chloride (Table 1) at a concentration of 5–200 g/L. The cultivation was carried out in the batch mode under intensive aeration (on a shaker) and under microaerobic conditions (without mixing the medium that filled 1/3 of the flask volume). Growth (physiological activity of the culture) was estimated by the change in the optical density of culture liquid recorded with KFK-56 at 610 nm in a 0.5-cm cuvette. Specific growth rate was calculated by the obtained batch growth curves [11].

## RESULTS AND DISCUSSION

Reconstruction of the composition of microbial coenoses in water samples based on the GC-MS chemodiagnostics has shown that the waters under study contain microorganisms capable of utilizing hydrocarbons as a sole carbon and energy source (HOB). These are members of the genera *Acinetobacter, Bacillus, Corynebacterium, Pseudomonas, Xanthomonas*, as well as actinobacteria of the genera *Mycobacterium, Nocardia, Rhodococcus*, and yeasts. In general, the percentage of HOB in microbial coenoses was 20 and 23% at the depths of 1900 and 3200 m, respectively; 17% of them are represented by actinobacteria. Among the latter, one should distinguish the genus *Rhodococcus* making up 43–51%. Representatives of this genus, according to the data of many authors, are characterized by a high level of adaptation to extreme habitats and utilization of both the higher gaseous homologues of methane and liquid n-alkanes as carbon and energy sources [12, 13]. The inoculation of water samples into nutrient media showed the presence of viable HOBs at all depths under study (Table 2).

The microorganisms utilizing liquid hydrocarbons were predominant among them; gasotrophic HOBs were found in negligible quantities only at the depth of 1900 m. The quantity of thermophilic HOBs was higher by one or two orders of magnitude compared to mesophilic HOBs, and their maximum quantity (up to 1000 cells/mL) was observed on a highly mineralized medium (V-1). The above data suggest that this group is indigenous in the microbial coenosis of the groundwater.

Hydrocarbon-oxidizing microorganisms of the association from the depth of 1900 m, isolated among other pure HOB cultures, are able to utilize gaseous alkanes (propane/butane) as the only carbon and energy source. As a result of subsequent reinoculation, the isolate was divided into several strains (GMB-1, KTB-2, KTB-4, and KB-1). Three of them were classified on the basis of chemo diagnostics and a number of morphological and biochemical characters as follows: (1) GMB-1 as basidiomycetous yeast *Rhodotorula rubra* (k = 0.604), (2) KTB-2 as a species of

**Table 2.** Quantity of microorganisms capable of utilizing hydrocarbons, cells/mL

| Depth, m | $t$, °C | Inoculation variant* | HOB medium containing as a carbon and energy source | | |
|---|---|---|---|---|---|
| | | | oil | hexadecane | propane/butane |
| 1900 | 32 | B-1 | $10^2$ | $10^1$ | + |
| | | B-2 | $10^1$ | $10^1$ | + |
| 3200 | 60 | B-1 | $10^3$ | $10^0$ | 0 |
| | | B-2 | $10^2$ | $10^1$ | 0 |

Note: *, mineralization level of 2000 (V-1); mineralization level of 1995 (V-2).

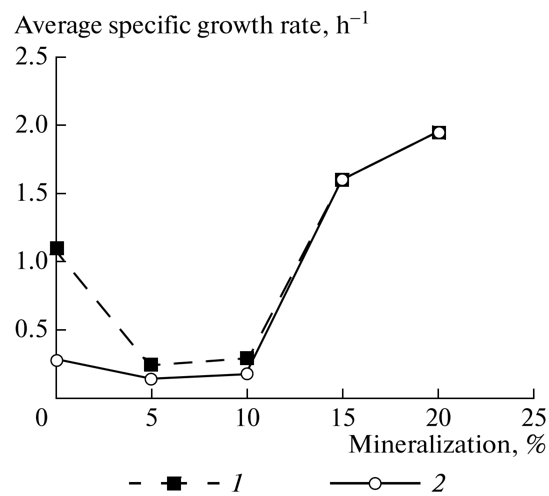
*Bacillus pumilus* (k = 0.579), and (3) KTB-4 as a species of *Bacillus subtilis* (k = 0.412). The strain KB-1 was assigned to the genus *Rhodococcus* based on the set of all morphological and biochemical characteristics. The results of species identification of the cultures are in agreement with the GC-MS analysis of the microbial community of groundwater from the given depth, which also showed the presence of the markers of bacilli, rhodococci, and microscopic fungi. During the study of the properties of pure cultures, the strains *Rhodococcus* sp. KB-1 and *R. rubra* GMB-1 showed lower viability outside the association and gradually lost the ability for growth and reinoculation in pure cultures. Close syntrophic relationships seem to exist between them in situ, which cannot be achieved under laboratory conditions during separate cultivation. The strains *B. pumilus* KTB-2 and *B. subtilis* KTB-4 proved to be viable and could be maintained in pure cultures. In general, the results of isolation of pure cultures lead to a conclusion that detection of the strains nonculturable under laboratory conditions is the evidence of uniqueness of groundwater microbial communities.

Physiological and biochemical properties necessary for identification were studied in isolated cultures (Table 3). It should be noted that all strains could utilize both gaseous and liquid hydrocarbons, as well as other carbon and energy sources (proteins, sugars, alcohols).

In view of the prospects of using the isolated strains for bioremediation, it is necessary to ascertain their ability to actively function under conditions of high mineralization of the medium that may be caused by combined pollution, as well as in the presence of oxygen, since aeration of the medium is a necessary condition for hydrocarbon oxidation.

The ability to grow under conditions of high mineralization has been studied in the facultative anaerobe *B. pumilus* KTB-2 in a batch culture. It has been shown that the rate of culture growth in a nonmineralized nutrient medium (without calcium chloride) with aeration was 4-fold higher than without aeration (figure). When mineralization was enhanced to 50 and 100 g/L, specific growth rate of the culture decreased approximately five times under aeration conditions and two times under micro-aeration conditions, compared to the nonmineralized medium. The high degree of aeration of the medium also resulted in more intensive growth of the culture: its growth rate was approximately 1.5-fold higher. With further enhancement of mineralization to 150 and 200 g/L, specific growth rate increased both with and without aeration, reaching the maximum of 1.95 $h^{-1}$ at 200 g/L. However, the maximum values of optical density of the culture, which is proportional to the number of cells, as well as the duration of its growth at such a high mineralization, were two and more times lower than in low- and nonmineralized media. In addition, the cells were reduced and changed their morphology. This is indicative of unfavorable conditions for culture growth at high mineralization. Thus, it may be concluded that the strain *B. pumilus* KTB-2 is halotolerant, its most favorable growth range is up to 10% of salts, and the presence of oxygen stimulates cell growth. It is most probable that bacteria are inactive under conditions of natural biotope at water mineralization up to 188 g/L and oxygen deficiency.

The findings are in agreement with the existing notions of the real rate of microbial metabolism in




Variation of the average specific growth rate of the strain *Bacillus pumilus* KTB-2 depending on mineralization and aeration of the medium: *1*, with aeration; *2*, without aeration.

**Table 3.** Some physiological and biochemical properties of isolated cultures

| Test | Strain | | | |
|---|---|---|---|---|
| | *Bacillus pumilus* KTB–2 | *Bacillus subtilis* KTB–4 | *Rhodococcus* sp. KB–1 | *Rhodotorula rubra* GMB–1 |
| Consumption: | | | | |
| Glucose | + | + | + | + |
| Sucrose | + | + | – | + |
| Maltose | + | + | + | + |
| Lactose | + | + | – | ± |
| Mannitol | + | + | + | – |
| Propane/butane | + | + | + | + |
| Hexadecane | + | + | + | + |
| Oil | + | + | + | + |
| Hydrolysis: | | | | |
| Gelatin | + | + | n/d | – |
| Urea | + | + | – | + |
| Casein | – | – | n/d | n/d |
| Starch | – | + | – | n/d |
| Amylogenesis | n/d | n/d | n/d | – |
| Growth on BEA | + | + | + | n/d |
| Sucrose fermentation | + | – | n/d | – |
| Voges–Proskauer reaction | + | + | n/d | n/d |
| Reference to $O_2$ | FA | OA | OA | A |
| Nitrate reduction | + | + | n/d | n/d |
| Presence of catalase | + | + | n/d | n/d |
| Formation of pseudomycelium | n/d | n/d | n/d | + |
| $t$, °C | 20–37 | 20–37 | 28 | 20–60 |

Note: FA, facultative anaerobe; OA, obligate aerobe; A, aerophile; n/d, not determined; $t$, °C is temperature.

deep layers being extremely low [14] and the functioning of microbiota being aimed at preservation and maintenance rather than growth of microbial population. It is demonstrated also by the results of GC-MS analysis of the microbial community of groundwater under study, according to which the substantial part of subsurface microbiocoenosis consists of spore-forming species [5]. It may be evidence of the fact that a considerable part of heterotrophic microorganisms is present in the highly mineralized oxygen-limited subsurface environment in the state of surviving unfavorable conditions, comprising the so-called microbial pool. However, as is shown by the studies of peculiar features of the strain *Bacillus pumilus* KTB-2, they can be activated by changes in environmental situation (reduced mineralization, enhanced partial oxygen pressure), which is evidence of considerable metabolic potential of bacteria from deep-seated extreme habitats [15].

Thus, the modern molecular and traditional microbiological methods of research made it possible not only to ascertain the presence of viable HOBs in the VDW groundwater from the depth of 1900 and 3200 m but also to isolate and identify a number of pure cultures capable of growth on laboratory nutrient media. Hence, it is possible to study the growth characteristics of isolated HOB strains under varied cultivation conditions (mineralization, aeration, etc.), bringing them closer to the natural conditions. Taking into account the possibility of using the isolated strains for purification of oil-polluted territories under salinization conditions, further experimental studies must be aimed at investigation of the ability of obtained cultures to oxidize hydrocarbons at different mineralization of the medium.

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