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Faculty of Science and Informatics

Ph.D. School in Biology

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Functional genomic analysis and biotechnological application of hydrocarboclastic *Rhodococcus erythropolis* strains

Ph.D. Thesis

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Background

In the last century, the industrial growth rate has been exponentially accelerated. Invention of the combustion engine was an important milestone in vehicle manufacturing. Utilization of crude oil derivatives in the transport sector has revolutionized traveling and transportation of goods which was fundamental driving force of the economic growth. Crude oil derivatives are still being widely used as fuel, lubricants and/or solvents.

Due to the fast development of technologies and increasing crude oil consumption, hydrocarbons and their derivatives also became potential threats for the environment. The ignition of oil-based fuels results in the emission of large amount of carbon dioxide having greenhouse effect. Moreover, accidents occurring during the extraction and transport of oil also have serious environmental impact [1]. It has been realized that crude oil derivatives - together with their all benefits – has represented enormous environmental risk, as well. Toxic components endanger both the nature and the health of people living in the area of the pollution.

Hydrocarbons are continuously released into the environment therefore, their elimination requires human intervention. In the last few decades, numerous methods were developed for the remediation of oil polluted sites. In contrast to physical and chemical methods, biological approaches are cheaper and environmentally sound solutions.

The microbial degradation of oil derivatives has already been recognized. The microbial contamination of oils is a serious problem for the petrochemical industry since biological activity can severely reduce the oil quality [2]. In the other hand, microbial hydrocarbon degradation is beneficial, since a number of hydrocarboclastic microbes are available for remediation of polluted sites.

Hydrocarbons can be degraded anaerobically or aerobically. The first step of the aerobic biodegradation is the oxidation of the hydrocarbon molecule. This reaction is catalysed by specific oxygenase enzymes. Based on the number of oxygen atoms incorporated into the substrate, oxygenases can be classified as mono- or dioxygenases. Both types of enzyme utilize molecular oxygen during the process. Alkanes, the major components of hydrocarbon fuels are oxidized mainly by monooxygenases [3].

Rhodococci can utilize a wide variety of environmental pollutants as carbon and energy source. Moreover, their remarkable adaptability, their compact hydrophobic cell wall and their surfactants make them excellent candidates for bioremediation purposes. These bacteria are able to utilize hydrocarbon derivatives efficiently [4–6], e.g. polychlorinated

biphenyls [7,8], persistent fungicides, such as carbendazim [9], or pesticides e.g. karbation [10].

In my work, two *R. erythropolis* strains were used as model organisms for studying the physiological and molecular biological background of hydrocarbon biodegradation. Deeper understanding of the ways how the strains utilize hydrocarbons might contribute to develop more efficient bioremediation technologies.

The *R. erythropolis* PR4 strain was isolated from the Pacific Ocean in 1000 m depth. The strain can utilize pristine as sole carbon source and it can convert other hydrocarbons, as well [4]. Furthermore, it was recently published that the strain could utilize other water insoluble pollutants e.g. animal fats, grease and plant oils [11]. The hydrophobicity of the *R. erythropolis* PR4 cell wall is usually increased in the presence of hydrocarbons for easier access to the organic molecule [12,13]. The genome of the strain has already been sequenced and it has been available since 2009. In addition to the chromosome, *R. erythropolis* PR4 harbours 3 plasmids; two of them are megaplasmids. The megaplasmids also contain genes coding for enzymes participating in hydrocarbon biodegradation and fatty acid metabolism [14]. Several gene products of *R. erythropolis* PR4 has already been characterized, for example, the industrially relevant multicopper oxidase [15] or a non-ribosomal peptide synthase having a role in siderophore biosynthesis [16].

The *R. erythropolis* MK1 strain is a soil bacterium which has been isolated by our group from industrial soil. It was identified by DSMZ taxonomical service based on 16S rDNA sequence and mycolic acid patterns. Similarly to PR4, *R. erythropolis* MK1 could efficiently utilize hydrophobic substrates in minimal medium [17]. However, the physiological and morphological properties of the two strains are apparently distinct. At the beginning of our work, the genome of *R. erythropolis* MK1 was not sequenced, therefore the *R. erythropolis* PR4 could be used as a reference genome and a model bacterium for the transcriptomic studies.

Methods

R. erythropolis strains were maintained on LB agar plates and cultivated in LB broth. Experiments were also carried out in minimal medium supplemented with various carbon sources (n-hexadecane, diesel oil, various kinds of hydrocarbon mixtures or sodium acetate) and salts when it was necessary. Soil experiments were carried out in commercial potting soil. For whole transcriptome analysis, cells were cultivated in bioreactors for optimal growth conditions.

Hydrocarbons were extracted from the samples by chloroform. Oxygen, CO_2 and hydrocarbon contents were followed by gas chromatography or gas chromatography coupled mass spectrometry. Statistical analysis of the data was carried out by student's t-test.

Genomic DNA was isolated according to the conventional phenol-chloroform extraction method. Whole genome sequencing was carried out by Illumina MiSeq next generation platform. *R. erythropolis* MK1 genome was assembled with MIRA 4 and CLC Genomic Workbanch 7.0. Automatic annotation of the assembled genome was carried out by the RAST annotation server.

For gene expression analysis, RNA was isolated with QiaGen RNeasy Mini Kit and a slightly modified protocol was used. Whole transcriptome analysis was carried out on SOLiD^{TM} next generation sequencing platform. Gene expression patterns were validated by RT-qPCR for a number of genes selected.

Results of the thesis

1) Two *R. erythropolis* strains, isolated from distinct environments: deep-sea water (PR4) and industrial soil (MK1), were studied and compared.
2) The two strains had different behaviours in the presence of hydrocarbons. Without shaking, the *R. erythropolis* PR4 emulsified the hexadecane in water while *R. erythropolis* MK1 formed biofilm on the n-hexadecane layer and grew on the interface of the two phases.
3) Light microscopic study of samples taken from shaken cultures revealed that both strains have grown on the surface of the n-hexadecane droplets but the shapes of the droplets were different. Oil droplets colonized by *R. erythropolis* PR4 had spherical shape, while droplets in the MK1 cultures were amorphous.
4) The *R. erythropolis* PR4 degraded 90 % of n-hexadecane in three days, while the strain MK1 decomposed only 50 % of the substrate in two weeks. The efficacy of biodegradation dropped under high salinity conditions for both strains. The strain PR4 reached 90% biodegradation yield only in the 7^{th} day, while strain MK1 was able to utilize only 31% of the n-hexadecane in 15 days. In potting soil, both strains had similar hydrocarbon degrading activity. Soil moisture influenced the efficacy of the degradation. At 50 % soil moisture, both strains reached 90-100% biodegradation yield. At 20 % and 30 % moistness the efficacy decreased to 60 %.
5) Basically, the hydrocarbons can be assimilated as cellular biomass or oxidized into CO_2. For both strains, approximately 2 % of the n-hexadecane was converted into carbon dioxide, while 98% of the substrate was utilized for biomass formation. The strains could utilize oxygen in various biochemical processes in similar ratios in soil: 70 % of the consumed oxygen is used for the monoterminal/subterminal oxidation of n-hexadecane, while 30 % is harnessed in other oxidative reactions. The *R. erythropolis* PR4 strain capitalized the oxygen in minimal medium similarly as in potting soil, however, the ratio mentioned above was shifted by high salinity. In salty medium, 89,5 % of the oxygen is used in the monooxygenase reaction. In contrast, this value is 90 % for *R. erythropolis* MK1 strain in both minimal and salty medium.
6) The two bacteria could distinctly grow on four gasoline fractions. The *R. erythropolis* MK1 could grow significantly slower on each fractions tested than the PR4 strain could. The growth rate of the MK1 is the slowest on the petroleum fraction, which can be interpreted by the absence of the *cyp153* genes (see below).

7) We performed a whole genome sequencing of *R. erythropolis* MK1 on Illumina MiSeq next generation sequencing platform, which yielded long paired end reads suitable for *de novo* assembly. The genome was assembled into 40 contigs and 6 252 orfs could be identified by RAST. The chromosomes of the two strains substantially resembled, however, no sequences similar to the plasmids harboured by the strain PR4 could be detected in the *R. erythropolis* MK1 genome.
8) Both genomes contain monooxygenase genes coding for proteins likely involved in the oxidation of hydrocarbons. The alkane monooxygenases (AlkB) are one of the most important enzymes in alkane biodegradation. There are 4 and 5 *alkB* genes in the PR4 and the MK1 genome, respectively.
9) Based on phylogenetic analysis, *alkB3* and *alkB4* are close to eachother, while the other two *alkB* genes are on distinct branches of the phylogenetic tree. This suggests the possibility of horizontal gene transfer of *alkB* genes.
10) The other enzyme group participating in hydrocarbon oxidation is the cytochrome P450 monooxygenase superfamily. The strain PR4 harbours 16, while strain MK1 posseses only 11 *cyp* genes. Two *cyp153*-type cytochrome P450 monooxygenase genes are localized on the large linear plasmid of *R. erythropolis* PR4. Sequencing data revealed that these genes are absent in the genome of *R. erythropolis* MK1.
11) In the *R. erythropolis* MK1 genome, contig 20 is likely a megaplasmid which does not resemble any of known rhodococcal plasmids.
12) The *R. erythropolis* PR4 was grown on hexadecane, diesel oil and acetate in bioreactor and whole cell transcriptome analyses of these cultures were performed. The expression changes were validated by RT-qPCR for numerous genes selected. Moreover, artificial hydrocarbon mixtures containing linear, branched and cyclic hydrocarbons were established and the expression levels of selected oxygenase genes were monitored in cells grown on such substrates.
13) As total, the expression level of 195 genes changed by more than three times in the presence of hexadecane and/or diesel oil as compared to the values obtained with acetate.
14) 26 oxygenase genes had elevated mRNA level in the presence of diesel oil and n-hexadecane. The *alkB1* had the highest expression level among monooxygenases on both carbon sources. Furthermore, the *alkB2* was substantially upregulated in the presence of hydrocarbons. The *alkB3* and *alkB4* genes were not induced on any hydrocarbon carbon sources [18].

15) The transcription of 8 *cyp* genes was upregulated in the presence of diesel oil. Transcript analysis of cells grown on artificial hydrocarbon mixtures revealed, that only the transcription of one *cyp* gene (*cyp153*) was induced by n-hexadecane, while the expression of 5 *cyp* genes was upregulated in the presence of aromatics and cycloalkanes. These five genes can be involved in the oxidation of aromatic and/or aliphatic rings [18].
16) A putative monooxygenase gene (RER_07440) had stronger expression in the presence of aromatics and cycloalkanes as compared to values obtained with hexadecane and branched chain alkanes. We concluded that this monooxygenase is also involved in the oxidation of aromatics and cycloalkanes [18].
17) Among the genes involved in fatty acid metabolism, the expression of those was upregulated which were involved in the β-oxidation. The transcription of the gene coding for the FASI enzyme, being responsible for fatty acid biosynthesis, was extremely downregulated in the presence of hydrocarbons. The expression level of FASII enzyme system and the polyketide synthase 13 was not changed in the presence of hydrocarbons [18].
18) We observed a transcriptional upregulation of the genes involved in exopolysaccharide biosynthesis in cells grown on all examined hydrocarbon carbon sources. The gene expression was 4-10 times higher in the presence of diesel oil and the artificial mixture containing aromatics and cycloalkanes than in the presence of linear/branched alkanes. These results can be explained by the higher toxicity of the aromatic, cycloalkane components [18].
19) In addition, the genes involved in iron transport and siderophore biosynthesis were also upregulated in the presence of hydrocarbons [18].
20) The whole cell trancript profiling disclosed global metabolic responses of the cells exposed to various hydrocarbons including linear, branched, cyclic, aromatic compounds and their mixtures. Beside well-known elements, novel components having a role in these processes were identified. It has been demonstrated that – in contrast to the "famous" oil degraders – *R. erythropolis* PR4 strain was able to catabolize various hydrocarbons (from linear to polyaromatic compounds) simultaneously, without substrate preference [18].

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