Investigation of aromatic hydrocarbon degrading microbial communities based upon the functional genes

Doctoral (Ph.D.) theses

András Táncsics

Eötvös Loránd University, Faculty of Science

Biology Doctoral School

Theoretical and evolutionary biology

Leader of the Doctoral School: Dr. Anna Erdei

Program leader: Dr. Eörs Szathmáry

Supervisor: Dr. Károly Márialigeti, head of department, associate professor

Department of Microbiology, Eötvös Loránd University

Budapest, 2009

INTRODUCTION

The microbial degradation of crude oil and oil compounds, like aromatic hydrocarbons, was first observed at the beginning of the 20th century. Several bacteria and fungi were found to use these xenobiotic compounds as sole source of carbon and energy. In 1946 Claude E. ZoBell was the first who summarized the knowledge about microbial degradation of hydrocarbons, and he revealed that hydrocarbon-degrading microbes are widely distributed in nature. He concluded that the biodegradation of hydrocarbons depends on the physical and chemical properties of the compounds in question moreover, the role of the environmental conditions were also recognized.

Because of the motorization and the quick growth of oil industry the oil drilling spread around the world, including the arctic regions as well. Due to this a vast amount of oil has emerged into the nature destroying untouched wildlife ecosystems. Recognizing this demolition increased investigation of microbial hydrocarbon degradation began at the second half of the 20th century. Since than several hydrocarbon-metabolic pathways and the key enzymes, involved in these processes, have been described. The number of known hydrocarbon-degrading microbes is still increasing, and their diversity in pristine and contaminated ecosystems is still under examination.

Why is it necessary after all to investigate these microorganisms and to reveal their metabolic capabilities? A significant part of existing microbial species has not been cultured yet, therefore their role in the microbial communities is not known. During the investigation of microbial communities of aromatic hydrocarbon contaminated environments with molecular methods it often occurs that the majority of detected microbes are uncultered and, there is no information about their metabolic features and their role in the community. In order to develop more effective and low-cost techniques for the bioremediation of hydrocarbon contaminated environments, it is crucial to be able to monitor the alteration of composition of microbial communities involved in the degradation of contaminants and, the changes of metabolic diversity. In my study i made an attempt to detect and monitor those functional genes, which play a key role in the degradation of monoaromatic hydrocarbons, in order to get a broader view about processes take part in aromatic hydrocarbon-degrading microbial communities.

AIMS

Nowadays analysis of the microbial communities of aromatic hydrocarbon contaminated soil and groundwater samples is based upon the detection and investigation of 16S rRNA genes. This method is used to reveal the diversity of microbes and the microbial processes take part in the contaminated ecosystem in order to choose the most effective bioremediation technique. Even so it occurs frequently that after gaining the 16S rDNA information it is not evident which bacteria take part actively in the degradation of the contaminant, because many bacteria are unknown, same as their metabolic capabilities. In this case it can be practical to detect and investigate the functional genes play a key role in the processes in question, because this makes possible to get a broad view about metabolic capabilities exist in the microbial communities of contaminated environments. The nucleotide sequence of some functional genes can be feasible for phylogenetic identification, same as the 16S rRNA genes, therefore based on this knowledge it could be define which microbes take part actively in the mineralization of the contaminant. Therefore, the investigation of functional genes play role in the degradation of aromatic hydrocarbons is in focus today. However, the genes in question – e.g. catechol dioxygenases – show high diversity, which makes their PCR based detection complicated.

The aim of my study was to investigate the diversity of catechol dioxygenase genes in smaller taxa, which included the designing of PCR primers for their detection and the development of a molecular monitoring technique. During my study i investigated the catechol 1,2-dioxygenase gene of aromatic hydrocarbon-degrading *Rhodococcus* species, than catechol 2,3-dioxygenase genes coded by species of the Beta-Proteobacteria.

METHODS

During my study I used both classical cultivation based and cultivation independent microbiological methods. Although mainly the latter methods were used during my works, cultivation dependent methods are still crucial to find aromatic hydrocarbon-degrading microbes and to reveal their metabolic capabilities.

1. Investigating aromatic hydrocarbon-degrading *Rhodococcus* strains we tried to detect the aromatic ring cleaving catechol 1,2-dioxygenase enzyme and its coding cat*A* gene.

Enzyme activity measurements were performed with catechol substrate using total enzyme extract from cells and the reaction was followed with spectrophotometer. The cat*A* gene was detected by PCR with newly designed, group specific PCR primers. To design primers catA gene sequences were retrieved from GenBank, then sequences were aligned with the ClustalW algorithm of MEGA3 software and homologous parts were selected for primer design. The known and newly identified *Rhodococcus* related cat*A* gene sequences were phylogenetically analyzed using the neighbour-joining method, and the functional gene based phylogenetic tree was compared to the 16S rRNA gene based tree of the investigated *Rhodococcus* strains.

2. Based on the cat*A* gene sequence data of the investigated *Rhodococcus* strains a single nucleotide primer extension (SNuPE) assay was developed for the detection and typing of aromatic hydrocarbon-degrading *Rhodococcus* strains. The SNuPE method is based on the hybridization of oligonucleotides to the target sequences and, the subsequent elongation of the oligos with one nucleotide. In the reaction solution all of the free nucleotides are ddNTPs, which means that they lack free 3' OH-groups therefore, the incorporation of a single nucleotide terminates the reaction. The four different ddNTPs (A, T, G, C) are differently labeled with a fluorescent tag therefore, the reaction results in a labeled product. Subsequent separation using capillary electrophoresis and laser induced fluorescence detection results in a very fast assay that is easy to interpret (Fig. 1.). Determination of the incorporated nucleotide may provide phylogenetic information.

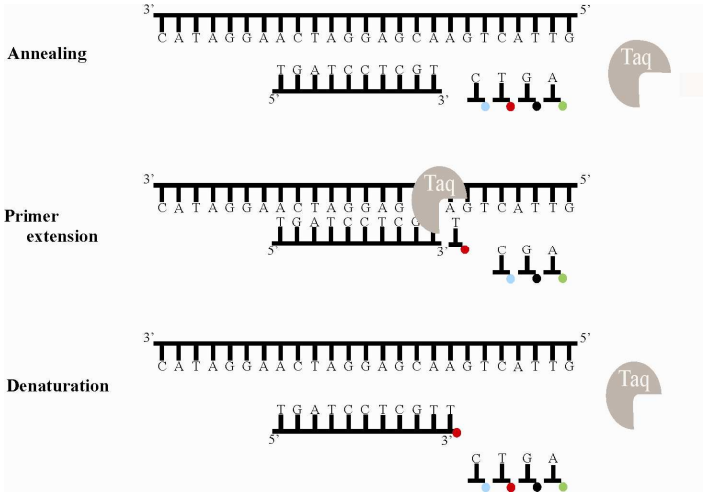

Fig. 1. The SNuPE reaction.

3. During my study I also investigated the microbial community of aromatic hydrocarbon contaminated, hypoxic groundwater based on the detection and phylogenetic investigation of catechol 2,3-dioxygenase (C23O) and 16S rRNA genes. Group specific PCR primers were designed to detect C23O genes mainly harbored by species of the family Comamonadaceae. The process of the primer design was the same as above. To investigate the diversity of C23O genes the PCR products obtained from community DNA samples were

analyzed with the method of terminal restriction fragment length polymorphism (T-RFLP). To identify the peaks obtained at the T-RFLP chromatograms the PCR products were cloned into p-GemT Easy vector and JM109 High Efficiency Competent *Escherichia coli* cells were transformed with the vector. The resulting C23O gene clone library was screened with T-RFLP and clones were grouped based upon the length of their terminal restriction fragment (TRF) then the sequence of each group representative was identified. The C23O gene sequences were aligned with their nearest neighbours and phylogenetic trees were constructed with the same method as detailed earlier to determine the phylogenetic position of the C23O clones. To investigate the species composition of the microbial community the 16S rRNA genes were amplified with *Bacteria* domain specific primers from the community DNA. T-RFLP analysis and cloning were performed as above.

RESULTS AND CONCLUSIONS

1. It is well known that species in the *Rhodococcus* genus can degrade a wide variety of xenobiotics, and they are often isolated from hydrocarbon contaminated environments. During this study the cat*A* gene was detected in 11 different *Rhodococcus* strains with newly designed group specific PCR primers. The phylogenetic investigation of the gene sequences showed that *Rhodococcu*s related cat*A* genes harbor phylogenetic information and can be used as a phylogenetic marker gene. Our opinion is that this fact may have resulted by the lack of recent lateral gene transfer among *Rhodococcus* species in the case of cat*A* gene. Because the cat*A* gene encodes protein its evolution is much faster than the evolution of the *rrna* operon which includes the ribosomal RNA genes. Therefore, the phylogenetic investigation of the cat*A* genes may give a higher taxonomical resolution. Due to this in case of closely related *Rhodococcus* species, which can not be separated based on the sequence of the 16S rRNA gene, the sequence of the cat*A* gene can be used for the exact identification.

2. Because the cat*A* gene of *Rhodococcus* species harbors phylogenetic information it can be used as a marker gene to monitor the activity of *Rhodococcus* strains used in bioaugmentation processes. If we detect this gene and the mRNA transcribed from the gene we can be sure not just about the presence of these bacteria but about their aromatic hydrocarbon degrading activity as well. To carry out a successful monitoring procedure it is crucial to develop quick and reliable molecular techniques. Nowadays conventional, cultivation based methods are still routinely used techniques to detect and monitor the

presence of strains used in bioaugmentation processes, but these methods are not efficient enough. The usually used cultivation independent molecular microbiological methods, like DGGE, cloning or T-RFLP are time consuming, labor-intensive methods. The method of single nucleotide primer extension (SNuPE) can be an adequate solution. Using this method we developed a procedure which enables the fast detection and typing of *Rhodococcus* strains based upon their cat*A* gene. This makes possible to monitor the presence and activity of *Rhodococcus* strains used in a bioaugmentation process. Moreover, during the analysis of microbial communities of aromatic hydrocarbon contaminated environments it makes feasible to investigate whether *Rhodococcus* strains with the ability of aromatic hydrocarbon degradation are present.

3. There can be found several well known aromatic hydrocarbon-degrading bacteria among the Beta-Proteobacteria as well, but their role in the degradation of these compounds and their diversity is still unrevealed. During this study group specific primers were designed for the detection of catechol 2,3-dioxygenase (C23O) genes mainly harbored by species of the family Comamonadaceae. These genes are considerably diverse and encode C23O proteins which belong to the subfamily I.2.C. of extradiol dioxygenases. These enzymes believed to possess a greater affinity for molecular oxygen than other extradiol dioxygenases therefore, they favour hypoxic conditions. Aeration of the contaminated area during *in situ* bioremediation of hydrocarbon contaminated subsurface soil and groundwater is a routine process. At the same time, it occurs often that the contamination reaches deep layers of the soil where oxygen still can be a limiting factor. Therefore, it is important to detect and reveal the diversity of microbes which harbor subfamily I.2.C. related C23O genes.

We investigated the microbial communities of hypoxic, aromatic hydrocarbon contaminated groundwater. It was found that Beta-Proteobacteria were dominant in the microbial communities and mainly subfamily I.2.C. related C23O genes were detected. These results confirm our hypothesis that under hypoxic conditions microbes possess these types of genes may play a significant role in the degradation of aromatic hydrocarbons.

PUBLICATIONS BASED ON THE STUDY

Táncsics A., S. Szoboszlay, B. Kriszt, J. Kukolya, E. Baka, K. Márialigeti, S. Révész (2008) Applicability of the functional gene catechol 1,2-dioxygenase as a biomarker in the detection of BTEX-degrading *Rhodococcus* species. *J Appl Microbiol*, **105**(4), 1026-1033.

Nikolausz M., A. Chatzinotas, **A. Táncsics**, G. Imfeld, M. Kästner (2009) Evaluation of single-nucleotide primer extension for detection and typing of phylogenetic markers used for the investigation of microbial communities. *Appl Environ Microbiol*, **75**, 2850-2860.

Nikolausz M., A. Chatzinotas, **A. Táncsics**, G. Imfeld, M. Kästner (2009) The single-nucleotide primer extension (SNuPE) method for the multiplex detection of various DNA sequences: from detection of point mutation to microbial ecology. *Biochem Soc Trans*, **37** (Pt 2), 454-9.