Occurrence of Hydrocarbon Degrading Genes in the Soils of the Republic of Tatarstan (Russia)

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Abstract
Oil pollution is one of the most serious environmental problems nowadays. The ability of soils for self-restoration is important, when choosing the strategy of pollution control. This ability depends on the pull of microbes able to decompose hydrocarbons that were present in the non-polluted soil prior to pollution. In this study, the occurrence of alkane degrading genes in the soils of the Republic of Tatarstan being one of the oil processing regions in Russia, was investigated. It was found that alkane degrading genes belonging to group I were present in 20 of the 25 soil samples, and their abundances ranged between 0.01 and 0.07%. Alkane degrading genes belonging to group II were not detected in the samples investigated, and those belonging to group III were present in all the samples, and their abundances ranged between 0.06 and 7.25%. No correlation between the alkane degrading gene copy numbers and pH and organic carbon content in soils was revealed.

1. Introduction
Taking into account the importance of soils for agriculture and food supply, their pollution with oil products is a serious threat [1,2]. When the soil is polluted, natural degradation of oil hydrocarbons is carried out by the microorganisms from the soil community present in it before the pollution. They are called hydrocarbon oxidizing microorganisms, capable of synthesizing enzymes to degrade alkanes and aromatic hydrocarbons – monooxygenases and dioxygenases [3].

There are microorganisms capable of synthesizing several enzymes to degrade hydrocarbons simultaneously, but usually only one enzyme can be synthesized by such microorganisms [4,5]. Alkane-monooxygenases complex consists of alkane-monooxygenase, rubredoxin-reductase and rubredoxin. Together with cytochrome P450-hydroxylase it is responsible for the degradation of alkanes [3,6–9]. The most widespread enzymic complex is alkane-monooxygenases enzymic complex, so the most researches are devoted to it. Gene alk responsible for encoding enzyme synthesis has an alternating structure, due to which enzymes possess different properties: for instance, alkB gene from Pseudomonas sp. is responsible for the synthesis of enzyme degrading short-chain alkanes C₆-C₁₂, alkM genes from Acinetobacter sp. encode the enzymes to decompose alkanes C₁₀-C₄₀, genes alkB₁ and alkB₂ from Rhodococcus sp. synthesize enzymes degrading alkanes C₈-C₃₂ [7,9–13]. Owing to the growth of the number of such genes, when the soil is polluted, the assessment of their number can be used to monitor oil pollution, both new and old [14,15].

In pollution-free soils alkane-degrading microorganisms can be found as well, which is connected with the presence of alkane-like substances in the environment [3]. Such soil characteristics as organic matter content, mineral and granulometric composition, acidity, humidity, etc. determine the structure of microbial coens [5,16,17]. The degradation of hydrocarbons is carried out by some representatives of soil microbial community (hydrocarbon degrading microbes), which can depend on soil properties [11]. Thus, studying the occurrence of genes in pollution-free soils can reveal the relation of this group.
of microorganisms and soil characteristics, and it can also show the soil of what type is more or less capable of self-restoration.

The objective of our investigation was to monitor the occurrence of different groups of alk genes (alkB, alkM and alkB1) in different soil types in the Republic of Tatarstan. To reveal the relation between the occurrence of genes and soil properties we have analyzed the following agrochemical characteristics: acidity and organic carbon content.

2. Materials and methods

2.1 Soil samples

Twenty-five pristine (uncontaminated) soils were sampled from various sites in republic of Tatarstan, far from residential areas, trails and oil production sites (Table 1). Prior to sampling, plants were removed and soil was sampled to a depth of 10 cm. Five replicates were sampled, and one representative sample was prepared. For chemical and physical analysis samples were air-dried, sieved (2 mm mesh size), and stored at 4 °C in the dark for further use. Subsamples for molecular biological analysis were collected with sterile spatulas, transported in coolers to the laboratory, and stored at -80°C until they were analyzed.

Table 1. Sampling Regions.

| Sample | Sampling Region | Soil Type          |
|--------|-----------------|--------------------|
| P1     | Sovetsky District, Kazan |                    |
| P2     | Sovetsky District, Kazan |                    |
| P3     | Pestrychinsky District |                    |
| P4     | Aviastroitelny District, Kazan | Eutric Podzoluvisols |
| P5     | Laishevsky District |                    |
| P6     | Zelenodolsky District |                    |
| P7     | Zelenodolsky District |                    |
| P8     | Arsky District |                    |
| G1     | Kamsko-Ustinsky District |                |
| G2     | Kamsko-Ustinsky District |                |
| G3     | Laishevsky District |                    |
| G4     | Laishevsky District |                    |
| G5     | Laishevsky District | Haplic greyzem     |
| G6     | Laishevsky District |                    |
| G7     | Kamsko-Ustinsky District |               |
| G8     | Kamsko-Ustinsky District |               |
| G9     | Kamsko-Ustinsky District |               |
| Ch1    | Nurlatsky District |                    |
| Ch2    | Nurlatsky District |                    |
| Ch3    | Buinsky District | Haplic Chernozems |
| Ch4    | Buinsky District |                    |
| Ch5    | Menzelinsky District |               |
| Ph1    | Apastovsky District |                    |
| Ph2    | Apastovsky District |                    |
| Ph3    | Apastovsky District |                    |

2.2 Chemical and biological analyses
Hydrocarbon content was assessed using IR-spectrometry with an AN-2 analyser (LLC Neftehimavtomatika-SPb, Russia). The total organic carbon content in waste samples was estimated according to ISO 14235:1998, pH according to ISO 10390:2005 [18,19].

2.3 Molecular analysis

Total genomic DNA was extracted from the samples using the FastDNA® SPIN Kit for Soil (Bio101, Qbiogene, Heidelberg, Germany) according to the manufacturer’s instructions. The DNA samples were stored at -20 °C or analyzed immediately.

The copy number of the *alk* genes in each sample was quantified with qPCR analysis. Real-time PCR was performed using a 0.1 U µl⁻¹ SynTaq Polymerase, 1x Buffer with SYBR Green, 2.5 mM MgCl₂, 200 µM dNTPs each, 0.2 µM primer each and 1 µl of DNA template in the CFX96 Touch Real-Time PCR Detection System (Bio-Rad, Munich, Germany). qPCR assays were performed with the group-specific *alk* primers listed in Table 2 [10,20]. PCR amplification was conducted using the following protocol: 15 min at 95 °C, followed by 39 cycles at 95 °C for 30 s, 30 s at 50°C, and 30 s at 72°C. Cycle thresholds were determined by comparison with standard curves constructed using several concentrations of a positive clone. The plasmid DNA concentration was determined on a Qubit 2.0 Fluorometer (Invitrogen, Thermo Fisher Scientific, Waltham, Massachusetts, USA), and the copy number of the target gene was calculated directly from the concentration of the extracted plasmid DNA. Ten-fold serial dilutions of a known copy number of plasmid DNA were subjected to real-time PCR in five replicates to generate an external standard curve.

To evaluate the abundance of n-alkane-degrading bacteria relative to total bacteria, the percentages of *alk* genes in proportion to 16S rRNA were also calculated. Bacterial 16S rRNA genes were quantified using 341f /534r primers [21,22]. The qPCR program consisted of initial denaturation at 95 ⁰C for 5 min followed by 39 three-step cycles of 95⁰C for 10 s, 55⁰C for 30 s, and 72⁰C for 30 s. The standard curves were generated for bacteria using serial DNA dilutions of DNA of *Pseudomonas putida*.

Three replicates for each sample were used for qPCR analyzes. All of qPCR assays performed with efficiency of more than 94%, R2 values greater than 0.99.

| Primers   | Sequence | Phylogenetic affiliation                  |
|-----------|----------|------------------------------------------|
| alk If    | 5’-CAT AAT AAA GGG CAT CAC CGT-3’        | *Ps. putida* *(alkB)*                     |
| alk Ir    | 5’-GAT TTC ATT CTC GAA ACT CCA AAC-3’    | *Stenotrophomonas* spp. *(alkB)*         |
| alk IIIf  | 5’-GAG ACA AAT CGT CTA AAA CGT AA-3’     | *Actinobacter* spp. *(alkB)*             |
| alk IIr   | 5’-TTG TTA TTC CCA CTA TGC TC-3’         | *Ps. fluorescens* *(alkB)*, *Ps. aeruginosa* *(alkB)*, *Rhodococcus* spp. *(alkB)*, *Burkholderia* spp. *(alkB)*, *Amycolatopsis* spp. *(alkB)* |
| alk IIIIf | 5’-TCG AGC ACA TCC GCG GCC ACC A-3’      |                                           |
| alk IIIr  | 5’-CCG TAG TGC TCG ACG TAG TT-3’         |                                           |

2.4 Statistical analysis

Sampling and chemical analyses were carried out in triplicate and biological analyses in quintuplicate, and all results were expressed on an air-dried sample basis. Statistical analyses were performed using Origin 8.0 (OriginLab, Northampton, USA) and R Statistical Software (R 3.0.0, R Foundation for Statistical Computing Version, Vienna, Austria) [23] packages. The confidence of data generated in the present investigations has been analyzed by standard statistical methods to determine the mean values and standard errors (SEs). The means were compared using Fisher’s least significant difference at α=0.05.

3. Results and Discussion

To test the hypothesis of relation between the distribution of genes responsible for alkane-degrading enzymes we have realized the analysis of agrochemical characteristics of soils under investigation.
Previous investigations showed that pH and organic carbon content were the main factors influencing the structure of soil microbial communities [3,11,24]. As a result of this, we have chosen the same parameters. The data obtained are presented in Table 3.

Table 3. Physical and Chemical Properties of the Soils.

| Sample | pH   | Organic C, % |
|--------|------|--------------|
| P1     | 5.45 | 0.76         |
| P2     | 5.99 | 0.72         |
| P3     | 5.85 | 0.92         |
| P4     | 5.05 | 2.17         |
| P5     | 5.88 | 1.66         |
| P6     | 5.84 | 1.18         |
| P7     | 5.82 | 2.53         |
| P8     | 6.23 | 1.77         |
| G1     | 6.40 | 1.76         |
| G2     | 6.10 | 1.08         |
| G3     | 5.49 | 1.07         |
| G4     | 6.40 | 1.27         |
| G5     | 5.70 | 1.08         |
| G6     | 5.64 | 1.99         |
| G7     | 6.64 | 2.97         |
| G8     | 6.78 | 1.92         |
| G9     | 6.60 | 2.11         |
| Ch1    | 7.29 | 4.54         |
| Ch2    | 6.21 | 3.49         |
| Ch3    | 6.60 | 3.21         |
| Ch4    | 6.82 | 4.51         |
| Ch5    | 7.25 | 3.10         |
| Ph1    | 7.15 | 3.90         |
| Ph2    | 7.30 | 4.52         |
| Ph3    | 7.20 | 3.00         |

As we can see in Table 3, soils belonging to the type of eutric podzoluvisols were characterized with lower pH values varying between 5.05 and 6.23, haplic greyzem – between 5.49 and 6.78, values close to neutral ones were detected in soils belonging to the types of haplic chernozems and luvic phaeozems. These results in whole coincide with the typical parameters of such soil types [25].

Organic carbon content also corresponds to the typical values for such types of soils. The lowest organic carbon content was found in samples P1 and P3 belonging to the type of eutric podzoluvisols (0.72 to 0.92%), and the highest – in samples Ph2 and Ch1 belonging to the type of luvic phaeozems and haplic chernozems (4.52 to 4.54%).

In all the samples the relation of alk-genes and the number of 16S rRNA gene copies was carried out using the qPCR method (Table 4).
Table 4. Percentages of $alkB$ genes copy numbers in proportion to 16S rRNA gene copy numbers.

| Sample | alkI/16S rRNA ratio (%) | alkII/16S rRNA ratio (%) | alkIII/16S rRNA ratio (%) |
|--------|--------------------------|--------------------------|---------------------------|
| P1     | 0.01                     | N/D                      | 0.80                      |
| P2     | 0.02                     | N/D                      | 0.47                      |
| P3     | N/D                      | N/D                      | 0.88                      |
| P4     | 0.02                     | N/D                      | 4.84                      |
| P5     | 0.05                     | N/D                      | 7.25                      |
| P6     | 0.05                     | N/D                      | 7.01                      |
| P7     | 0.04                     | N/D                      | 7.04                      |
| P8     | 0.04                     | N/D                      | 1.61                      |
| G1     | 0.01                     | N/D                      | 0.06                      |
| G2     | N/D                      | N/D                      | 0.06                      |
| G3     | N/D                      | N/D                      | 0.72                      |
| G4     | 0.01                     | N/D                      | 1.49                      |
| G5     | 0.01                     | N/D                      | 1.16                      |
| G6     | 0.02                     | N/D                      | 1.38                      |
| G7     | 0.01                     | N/D                      | 0.26                      |
| G8     | 0.04                     | N/D                      | 3.15                      |
| G9     | 0.04                     | N/D                      | 4.88                      |
| Ch1    | N/D                      | N/D                      | 0.92                      |
| Ch2    | 0.01                     | N/D                      | 0.68                      |
| Ch3    | N/D                      | N/D                      | 0.93                      |
| Ch4    | 0.02                     | N/D                      | 0.75                      |
| Ch5    | 0.05                     | N/D                      | 4.10                      |
| Ph1    | 0.07                     | N/D                      | 0.92                      |
| Ph2    | 0.02                     | N/D                      | 2.06                      |
| Ph3    | 0.06                     | N/D                      | 6.58                      |

$^a$ – not detected

Relative abundance of genes belonging to group alk I varied in the range of 0.01 to 0.07% in samples P1 and Ph1, correspondingly. In samples P3, G2, G3, Ch1 and Ch3 genes of the group haven’t been detected.

Some authors note that the number of $alk$-genes grows, when the amount of organic matter grows, and the maximum variety of the community is observed in soils, where pH is close to neutral values [16,26]. Others note the wide-spread occurrence of alkane degrading bacteria in acid soils with low organic matter content [11]. However, in our case, the influence of agrochemical properties of soil on the occurrence of $alk$-genes belonging to group one (alk I), associated with $alkB$ gene from *Pseudomonas putida* and *Stenotrophomonas* spp. hasn’t been detected ($R^2=0.23$). Absence of significant relation can be connected with a narrow range of differences in agrochemical characteristics of the soils sampled.

Genes belonging to group two (alk II) being the gene of $alkM$ and the genus of *Acinetobacter* spp. haven’t been detected in the pollution-free samples under analysis. Similar results were obtained in the research realized by Luz (2004), where $alkM$ genes haven’t been found in pure soils, either [13].

$AlkB$ and $alkB1$ genes responsible for the synthesis of alkane-monoxygenases enzyme in group three (alk III) are more often found in the representatives of *Pseudomonas* spp. (*Ps. fluorescens*, *Ps. aeruginosa*), *Rhodococcus* spp., *Burkholderia* spp. and *Amycolatopsis* spp. genus. This group of genes
was the most highly represented in all the samples, being 10 to 100 times higher than the presence of alk I group. Relative abundance of alk genes varied from 0.06 to 7.25% in samples G1 and P5, correspondingly. The highest presence of genes belonging to this group was registered for the soil type of eutric podzoluvols and, the lowest – for the soil type of haplic greyzem.

The analysis of the data received hasn’t revealed any connection between the agrochemical characteristics of soil and the spread of that group of genes (R²=0.21). The abundance of genes of the group connected with the presence of certain representatives of Pseudomonas spp., Rhodococcus spp., Burkholderia spp., and Amycolatopsis spp. genus can be explained by the variety of the group presented and the wide occurrence of the carriers of such genes in soil [20,27,28]. For example, in many investigations it is registered that the representatives of Pseudomonas spp. and Rhodococcus spp. genus are widely spread ones in soil communities in cold and moderate climate zones [27–29].

It can be pointed out that the most numerous group is that of alk III genes, genes belonging to alk I group were found in 20 samples, and gene carriers of alkM (alk II group) haven’t been detected. Our results coincide with those received by Margesin et al. (2003) [20]. The authors point out that pure soil is characterized by low amounts of representatives of alk genes from Pseudomonas spp. and Acinetobacter spp., while alkB1 and alkB2 from Rhodococcus spp. are found in large quantities in pollution-free soils.

Conclusions

Thus, it has been discovered that the greatest amount of genes detected belongs to alk III group (alkB and alkB1), genes belonging to alk I group (alkB) were also found. Those genes belong to Pseudomonas spp., Rhodococcus spp., Burkholderia spp. and Amycolatopsis spp. genus. AlkM genes from Acinetobacter spp. have been detected in none of the 25 samples. No relation between the characteristics of soil (acidity and organic carbon content) and the distribution of genes responsible for alkane degradation was revealed either.

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