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Thermophilic aerobic organoheterotrophic soil bacteria from anthropogenically changed territories of Saint Petersburg and Leningrad region

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Anthropogenically altered soils of Saint Petersburg and Luga (Leningrad Region) were investigated for the presence of thermophilic aerobic chemoorganoheterotrophic bacteria, potentially capable of decomposing hydrocarbons at elevated temperatures (60°C). 6 strains of pure spore-forming cultures of bacteria were isolated. Analysis of the nucleotide sequences of the 16S rRNA genes showed that they belong to the genera *Geobacillus* and *Aeribacillus*. For the first time, we obtained information on the presence of representatives of the genus *Aeribacillus*, which are typical inhabitants of hot springs and zones with geothermal activity, in the soils of the regions of Saint Petersburg and the Leningrad Region.

Keywords: aerobes; thermophilic bacteria; acetate-decomposing bacteria; oil pollution; anthropogenic soil pollution.

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Термофильные аэробные органогетеротрофные бактерии антропогенно измененных территорий Санкт-Петербурга и Ленинградской области

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Исследованы антропогенно измененные грунты Санкт-Петербурга и Луги (Ленинградская область) на предмет присутствия термофильных аэробных хемоорганогетеротрофных бактерий, потенциально способных к разложению углеводородов при повышенной температуре (60 °С). Выделено 6 штаммов чистых культур спорообразующих бактерий. Анализ нуклеотидных последовательностей генов 16S рРНК показал их принадлежность к родам *Geobacillus* и *Aeribacillus*. Сведения о присутствии представителей рода *Aeribacillus*, типичных обитателей горячих источников и зон с геотермальной активностью, в почвах регионов Санкт-Петербурга и Ленинградской области получены нами впервые.

Ключевые слова: аэробы; термофильные бактерии; ацетатразлагающие бактерии; нефтезагрязнение; антропогенное загрязнение почв.

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INTRODUCTION

Numerous studies of anthropogenically modified soils and grounds in different climatic zones have shown that the composition of the microbial community, in particular, the consortium of crude oil degraders, is different [1–4]. Most of the microorganisms inhabiting places remote from geothermal areas are mesophiles growing at temperatures from 0°C–10°C to 40°C–45°C, with the natural optimal temperature of 37°C [5, 6]. Thermophilic microorganisms are considered as an ecologically separate group of microorganisms; the optimum temperature for the growth of moderately thermophilic bacteria is 50°C–75°C, and for extreme thermophilic and hyperthermophilic bacteria and archaea, it is within the range of 75°C–105°C, with the maximum temperature reaching 100°C and above [5, 6]. The main distinguishing traits of these groups are the specific chemical composition and strength of cell membranes, thermostable proteins and enzymes, increased lipid content in the cell wall, saturated fatty acid content, and accelerated metabolism, which, among other things, enables restoration of quickly damaged cell structures [6].

The natural habitats of thermophilic microorganisms in the contemporary biosphere are thermal springs, deep-sea hydrothermal springs, high-temperature underground biosphere, as well as ecosystems associated with human activities, namely, composts, waste water, storage sites for manure, peat, and hay [5]. Numerous studies have shown the presence of thermophilic microorganisms in various geographic areas such as in Indonesia, New Zealand, Mexico, China, Nepal, Thailand, India, People's Republic of China, Japan, Turkey, Algeria, Saudi Arabia, Iran, Tunisia, Germany, Italy, Bulgaria, Russia, Armenia, Kazakhstan, and Antarctica [6–37]. Microorganisms discovered are mainly representatives of the microbiota of thermal springs, reservoir waters of high-temperature oil fields, and ecosystems associated with them; however, they also include inhabitants of soils and even subglacial lake waters [21], which indicate the adaptability of these microorganisms to various habitat conditions. Thus, according to the literature, the presence of thermotolerant bacteria was also confirmed in the soils of the northern regions [38], which is presumably associated with anthropogenic effect and climate change. The study of microorganisms adapted to the climatic conditions of a particular region must be taken into account for the selection of a system for bioremediation of oil-contaminated soils and the use of agents in it based on the application of microbial cultures [19]. In addition, thermophilic microorganisms, one of the main characteristics of which is rapid metabolism, can accelerate significantly the decomposition of oil products in the soil. We have revealed previously the presence of thermophilic hydrocarbon-oxidizing microorganisms in the soils of St. Petersburg [39]; nevertheless, in general, their representatives living in the soils of the northern regions free of geothermal activity are still under-investigated.

Under natural conditions in the southern regions, where thermophilic microorganisms are typical inhabitants, the soil surface can be heated by solar radiation up to 60°C–75°C [40, 41], and in the northern regions, it can be heated up to 46°C–57°C [42, 43]. While heating is also naturally influenced by the state of the vegetation cover, the dry-weight percentage of the soil, and its moisture capacity [44], sandy soils warm up to a greater extent than cold ones and cool faster; the contrast ratio of the temperature regime is also typical for anthropogenically modified territories with deterioration or absence of soil and vegetation cover [45], as well as for areas where the soil cover is completely replaced by bulk ground. Landfills and areas of railways represent such anthropogenically altered landscapes with a locally increased ambient temperature and the regular introduction of alien flora and microflora into the ecosystem [46, 47]. This prompts the hypothesis that thermophilic microorganisms that are resistant to pollution and able to adapt to the conditions of a lack of organic compounds can inhabit the surface layer of soils of such altered landscapes.

Thus, *this work aimed* to reveal the presence of thermophilic bacterial crude oil degraders in anthropogenically altered soils of St. Petersburg and Leningrad region as well as to isolate pure cultures and characterize and identify strains.

MATERIALS AND METHODS

The study objects were samples of anthropogenically contaminated soil from the landfill in St. Petersburg (samples K2 and K6) and soil samples from the Luga railway tracks (samples L1 and L2) taken in 2019. Sample K2 was soil from the landfill in the Kudrovo area, sample K6 was the background soil near the landfill, sample L1 was the background soil on the slope of the railway in Luga, and sample L2 was the sandy soil from the railway track in Luga with visible oil contamination. Samples of the surface layer of soil or ground (0–5 cm) were taken into sterile vessels at 5 points on an area of 1 m^2, and then they were mixed under sterile conditions and solid inclusions and plant residues were manually removed.

For identification of the presence of thermophilic bacteria and obtaining enrichment cultures in averaged samples, the samples were inoculated into a modified Voroshilova–Dianova (VD) liquid mineral nutrient medium [48] with the addition of Na acetate as the only source of energy and carbon for the growth of microorganisms. The VD medium (per 1 L) was composed of 1.0 g of NH_4Cl,

1.0 g of NaCl, 1.0 g of K_2HPO_4, 1.0 g of KH_2PO_4, 0.2 g of $MgSO_4$, 0.02 g of $CaCl_2$, and 1.36 g of $CH_3COONa \cdot 3H_2O$. The medium pH was adjusted to 7.0–7.4. The medium was sterilized by autoclaving for 30 min at 1 atm and 121°C. Trace element solutions prepared as described in the source [49] were added at 1 ml/L of each to a sterile medium before inoculation. The bacteria were cultivated aerobically in a thermostat at a constant temperature of 60°C. Initially, 0.5 ml of an aqueous extract of soil was added to a test tube with VD medium of 5 ml [1 g of soil was introduced into a test tube with 10 ml of distilled water, shaken on a Vortex V-1 plus (Biosan). Inoculation with a pipette from the upper part of the extract was performed after 15-min settling of sedimentation of soil particles]. Subsequently, the method was adjusted, and 0.05 g of a soil sample was introduced directly into a test tube with the medium.

To isolate pure cultures, enrichment cultures were subcultured in test tubes with a liquid VD medium with sodium acetate (method of serial dilutions) and cultured in a thermostat. After shaking on a Vortex using a pipette, 0.1 ml of the culture liquid was inoculated from the upper part of the test tube onto plates with solid fish meal hydrolyzate (FMH) medium with agar (composition per 1 L included 12.0 g of pancreatic FMH, 12.0 g of enzymatic peptone, 6.0 g of NaCl, 12.0 g of microbiological agar) and triturated evenly with a spatula, and cultivation took place within 1 day, after which pure cultures were isolated from the grown colonies by the bacto-streep method.

Microscopic examination to check the purity of the cultures was performed on a Zeiss Axiostar plus microscope equipped with a phase-contrast device at a total magnification of ×400. A day-old culture grown on solid FMH medium was used.

To test the ability of the cultures to use oil as the only carbon source, isolated cultures were inoculated in a liquid VD medium with the addition of oil as a substrate (10 ml/L). After the surface introduction of oil into the medium, the bacterial suspension was inoculated and cultivated in a thermostat at a constant temperature of 60°C. To confirm the growth of bacteria using oil hydrocarbons as the only carbon source, the bacterial suspension obtained from oil was subcultured three times on a similar medium with oil, followed by cultivation with measurement of optical density.

The dynamics of culture growth in a liquid medium was determined by the change in the optical density of the medium using a PE 3000-UV spectrophotometer (Promecolab) at a wavelength of 570 nm in absorption units (abs). Based on the results of measurements for 2.5 days (until the growth curve reached a plateau), the culture growth curve was plotted. The experiment was performed in triplicate, and the culture optical density for each point was measured twice in a 2.5-ml cuvette; statistical processing and plotting with the display of the relative error were performed in MS Excel 2007. The range of values lies within the error limits ($\Delta = 0.05$).

Strains were identified by molecular genetic methods at Evrogen (Moscow) based on the analysis of the nucleotide sequence of the 16S rRNA gene obtained using standard primers 27F (5'-AGAGTTTGATCCTGGCTCAG3') and 1492R (5'-ACGGYTACCTTGTTACGACTT3').

For analysis of nucleotide sequences and construction of a phylogenetic tree, we used the NCBI BLAST libraries and neighbor-joining and maximum likelihood methods [49–53] (MUSCLE, Gblocks, PhyML, TreeDyn programs). The length of the sequences analyzed was 1439–1444 nucleotides.

Nucleotide sequences of 16S rRNA gene fragments were deposited in the GenBank NCBI database under numbers MW676172–MW676177.

RESULTS AND DISCUSSION

Isolation of pure cultures

Inoculation of water extracts of soil in variants K2 and K6 on a liquid VD medium and subsequent incubation did not lead to the expected result, the growth of thermophilic microorganisms was not detected, probably due to a small number of their cells in the soil. Therefore, the technique was subsequently modified, and 0.05 g of a soil ground sample was used as an inoculum, which was introduced directly into a test tube with the medium. In this case, the growth of microorganisms started 1 day after inoculation. Microorganisms capable of growing at a temperature of 60°C were detected in all studied samples K2-2, K6, L1, and L2.

As a result of several serial dilutions and successive transfers of microorganisms grown from the studied soil samples (K2 and K6, and L1 and L2), stable growing enrichment cultures of thermophilic bacteria were obtained. Subsequently, pure cultures K2-2, K6, as well as L1, L2-1, L2-2, and L2-3 were isolated from enrichment cultures. The L2-1 culture stopped growing after several reinoculations and was not used in further experiments. Isolation of enrichment cultures K2 and K6 was described by Volkova et al. [39]. The isolation of pure cultures of bacteria from the Kudrovo and Luga samples is described in this publication.

Inoculation of the L2 enrichment culture on the surface of the agar medium of FMH resulted in the growth of bacterial colonies visually different in color and growth pattern. Three pure cultures of bacteria, namely, L2-1, L2-2, and L2-3, were isolated by the depletion bacto-streep method on this medium, while the L1 culture was initially a homogeneous monoculture. A greater diversity of cultivated thermophilic microorganisms was detected in a sample of contaminated soil compared with

the background, which indicates a greater adaptability of the microbial community under conditions of technogenic pollution.

Characteristics of cultures

Microscopic examination of the cultures showed that cultures K2–2, K6, L1, L2–2, and L2–3 are immobile, sporulating small thin rods, and L2–1 are immobile sporulating long rods.

The general view of microbial cells and spores is presented in Fig. 1.

All isolated cultures were able to grow by using acetate as a source of energy and carbon. The growth dynamics of cultures K2-2 and K6 are demonstrated in Figs. 2 and 3. The maximum optical density (0.25 ± 0.012) was registered after 2–3 days for the K2-2 culture and 1.7 days for the K6 culture.

The isolated bacterial strains were tested for their ability to grow by the use of oil. Strains K2-2 and K6 were able to grow by using petroleum hydrocarbons. The ability to use petroleum hydrocarbons by strains isolated from Luga's samples requires further study.

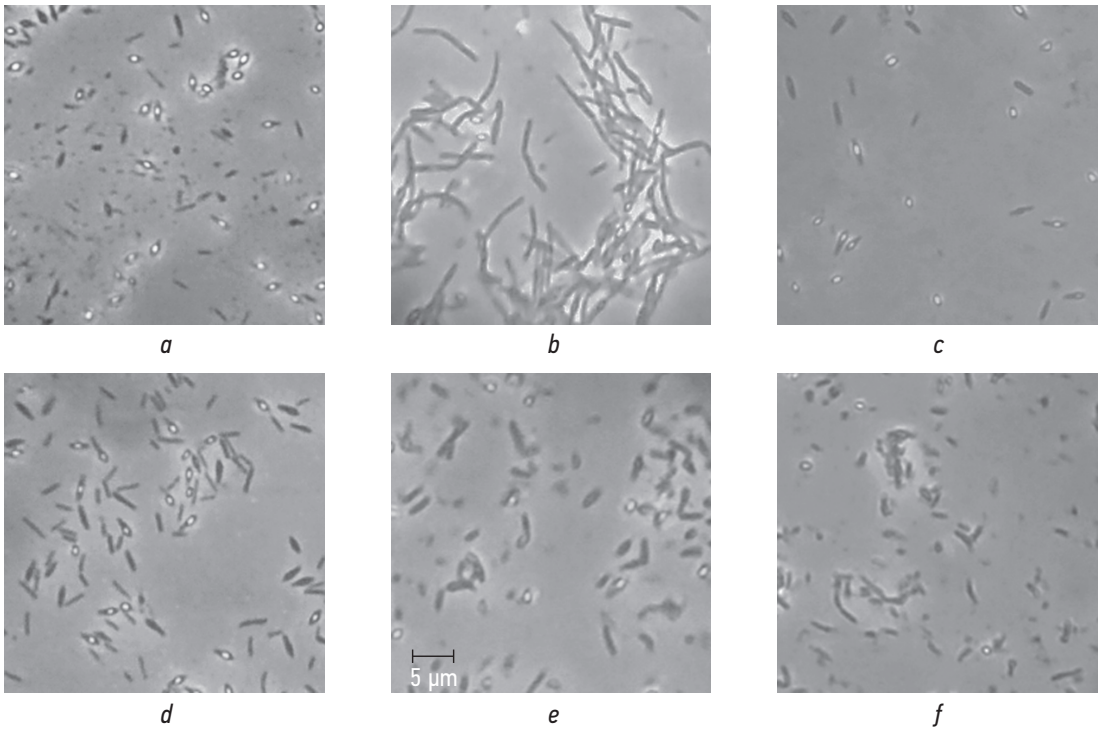

Fig. 1. General view of cells and spores: *a* – L1, *b* – L2-1, *c* – L2-2, *d* – L2-3, *e* – K2-2, *f* – K6

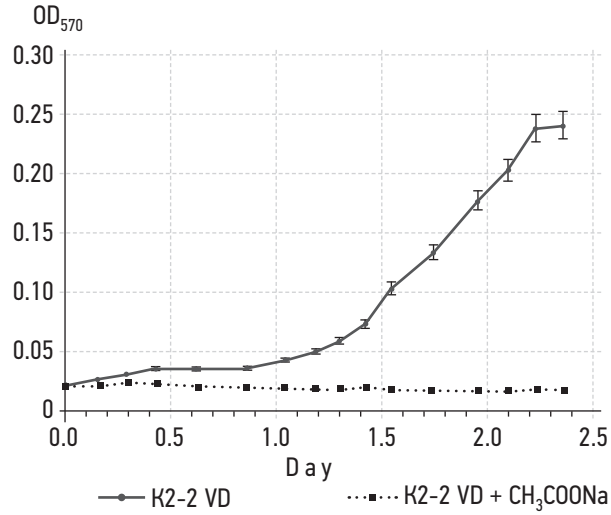

Fig. 2. Growth curve of a bacterial culture K2-2 in a liquid VD medium with sodium acetate and without an organic substrate for 2.5 days. OD, optical density of the culture

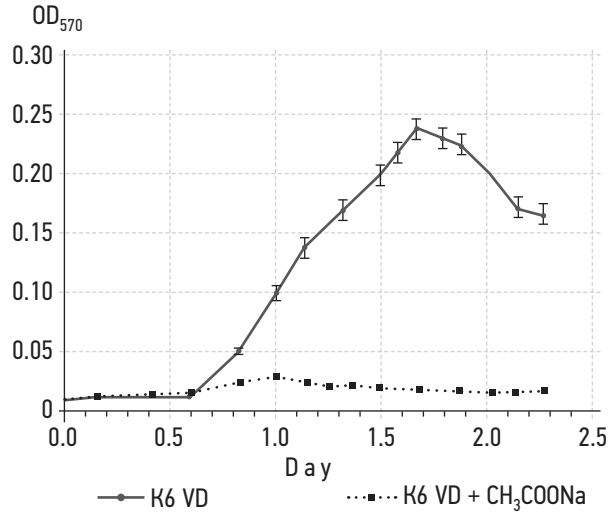

Fig. 3. Curve of the change in the optical density of the bacterial culture K6 in VD medium with sodium acetate and without an organic substrate for 2.5 days. OD, optical density of the culture

The maximum optical density of the K2-2 and K6 cultures at the end of the experiment during their cultivation for 2.5 days on a liquid VD medium added with oil was 0.143 ± 0.011 for the K2-2 culture and 0.064 ± 0.005 for the K6 culture, which indicated the ability of these cultures to use various petroleum hydrocarbons as a growth substrate. The K2-2 strain isolated from the contaminated soil at the landfill territory is possibly capable of decomposing more oil components and, accordingly, appears more promising for further research in this field.

Identification of strains

The study of the phylogenetic position of the isolated cultures based on the analysis of the nucleotide sequence of the 16S rRNA genes obtained with universal primers 27F and 1492R revealed that they represent different genera of gram-positive spore-forming bacteria belonging to *Firmicutes* of the *Bacilli* class of the order *Bacillales* of the *Bacillaceae* family. Thus, strain L2-1 belongs to the genus *Geobacillus*, and strains K2-2, K6, L1, L2-2, and L2-3 belong to the closely related genus *Aeribacillus* (Fig. 4), which was relatively recently separated from *Geobacillus* [10]. This genus currently comprises two species [10, 55].

According to the literature, strains *Aeribacillus pallidus* and *Geobacillus thermodenitrificans*, which are bacterial species most similar to those we found based on the results of 16S rRNA sequencing, are inhabitants of hot geographic regions, and they were found in Mexico [11], Iran [35], and Turkey [9]; there is little information about their ability to decompose hydrocarbons. Moreover, some strains of these species are capable of decomposing petroleum hydrocarbons (alkanes <C17, aromatic hydrocarbons, naphthalene, phenanthrene), namely, *Aeribacillus pallidus* 8m3, SL1, VP3, XS2, and XS3 [20, 24, 56–59] and *Geobacillus thermodenitrificans* NG80-2 [60]. As a rule, thermophilic bacilli of the genus *Aeribacillus* were found in thermal springs and geothermal activity zones [12, 13, 20, 61]; we obtained information on their presence in the soils of the regions of St. Petersburg and Leningrad for the first time. According to literature data, some other thermophilic representatives of the genus *Geobacillus* were revealed on the territory of Russia [18, 62, 63], and these species were also found mainly in thermal springs. While there is very little information

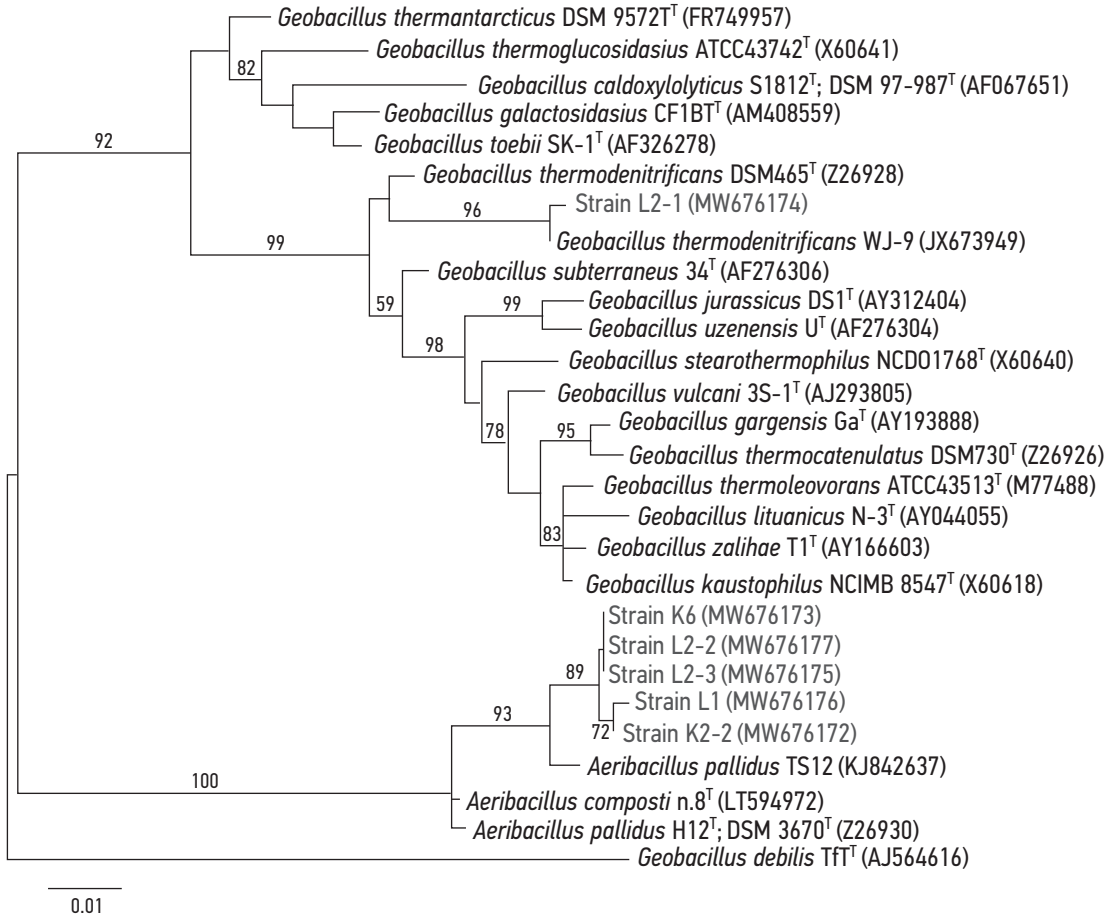

Fig. 4. Phylogenetic tree of closely related strains obtained by applying the neighbor-join and BioNJ algorithms to the matrix of pairwise distances [50–54]. Branch length is presented to scale and is measured by the number of nucleotide substitutions per site

nowadays on their presence in soil on the territory of Russia, there are only a few data on the presence of thermophilic microorganisms in anthropogenically modified soils, such as timber stockpiling areas [64], as well as of agricultural soils after the application of wastewater treatment products [65].

Thus, the hypothesis of the presence of thermophilic bacteria in anthropogenically altered soils of St. Petersburg and Leningrad region was confirmed, and the results of this study demonstrated that, in general, representatives of thermophilic bacteria with rapid metabolism, atypical for the northern regions, are capable of adapting and surviving under conditions of anthropogenic contamination, using a short period of elevated temperatures for reproduction and waiting out of unfavorable periods in a state of spores.

CONCLUSION

The microflora of anthropogenically altered soils of the Kudrovo region (St. Petersburg) and the city of Luga (Leningrad region) was investigated. This study revealed the presence of thermophilic aerobic bacteria with growing capability due to the destruction of acetate at an elevated temperature (60°C). Pure bacterial cultures (6 strains) were isolated and described, and the obtained strains were identified based on molecular genetic methods, which showed that they belong to the genera *Geobacillus* and *Aeribacillus*. For the first time, we obtained information about representatives of the genus *Aeribacillus* in the regions of St. Petersburg and Leningrad.

Future studies should identify the adaptability of bacterial cultures obtained under different temperature conditions, as well as the ability to decompose individual hydrocarbons.

ADDITIONAL INFORMATION

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