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CHARACTERIZATION OF THE *RHODOCOCCUS* SP. MK1 STRAIN AND ITS PILOT APPLICATION FOR BIOREMEDIATION OF DIESEL OIL-CONTAMINATED SOIL

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Petroleum hydrocarbons and derivatives are widespread contaminants in both aquifers and soil, their elimination is in the primary focus of environmental studies. Microorganisms are key components in biological removal of pollutants. Strains capable to utilize hydrocarbons usually appear at the contaminated sites, but their metabolic activities are often restricted by the lack of nutrients and/or they can only utilize one or two components of a mixture. We isolated a novel *Rhodococcus* sp. MK1 strain capable to degrade the components of diesel oil simultaneously. The draft genome of the strain was determined and besides the chromosome, the presence of one plasmid could be revealed. Numerous routes for oxidation of aliphatic and aromatic compounds were identified. The strain was tested in *ex situ* applications aiming to compare alternative solutions for microbial degradation of hydrocarbons. The results of bioaugmentation and biostimulation experiments clearly demonstrated that – in certain cases – the indigenous microbial community could be exploited for bioremediation of oil-contaminated soils. Biostimulation seems to be efficient for removal of aged contaminations at lower concentration range, whereas bioaugmentation is necessary for the treatment of freshly and highly polluted sites.

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Introduction

Diesel oil consists of various hydrocarbons that have serious toxic effects on living beings [1]. Among the numerous available strategies for removal of hazardous pollutants, the bioremediation methodologies [2, 3] are environmentally sound, reliable, cost-effective, and widely applicable approaches in certain concentration range of contaminants [4–7]. Several types of biotreatments [8] are known, such as composting [9], biopiling [10], biosparging, landfarming, or bioreactor-based conversion of contaminants [1, 5, 6, 11]. Depending on the nature and the level of the pollution, either *ex situ* or *in situ* treatment can be applied [6]. At and above a certain concentration, the contaminants may become toxic to the organisms, thus the biological approach could not be effective. Application of immobilized cells may lead to a rapid and complete biodegradation owing to the protection of cells against toxic environment by the immobilization matrices [12, 13].

The indigenous microorganisms in soils must adapt to the rapidly changing environmental conditions. Some of them might be able to utilize the newly appearing organic compounds provided that the micronutrients are available [14, 15]. The biostimulation might enhance the natural biotic decomposition by supplementing nutrients and other relevant materials [16, 17], e.g., the addition of biosurfactants to the contaminated sites [18]. However, in many cases, other methods improving the biodegradation efficacy should be included [19]. In such cases, bioaugmentation using preadapted microorganisms might be used to promote the bioconversion rate and yield [5, 14, 20, 21]. An alternative strategy might be the usage of a remediated soil as an "inoculums," since it might contain hydrocarbon-adapted microorganisms for proper biodegradation [22]. However, only a few studies were published on the bioremediation of freshly contaminated site [16] and limited information is available about the usage of previously bioremediated soil to enhance the degradation of hydrocarbons [22].

Bioremediation of hydrocarbon-contaminated sites has been in the focus of numerous studies because of frequent occurrence of oil catastrophes [23] and the daily emitted commercial oily wastes. Both aliphatic and aromatic hydrocarbons can be degraded under either aerobic or anaerobic conditions [6]. The aerobic degradation is more efficient. Most of the hydrocarbons are water-insoluble, which is one of the main reasons that are not easily metabolizable for microorganisms. A frequent mode to enhance the availability of hydrophobic organic compounds is the application of surfactants, which might also be produced by microorganisms [24, 25].

Several species of the genus *Rhodococcus* were applied in a number of environmental and industrial biotechnology studies because of their ability to degrade a wide range of chemicals [26–30]. Rhodococci are naturally widespread in various contaminated environments due to their wide range of enzymes, surfactants producing capability, and tolerance against several environmental conditions, so they might be commonly used for rehabilitation of soil and aquatic habitats. The presence of exopolysaccharides and mycolates in the cell wall of rhodococci might promote the degradation of pollutants by emulsification of the substrates and protecting the cells [27, 31].

In this study, we present the characterization and draft genome sequence of a novel *Rhodococcus* sp. MK1 isolate capable to degrade various types of hydrocarbons in diesel oil. The strain was also tested in comparative biostimulation and bioaugmentation experiments performed in laboratory and on field.

Materials and Methods

Chemicals and contaminated soils

Summer diesel oil was provided by MOL Group (Hungary), analytical grade dichloromethane (DCM) (>99.8% purity) was purchased from Sigma-Aldrich. Na-alginate HF 250 was obtained from Protanal (Norway). All other chemicals were derived from standard commercial suppliers (Reanal, VWR International, Merck, Biolab).

The diesel oil-contaminated soil was collected from a contaminated area close to Szeged, Hungary. The contamination had stably existed there for decades (since 1980s). Prior to bioremediation, the total hydrocarbon concentration was 670 mg/kg soil. "Freshly" contaminated soil was modeled by artificial addition of diesel oil to soil up to total petroleum hydrocarbon (TPH) = 3,480 mg/kg. It was measured after contamination.

Characterization of the hydrocarbon-degrading strain

The *Rhodococcus* sp. MK1 strain was isolated from an industrial waste of a chemical company (Nitrokémia Corp., Balatonfűzfő, Hungary) using selective medium containing 1% (v/v) diesel oil as carbon source.

Morphological, chemotaxonomical, physiological, and biochemical characterization of the strain was performed. The morphological and chemotaxonomic marker analyses [the fatty acid, mycolic acid patterns were determined by Deutsche Sammlung von Mikroorganismen und Zellkulturen GmbH (DSMZ)]

were investigated after the cells had been grown on Tryptone Soya agar (15 g/L tryptone, 5 g/L soytone, 5 g/L sodium chloride, and 15 g/L bacto agar). Typical biochemical and physiological characteristics of the strain MK1 were determined by the following assays: oxidase-; catalase tests; urease activity; gelatine-, casein-, Tween 80-, starch hydrolysis tests; nitrate-, nitrite reduction tests, indole production, and methyl-red reactions performed according to Cowan et al. [32]. The growth characteristics were tested in Luria-Bertani broth (LB) medium at different temperatures (4, 10, 15, 20, 25, 30, 35, 37, 40, and 42 °C), at different pH (5.0, 6.0, 7.0, and 8.0), and the salt tolerance [1.0%–5.0% (w/v) NaCl] was determined at 24 °C for 24 h.

Growth conditions and inoculum preparation

The strain MK1 was grown in 20 ml LB medium (10 g/L tryptone, 5 g/L yeast extract, and 10 g/L NaCl) at 24 °C overnight shaking at 150 rpm. Then, the culture was centrifuged (20,200 rcf for 10 min), and the pellet was suspended to an optical density of 1.0 at 600 nm in physiological salt solution (9 g/L NaCl). This inoculum was used in subsequent biodegradation experiments.

DNA isolation

Genomic DNA was isolated with the standard phenol–chloroform method [33] with small modifications. Briefly, cells were cultivated in LB medium overnight. To weaken the cell wall, grown cultures were diluted to $OD_{600} = 0.700$ and were supplemented with 1 mg/ml ampicillin as it was described earlier by Nagy et al. [34]. Cultures were agitated for 2 h at room temperature in the presence of ampicillin. Pellet of the culture was treated with 50 mg/ml lysozyme at 37 °C. Cells were resuspended in Genomic I. solution (10 mM NaCl, 2 mM Tris HCl pH = 8.0, 1 mM EDTA, and 0.5% SDS) and mixed on a Biosan RS-60 rotary shaker (Biosan, Riga, Latvia) for an hour. After proteinase K and RNase A treatment, the mixture was treated five times with 1:1 phenol:chloroform then precipitated with ice–cold 90% (v/v) ethanol in the presence of 300 mM sodium acetate and washed with 70% (v/v) ethanol. Purified DNA was dissolved in molecular biology grade water and stored at −20 °C until use.

Sequencing gDNA

Genomic DNA was sheared by nebulization according to Roche (Rapid Library Preparation Manual GS FLX+/XL+ version: May 2011). DNA library

was prepared using the TruSeq DNA PCR-Free LT Library Preparation Kit (Illumina Inc., San Diego, CA, USA) following the instructions of the manufacturer. Paired-end sequencing was carried out on Illumina MiSeq bench top sequencing platform with MiSeq reagent kit v3 (2×300 cycles).

Bioinformatics

Sequencing data obtained in FASTQ format were processed with MIRA 4 assembler [35] running in Debian 7.10 operation system. Quality filtering of reads was automatically performed by the assembler with default options. Read mappings and QC reports were generated by CLC Genomic Workbench 7.5 (QIAGEN Arhus A/S, Arhus, Denmark). All mappings were performed by applying the default parameters. Annotation of the assembled sequences was carried out by the RAST program (version 2.0) [36] with default settings and the classic RAST annotation scheme. Basic Local Alignment Search Tool (https://blast.ncbi.nlm.nih.gov/Blast.cgi) was used for sequence database search.

Deposition of genome-sequencing data

This whole genome shotgun project has been deposited at DDBJ/ENA/GenBank under the accession MUBD00000000. The version described in this paper is version MUBD01000000.

Diesel oil degradation in liquid phase and soil at laboratory scale

The hydrocarbon-degrading capability of the MK1 strain was tested in mineral medium (MM) described in [37] and soil. Diesel oil (1 ml diesel oil was used with 100 ml MM or 10 g soil, and there was no other carbon sources) containing MM (20 ml) or soil (50 g, sterilized for 1 h, at 100 °C) was inoculated with 1% (v/v) starter culture of the MK1 strain in gas tight serum bottles (volume 50 ml). The soil water content was adjusted to 20%.

Soil dry matter and water content

The weight of soil samples was measured and they were dried in a baker at 105 °C until constant weight. The 20% (w/w) water content means 20% loss in weight during the drying process [38].

Experiments with immobilized/entrapped cells

The diesel oil biodegradation efficiency of immobilized cells was also tested in aqueous phase. The method of immobilization into Na-alginate HF 250 was described earlier by Perei et al. [37]. The ratio of 1.5% (w/v) Na-alginate solution and cell suspension (10^6 cell/ml) was 5:1. Finally, 20 ml of hydrocarbon containing MM was inoculated with 1 g (wet weight) of immobilized cells. The cultures were incubated at room temperature with shaking at 150 rpm for a week. The hydrocarbon content of the samples was extracted with DCM (the volume ratio of the sample and DCM was 1:1). The analysis of the extracts was performed by a gas chromatograph-coupled mass spectrometry (GC-MS) (see below).

Inocula preparation for bioaugmentation processes

For the field experiments, the MK1 strain was grown in 5 L fermenters to get large amount of inoculum for bioaugmentation processes. The strain was grown up in LB medium at 25 °C for 1 day. The viable cell counts (CFU) were established by plating. The cultures were centrifuged at 13,000 rpm for 10 min and the pellets were resuspended with proper amount of MM to reach the optical density of 1.0 at 600 nm.

Field experiment

The field experiment was performed as follows: a 25 m × 35 m working area was covered with PVC foil for preventing the leakage of hydrocarbons. Five piles (each was 2 m^3) were created (Table I) from old contaminated soil previously mixed with 5 g/kg soil wheat-straw (to lose the soil structure) using a MLT731

Table I. Experimental strategy for comparison of the *on site* biostimulation and bioaugmentation approaches

	Old contamination	Old + artificial contamination	Bioaugmentation	
Piles	TPH = 670 mg/kg	TPH = 3,480 mg/kg	MK1 strain	Biostimulation
1	+	−	−	−
2	+	−	−	+
3	+	+	−	+
4	+	−	+	+
5	+	+	+	+

Note: +: treated; −: untreated; TPH: total petroleum hydrocarbon.

Manitou compact loader. The distance of the neighboring piles was 30 cm. Holes of 30–35 cm depth and of 5 cm diameter were drilled at 20 cm regular distance at the top of the mounds. Diesel oil was added to the piles via these holes (Piles 3, 5). The experimental setup for the piles is presented in Table I. To achieve natural water content (about 20%), all piles were irrigated with tap water. An amount of 21 L of mineral salt solution (MM) was added to the Piles 2–5 for modeling the biostimulation process. The Piles 4 and 5 were inoculated with 5×10^8 CFU ml^{-1} MK1, which were previously suspended in 21 L MM (5×10^8 cell/ml final concentration).

Determination of the hydrocarbon content of soil

The samples were taken from the piles by 3 weeks in a total 9-week period. Each sample was a mixture of 100–100 g of soils collected from three different areas/depth of a pile. This step was critical because of the heterogeneity of contamination [39]. About 10 g soil from each homogenized sample was used for the determination of hydrocarbon content in three replicates. The hydrocarbons were extracted with DCM [1:1 (w:v) ratio] and 1 µl from the organic phase was injected into a gas chromatograph. The hydrocarbon concentrations were calculated from the peak areas. The analytical procedure was validated by comparing the theoretical and experimentally measured hydrocarbon concentrations in soils freshly contaminated with diesel oil (the relationship was linear with the range of 0–10.000 mg/kg range with a square of the correlation coefficient: $r^2 = 0.9988$). Each sample was measured in triplicates.

Gas chromatography (GC) analysis

Diesel oil contents of the samples were measured on Agilent 6890 GC (Agilent Technologies Inc., Santa Clara, CA, USA) coupled with an Agilent 5975 C EI-simple quadrupole mass spectrometer. GC was equipped with 30 m × 250 µm × 25 µm HP-5MS capillary column (Agilent Technologies Inc., Cat. No.: 19091S-433). Samples of 1 µl volume were injected with an Agilent 7683 B autosampler unit. Split ratio was set to 15:1 and the column flow rate was 1.2 ml/min. Oven program was as follows: initial temperature was set to 65 °C and held for 4 min subsequently increasing the temperature until 290 °C by 15 °C/min. Final temperature was kept for 5 min. Ionization energy was 70 eV. Data were obtained with single ion monitoring following the most abundant ions in the diesel oil main components (m/z = 57 and 71). Samples were quantified by considering summed peak area of the chromatogram.

The respiration activities were determined according to the protocol described in [51].

Data analysis

Each experiment was repeated thrice. The bioconversion percentage of diesel oil degradation was calculated by the following expression:

$$[(\text{Control(cell free)} - \text{Treated})/\text{Control(cell free)}] \times 100.$$

Results and Discussion

Strain characterization

The MK1 strain is a Gram-positive, aerobic bacterium with oxidase-, urease-, catalase- and protease activity; starch was not hydrolyzed and there was no sign of nitrate-, nitrite-reduction activity, acid-fast. The MK1 strain was grown at temperatures ranging from 4 to 33 °C; however, the optimal growth was observed at 25 °C. The cells could tolerate 5% (w/v) salt concentration. The pH range for growth was 6.0–8.0, but optimum was at pH = 7.0. The major fatty acids (1%–5%) of the cells were $C_{16:0}$ (>30%), $C_{16:1}$ (15%–30%), $C_{18:0}$ (15%–30%), $C_{18:1}$ (>30%), and 18-Me (>30%). One of the main characteristics for rhodococci is the mycolic acid content. In the MK1 strain, mycolic acids were built up with 32–44 carbon atoms in our isolate. The DNA G + C content of the MK1 strain was 63 mol %. According to the classical taxonomical classification, the strain was identified as *Rhodococcus erythropolis* MK1 (DSMZ GmbH).

Sequencing *Rhodococcus* sp. MK1 genome

The whole genome sequencing of the MK1 strain resulted in 978,788 paired-end reads. About 97.35% of the reads were between 299–301 bp in length. About 96.75% of the reads had an average Phred score above 20 and the ratio of ambiguous base containing sequences was 0.07%.

The genome sequence of the strain has been deposited in the DDBJ/ENA/GenBank. The genome comparisons revealed that the closest reference genome was the *R. erythropolis* CCM2595 (Bioproject PRJNA81583) genome with 87.3% symmetrical identity value (SIV), the closest complete genome was the genome of

Rhodococcus sp. BH4 (Bioproject: PRJNA313101, 88.7% SIV), whereas the closest draft genome was that of *Rhodococcus qingshengii* CW25 (Bioproject: PRJNA316739, 94.4% SIV). According to these comparisons, the GenBank classified our strain as *R. qingshengii* MK1. Still, there is no fully assembled *R. qingshengii* genome available. Nevertheless, the reads of the MK1 were mapped onto the *R. qingshengii* CW25 and *R. qingshengii* CS98 contigs and 92.8% and 90.0% of the reads could be mapped onto their contigs, respectively.

However, four fully sequenced genomes of *R. erythropolis* are available in the database [40–43]. *R. erythropolis* CCM2595 has one, the other three strains (BG43, R138, and PR4) possess three plasmids of 3.7–478 kb size (Table II). Moreover, three other plasmids have been fully sequenced (without the chromosome) [44–46]. Mapping the reads onto the existing genomes resulted 30 times average coverage and 87%–88% of the reads were mapped onto all chromosomes. This is in concordance with our presumption of close relationship between MK1 and the other four strains. On the other hand, less than 0.6% of the reads could be mapped onto the 12 plasmid sequences. Harboring multiple plasmids of a variety of size in either linear or circular form in within the same cell is a common feature among rhodococci (Table II) [40, 47].

The assembly of the reads of the MK1 genome with MIRA 4 resulted in 40 contigs, the total size of the contigs was 6,469,205 bp. GC content was 62.5%.

Table II. Mapping statistics of the reads derived from the genome sequencing of the MK1 strain onto the other known *R. erythropolis* genetic elements derived from complete genome sequencing

Organism	Molecule name	Type	Length (bp)	Reads mapped (%)
R. erythropolis CCM2595		Chromosome	6,281,198	87.43
	pRECF1	Plasmid	90,223	0.33
R. erythropolis BG43	–	Chromosome	6,334,075	87.81
	pRLCBG43	Plasmid	240,129	0.19
	pRLLBG43	Plasmid	261,537	0.59
	pRSLBG43	Plasmid	29,464	0
R. erythropolis R138	–	Chromosome	6,236,682	87.45
	pCRE138	Plasmid	477,915	0.56
	pLRE138	Plasmid	91,729	0.16
R. erythropolis PR4		Chromosome	6,516,310	87.27
	pREC1	Plasmid	104,014	0.09
	pREC2	Plasmid	3,637	0
	pREL1	Plasmid	271,577	0.23
R. erythropolis (other strains)	pDB2	Plasmid	210,205	0.15
	pRE8424	Plasmid	5,987	0
	pFAJ2600	Plasmid	5,936	0

Based on RAST results, 6,252 open reading frames (ORF) were identified in the assembled genomes. About 36% of the annotated features could be metabolically classified by RAST. The metabolic distribution of the genes is shown in Figure 1.

We mapped the reads that are not matching to the reference genomes on each individual contig. Out of these, 18.31% of the reads were mapped on a 105,331-bp long sequence (contig 20) and the read numbers were evenly distributed along the sequence. Average coverage was 46.3. BLASTn analyses against the non-redundant nucleotide database revealed that this contig shares partial sequence identity with distinct rhodococcus plasmids. The annotation process disclosed 118 ORFs coding DNA-modifying enzymes, integrase, and mobile elements, which are common among *Rhodococcus* plasmids. It also has its own *parAB* genes, which are responsible for plasmid partitioning. Based on these results, we assume that contig 20 can be a novel *Rhodococcus* plasmid. Around 58% of the ORFs are hypothetical; however, contig 20 contains genes encoding mercury and cadmium-resistance proteins, glucoamylase, glucose-6-phosphate dehydrogenase, and high-affinity carbon uptake protein, as well. In addition, it does not contain any genes coding for hydrocarbon biodegradation, all of these enzymes seem to be localized on the chromosome.

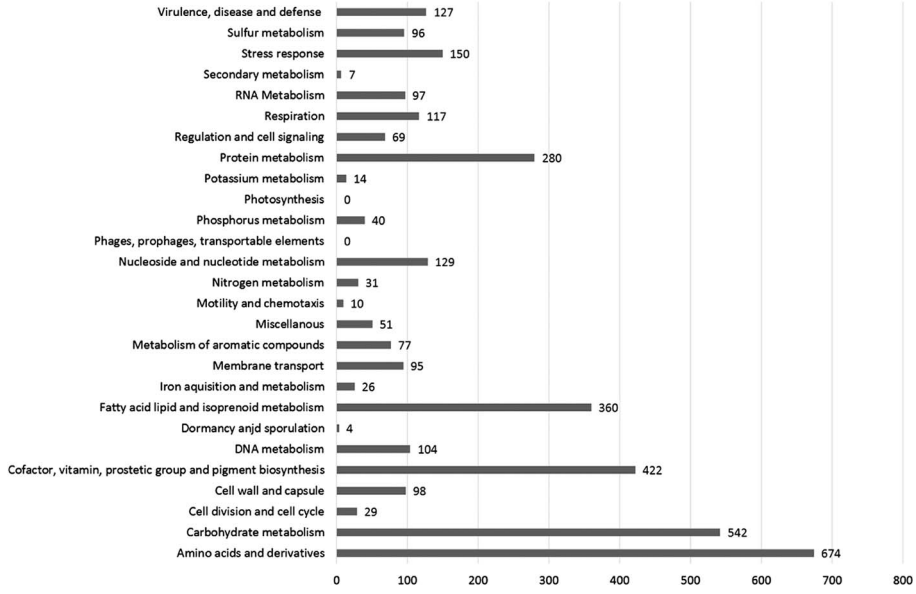

Figure 1. Features in subsystems in *Rhodococcus* sp. MK1. The numbers on the figure represent the number of genes involved in the corresponding metabolic pathways

Genes involved in hydrocarbon oxidation

The major components of diesel oil are alkanes. Oxidation of these compounds is the key step of their bioconversion. Bacteria have evolved a number of oxygenase enzymes catalyzing this crucial step on alkanes of various chain lengths [48].

In the genome of the MK1 strain, five genes coding for alkane-1-monooxygenase (AlkB) enzymes have been found. These are non-hem iron proteins integrated into the cell membrane. Usually, AlkB enzymes use rubredoxin as electron carrier in the oxidation reaction, but in our case, only two *AlkB* genes are associated with rubredoxin and *tetR* type regulator genes. The latter might act as transcriptional regulator. Rubredoxin reductase is present only in one *AlkB* gene cluster. In our recent study, we have shown that only those *AlkB* genes have highly elevated transcription in the presence of alkanes, which have rubredoxin and *tetR* regulator genes in their proximity in *R. erythropolis* PR4 [49], thus these can be the key enzymes in medium chain alkane oxidation.

In *R. erythropolis* PR4, there are two paralogues of *cyp153* genes encoding cytochrome P450 enzymes, which are responsible for oxidation of short chain alkanes [40, 48–50]. On the contrary, the MK1 strain does not possess a copy of the gene, since it lacks the pREL1-type plasmid, which encodes the *cyp153* genes. CYP153 enzymes are usually responsible for oxidation of short chain alkanes. Apparently, the strain MK1 could utilize short chain alkanes only in the presence of longer alkanes (data not shown), which might indicate the role of other enzymes, such as AlkB [48]. There are 11 other cytochrome P450 proteins encoded in the MK1 genome alongside numerous other genes coding for oxygenase likely catalyzing the oxidation of a variety of aliphatic and aromatic compounds.

Based on RAST SEED viewer, there are 77 genes belonging to the subsystem for aromatic compound metabolism (Figure 1). Twelve genes participate in the peripheral and 57 genes in the central catabolic pathway. Eight genes are related to the metabolism of aromatics but cannot be classified in any of the two subcategories above.

Biodegradation of diesel oil in liquid phase and soil under laboratory conditions

In the preliminary study, the diesel oil degradation activity of the MK1 strain was tested by monitoring of cell respiration activity in liquid culture for 1 week [51]. Respiration of the cells was intensive, almost all of the available oxygen was consumed and large amount of carbon dioxide was produced. These results and genome data suggest that the MK1 strain can efficiently convert at least one component of diesel oil contaminations.

The diesel oil concentration was followed by GC-MS to evaluate the hydrocarbon consumption. In liquid phase, both free and immobilized cells were applied. Without immobilization, almost 70% of the diesel oil was removed by cells as compared with cell-free control samples, while a lower degradation rate was obtained in the case of immobilized cells (Figure 2). This latter result might be surprising, since the immobilization of cells is expected to have a protective effect, which usually improves the bioremediation efficacy. Presumably, the cell wall-bound surfactants of the strain MK1 entrapped were hardly able to interact with the hydrophobic components floating on water surface leading to a reduction in hydrocarbon degradation. This coincides with the observation, that in a biphasic liquid (oil – water after stopping the shaking) culture, the cells were strongly attached to the upper organic phase, whereas the aqueous phase remained clear (data not shown).

A spectacular change in practically all peak height could be observed after 1 week of cell growth on diesel oil (Figure 3A and B). Apparently, the height (area) of all peaks decreased simultaneously, indicating the broad substrate specificity of the strain. Four chemically distinct components of diesel oil (xylene, n-nonane, n-hexadecane and 2,6,10-trimethyltetradecane, indicated by arrows in Figure 3A and B) were chosen and their biodegradation rate was quantified (Figure 4). The bioconversion rates were calculated from the consumption of substrates related to their initial concentrations. It can be clearly seen that each compound could be converted by the strain MK1, their quantities were dropped to 30%–40% of the initial concentration in a week. This feature is an important advantage in a comparison with *Alcanivorax borkumensis*, which is considered to be one of the main oil degraders, however, it predominantly degrades n-alkanes only [52, 53].

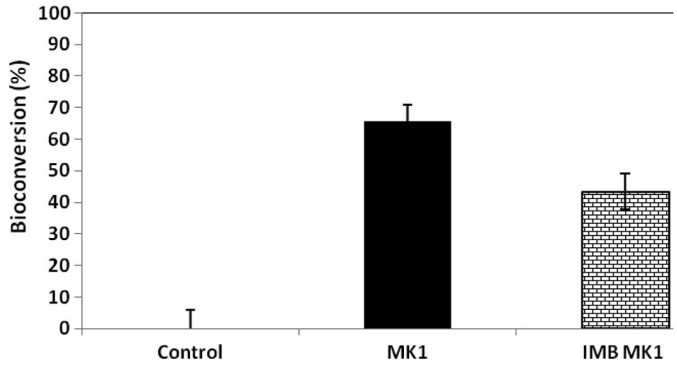

Figure 2. Bioconversion (%) of diesel oil in minimal medium using free and entrapped cells. Control: cell-free, MK1: sample containing freely moveable cells, IMB MK1: immobilized MK1

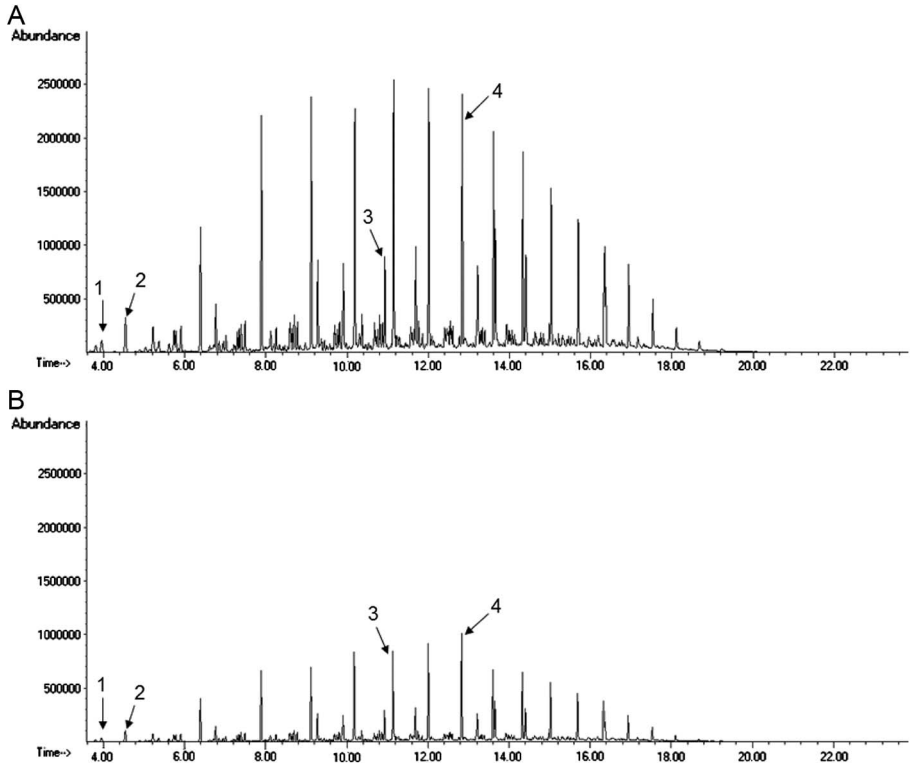

Figure 3. Chromatogram of cell free control (A) and MK1 culture (B) grown on diesel oil. The arrows indicate the following components, which were further analyzed (see Figure 4). 1: p-xylene (RT: 3.96 min), 2: n-nonane (RT: 4.54 min), 3: 2,6,10-trimethyltetradecane (RT: 11.69 min), and 4: n-hexadecane (RT: 12.83 min)

The hydrocarbon-degrading activity of the MK1 strain was also characterized in contaminated soil under laboratory conditions. The soil was supplemented with 1% (w/v) diesel oil; the biodegradation was monitored for 1 week by measuring the TPH concentrations. The efficacy of diesel oil biodegradation in soil (66%) was similar to the values obtained in the liquid culture.

Field application

To test our strain in real *ex situ* bioremediation technology, biopiles were constructed using "old" and artificially polluted soil. The experimental design can be seen in Table I. The Piles 1, 2, and 4 were heaped from old contaminated soils. The diesel oil was released into the field more than 20 years ago and its

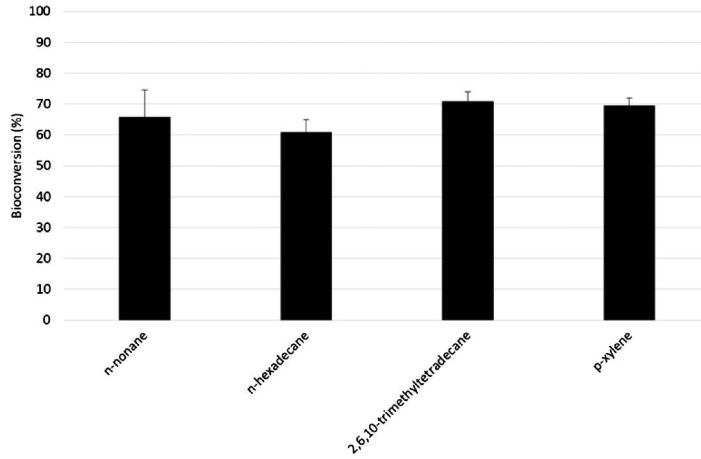

Figure 4. The bioconversion of individual compounds indicated in Figure 3

concentration was 671 mg/kg in the soil (old contaminated soil) at the beginning of the experiments. Piles 3 and 5 heaped from old contaminated soil polluted additionally with "fresh" diesel oil (referred as artificially or freshly contaminated soil), where the concentration of the pollutant was five times higher (3,480 mg/kg soil) than in the old contaminated ones. These piles will be referred as freshly contaminated sites.

Neither nutrients nor MK1 was added to the control pile (Pile 1). The indigenous microflora was stimulated using mineral solution (MM) in the biostimulated piles (Piles 2–5). The strain MK1 was inoculated into the bioaugmented piles (Piles 4 and 5). Biodegradation processes were followed by measuring hydrocarbon concentration.

There were no changes in the control pile (Figure 5A, Pile 1) in 9 weeks. However, the quantity of the hydrocarbons rapidly decreased in the biostimulated pile (Figure 5A, Pile 2) containing exclusively old contamination. It might be concluded that the contaminated soil might have been depleted in components essential for microbial life, since the microbial activity could be simply stimulated by supplementing the Pile 2 with minerals. Comparing the results of the biostimulation (Figure 5A, Pile 2) and bioaugmentation (Figure 5A, Pile 4) experiments, no significant differences between the degradation rates in Piles 2 and 4 could be observed. According to these results, it is presumable that inactive local microflora has accumulated at the old contamination, which could be activated by providing minerals. In other words, the MK1 strain could not further accelerate the degradation of aged pollutants in the concentration range applied.

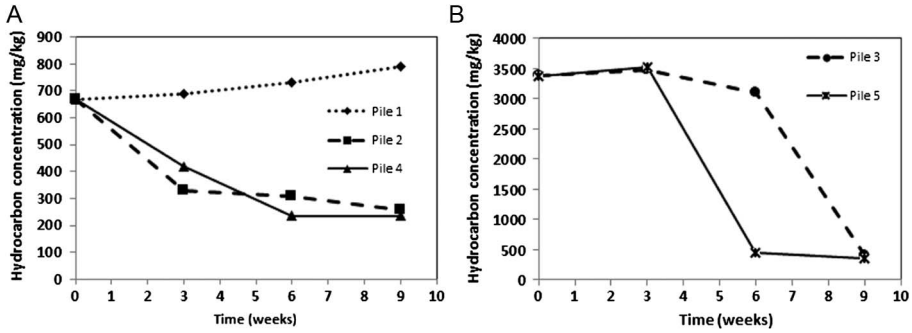

Figure 5. TPH in piles containing old (A) (TPH = 670 mg/kg) and fresh (B) (TPH = 3480 mg/kg) contamination during bioremediation. Pile 1: diamond, dotted (A); Pile 2: square, dashed (A); Pile 3: circle, dashed (B); Pile 4: triangle, continuous (A); Pile 5: asterisk continuous (B). The curves illustrate representative data of the experiments. In each case, in each sample, similar tendencies were observed

Two piles were used to model fresh contamination in field experiments (Figure 5B). The old contaminated soil was used to heap these piles (Piles 3, 5) that have been polluted artificially/freshly with diesel oil. Nutrients were added to all of these piles, while the Pile 5 was bioaugmented with the strain MK1.

The biostimulation and bioaugmentation approaches were compared for soils freshly polluted with diesel oil at much higher concentration. It can be seen in Figure 5 that the indigenous microorganisms in Pile 3 had a longer lag period in spite of the fact that the pile was supplemented with nutrients. Biodegradation of hydrocarbons was visible after 3 weeks in the case of the bioaugmented Pile 5. The degradation rate was apparently accelerated in the biostimulated pile (Pile 3) after 6 weeks. At the end of the experiment (9 weeks), the concentration of hydrocarbons was less than the original concentration of the old contamination in both piles. Consequently, the fresh contamination could be apparently removed in 3 months by stimulating local microorganisms; however, a much faster degradation could be achieved by applying bioaugmentation. Nevertheless, the final bioconversion yields were approximately the same for both approaches. Thus, biostimulation is cheaper while bioaugmentation is faster technique for bioremediation of fresh pollutants.

Conclusions

The strain MK1, isolated from an industrially contaminated site, was identified on the basis of morphological, chemotaxonomical, physiological, and biochemical properties as *R. erythropolis* MK1. Comparison of the draft genome

sequence of the MK1 strain to the genomes in the databases revealed the genome of *R. erythropolis* CCM2595 and *R. qingshengii* CW25 as the closest species reference genome and closest draft genome, respectively. Thus, the strain appears in the genome databases as *R. qingshengii* MK1. In the MK1 genome, numerous genes coding for enzymes likely participate in hydrocarbon biodegradation. Moreover, a novel extrachromosomal element was likely identified. The *R. erythropolis* MK1 proved to be very adaptive and it could efficiently decompose numerous components of diesel oil in liquid or soil phase at laboratory conditions.

Little is known about the hydrocarbon-degradation activity of the immobilized rhodococci. It was shown that entrapment of the strain MK1 led to reduced degradation ability indicating that the bacterium had cell wall-bound surfactant. The alginate layer prevented the direct connection of the cells to the hydrocarbons and in the absence of "mobile" surfactants, the substrates could not be efficiently emulsified.

Accidental oil leaks/spills are common during the transportation of fuels, which require a rapid and clean pollution removal process especially close to dwelling places.

The processes developed in laboratory sometimes do not work well in field under uncontrolled conditions. The data obtained from the comparison of *ex situ* bioaugmentation and biostimulation experiments suggested that the indigenous strains could be efficiently activated for bioremediation of oil contaminated soils in certain cases. Biostimulation might be sufficient for old contaminations, whereas bioaugmentation is necessary for freshly and highly polluted sites.

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Conflict of Interest

The authors declare no conflict of interest present in this work.

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