Groundwater microcosm studies on the microbial biodegradation of aromatic hydrocarbons under hypoxic conditions

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Final Research Report

I. Establishment of the Single-Nucleotide Primer Extension Assay for monitoring of subfamily I.2.C catechol 2,3-dioxygenase genes in the investigated BTEX-contaminated environment

One of the main tasks of the research was to develop a Single-Nucleotide Primer Extension (SNuPE) assay to detect the expression pattern changes of subfamily I.2.C catechol 2,3-dioxygenase (C23O) genotypes that may occur during the microcosm experiments. To develop this assay we used community RNA samples isolated from contaminated groundwater of the "Siklós" BTEX-contaminated site. Groundwater samples were taken between May 2010 and May 2011. Therefore, samples of 13 consecutive months were analyzed. Microbial community compositions were first analyzed by Terminal Restriction Fragment Length Polymorphism (T-RFLP). In order to get species level information on the community compositions and to identify the peaks of the T-RFLP electropherograms, 16S rRNA based clone libraries were established in case of representative samples May 2010, December 2010 and May 2011. These clone libraries were characterized by a high ratio of *Betaproteobacteria*, and mainly members of the genera *Rhodoferax*, *Albidiferax* and *Azoarcus* and a yet unknown Rhodocyclaceae bacterium were dominant throughout the monitoring period. Although the community composition of the samples groundwater well was quite stable, remarkable shifts in the dominancy of these major taxa were observed. Diversity of subfamily I.2.C C23O genes was assessed in case of the same representative samples. To cover as large diversity as possible the subfamily I.2.C specific primers were used designated as the XYLE3 primer set. This primer set allowed the PCR amplification of *Beta-*, and *Gammaproteobacterial* I.2.C C23O genes. Clone libraries of the three representative samples revealed the presence of six I.2.C C23O clusters. Unfortunately only two of these clusters could be assigned to known bacterial genera (genera *Pseudomonas* and *Pseudoxanthomonas* of class *Gammaproteobacteria*). The other four clusters could not be affiliated to any known bacterial species. SNuPE primers were developed for the detection of these six distinct clusters. After testing the specificity of the primers, activity of the targeted clusters was investigated in all of the 13 samples. It was observed that only two of the clusters (clusters B and C) were constantly detectable. Consequently; their considerable role in the microaerobic degradation of BTEX-compounds can be hypothesized. Unfortunately these clusters cannot be clearly linked to any cultured bacterium. On the other hand, results of the SNuPE assay provided possibility to assign phylogenetically these clusters. We statistically evaluated the 16S T-RFLP and SNuPE data to look for correlations between the presence and activity of

certain 16S T-RFs and the presence and activity of I.2.C C23O clusters. As a consequence we suggested that one of the significant clusters (cluster B) is probably affiliated with an unclassified Rhodocyclaceae bacterium, while another one (cluster C) is most probably affiliated with a yet uncultured member of the genus *Rhodoferax* (genus Comamonadaceae of class Betaproteobacteria). These results were published in a research article in the journal of *Systematic and Applied Microbiology* (Táncsics et al. 2013, *Syst. Appl. Microbiol.* 36:339-350).

II. The microcosm experiments

By using the newly designed SNuPE assay we investigated the dynamics of the bacterial community structure and the expression pattern of subfamily I.2.C C23O genes under decreasing dissolved oxygen (DO) concentration in microcosm experiments. Parallel microcosms were used to investigate these issues under 0.5 and 0.1 mg/L DO concentrations and three days after oxygen depletion. DO concentrations in the microcosms were measured non-invasively by using a Fibox 3 trace v3 fiber optic trace oxygen sensor with Pst 3 sensor spots. To reveal the community composition of the initial groundwater sample a metagenomic approach was used. More than 90 000 DNA sequences were gained and analyzed by using an Ion Torrent platform (Life Technologies) and the results showed the overwhelming dominance of Betaproteobacteria in the bacterial community. The most dominant bacteria belonged to the genus *Azoarcus* followed by genera *Thauera*, *Acidovorax*, *Rhodoferax*, *Polaromonas* and *Dechloromonas*. The class Gammaproteobacteria was mainly represented by members of the genus Pseudomonas. The effect of decreasing DO concentration on the microbial community composition was assessed by T-RFLP both on DNA and RNA level. It was found that the decreasing DO concentration had no significant effect on the community composition and remained practically the same throughout the experiments regardless of the availability of oxygen. Diversity of subfamily I.2.C C23O genes was investigated in case of the initial sample by cloning and sequencing. It was found that some of the formerly detected clusters disappeared since May 2011 while new clusters emerged dominantly. This led us to modify the formerly developed subfamily I.2.C C23O SNuPE assay. Subsequently, with this modified SNuPE assay we investigated how the decreasing DO concentration affected the presence and activity of the detected C23O gene clusters. On DNA level it was found that there was no change in the presence of subfamily I.2.C C23O gene clusters throughout the experiment. This is not a surprise since the bacterial community composition was stable as we

noted this above. On RNA level it was found that the decreasing DO concentration had significant effect on the activity of some subfamily I.2.C C23O genes, by showing decreasing activity with decreasing DO concentration. These genotypes in question could be loosely assigned to members of the genus Pseudoxanthomonas (class Gammaproteobacteria) On the other hand; some C23O clusters were constantly active regardless of the concentration of oxygen. These latter C23O genotypes might be associated with members of betaproteobacterial genera, such as *Rhodoferax* and might be key players in microaerobic degradation of aromatic hydrocarbons. It can be also presumed that there is a difference in the transcriptional regulation of subfamily I.2.C C23O genes between Gamma-, and Betaproteobacteria. Nevertheless, further investigations are needed to clarify this question. Beside subfamily I.2.C C23O genes, we also investigated the effect of dercreasing DO concentration on the presence and activity of benzylsuccinate synthase (bssA) genes, key genes of anaerobic degradation of toluene. Diversity of bssA genes was investigated in case of the initial sample by cloning and sequencing. To investigate the effect of decreasing DO concentration on the transcription activity of the detected bssA clusters a SNuPE assay was developed. On DNA level similar observation was made as earlier in case of 16S rRNA and C23O genes. Bluntly; there was no change in the bssA gene diversity throughout the experiment. On RNA level it was found, that one of the detected bssA clusters (most closely related to bssA gene of *Thauera aromatica* strain K172 – 98% nucleotide sequence similarity) was detectable only on DNA level, while the other clusters were constantly active regardless of the presence of oxygen. This observation is in accordance with data can be found in the respective literature. Benzylsuccinate synthase is a glycyl radical enzyme inactivated by oxygen (its half-life under truly oxic conditions is 30 seconds) and supposed to be active only under true anaerobic conditions. Therefore, *Thauera aromatica* strain K172 stops the transcription of its bssA gene in the presence of oxygen. On the other hand; it is known that other Thauera-relatives (e.g. *Thauera* sp. DNT-1) maintain the expression of their bssA genes even under (micro)aerobic conditions. Concluding everything it can be assumed that aerobic and anaerobic degradation pathways of aromatic hydrocarbons are both active under microaerobic and anaerobic conditions. Nevertheless; further investigations are needed to reveal their actual contribution to aromatic hydrocarbon degradation under microaerobic and anaerobic conditions. Results of the above detailed experiments have been demonstrated in the form of an oral presentation at the Ninth International Symposium on Subsurface Microbiology, October 5-10, Pacific Grove, California, USA. Besides, a manuscript with a

portion of results is still under preparation and planned to be submitted to a peer-reviewed journal of the field of environmental microbiology.

In the second round of microcosm experiments we investigated the dynamics of BTEX degrading bacterial communities under decreasing dissolved oxygen (DO) concentration in microcosms amended with nitrate or Fe(III). The effect of decreasing DO concentration on the microbial community composition was assessed by T-RFLP both on DNA and RNA level. Similarly to the previous microcosm experiment the decreasing DO concentration had no significant effect on the community composition and remained practically the same throughout the experiments regardless of the availability of oxygen, nitrate or Fe(III). This is most probably due to the short (5 days) time frame we used during the experiment. Regarding the composition of the microbial community of the groundwater used to establish the microcosms was highly similar to the community observed in the previous experiment (Groundwater sample was taken from the same well of the Siklós BTEX contaminated site as in the previous year!). Regarding the depletion of DO it was observed, that the process of depletion was much more quick in case of nitrate or Fe(III) amended microcosms then in the control [no nitrate/Fe(III) amendment] microcosms. Presumably, the initial groundwater was highly electron acceptor limited, and the amendment with nitrate or Fe(III) caused an increase in the activity of facultative anaerobic bacteria, and accompanying processes promoted the depletion of DO. However, the elevated metabolic activity of bacteria did not associate with increased degradation of BTEX compounds. Moreover, amendment with Fe(III) had detrimental effect on the degradation. In the control and nitrate amended microcosms similar degradation rates were detected. However, it was also observable that the addition of nitrate slightly facilitated the degradation of toluene and ethyl-benzene (possibly by facilitating the activity of *Azoarcus* and *Thauera* related bacteria), while it decreased the degradation rate of benzene and xylenes. Regarding Fe(III) amended microcosms, only ethyl-benzene was depleted, while degradation rate of other BTEX compounds was much lower compared to the control microcosms. Based on the degradation results it can be assumed that significant degradation of xylenes took place in the microcosms until DO was present. Addition of nitrate or Fe(III) to the microcosms decreased the degradation rate of xylenes by promoting the activity of facultative anaerobic bacteria, thus facilitating DO depletion. Similar process was observed in case of benzene. Since only few anaerobic benzene or xylene degrading bacteria are known, the persistence of these aromatic compounds in the microcosms under anaerobic conditions is not striking. Consequently, microaerobic degradation seems to be an effective process to eliminate all of the BTEX compounds in oxygen limited subsurface environments.

Regarding subfamily I.2.C C23O genes we found the same genotypes in the initial groundwater as in case of the previous microcosm experiment, which is not a surprise, since the bacterial community structure of the groundwater well used to collect sample (Siklós BTEX contaminated site) is rather stable. Furthermore, the same expression pattern changes were observed with the SNuPE assay developed in the first research period. Nevertheless, the studied C23Os most likely contribute to BTEX degradation under microaerobic conditions.

III. Enrichment approaches

Another cardinal point of our research was to try to enrich Fe(III)-reducing microbes (focusing on *Rhodoferax* species) from the investigated groundwater. To reach this goal four slightly different enrichment media were used, which were common in that the only available electron acceptor was Fe(III)NTA in them, while the sole source of carbon was acetate (10 mM). The basis of the enrichment media was always a mineral medium without any fix nitrogen form. To create slightly different enrichments one medium was amended both with 0.05% (w/v) yeast extract and a fix nitrogen form (ammonium-chloride), one was amended solely with yeast extract, another one solely with the fix nitrogen form, while the 4th type lacked any of these amendments. These media were transferred into 100 ml serum bottles (50 ml medium and 50 ml headspace) which were sealed with butyl rubber stoppers and crimp sealed. Subsequently bottles were sparged with nitrogen to ensure anoxic conditions and inoculated with 1 ml groundwater sample. Enrichment cultures were incubated at 15 °C in the dark without shaking for one week. The color change of the enrichment media from dark orange [due to the presence of Fe(III)] to grayish green or to colorless indicated the enrichment of Fe(III)-reducing microbes. Subsequent passages were performed by transferring 1 ml of the enrichments to 50 ml new medium. After 5 consecutive transfers microbial community composition of the enrichments was investigated by the methods of T-RFLP and molecular cloning. Furthermore, microbial structure of the groundwater sample which was used to initiate the enrichments was revealed by using a metagenomic approach. More than 250 000 DNA sequences were gained and analyzed by using an Ion Torrent PGM system (Life Technologies) and the results showed the overwhelming dominance of Beta- and Gammaproteobacteria in the bacterial community. The most dominant bacteria belonged to the genus *Dechloromonas* followed by genera *Pseudomonas*, *Acidovorax*, *Geobacter*, *Azoarcus*, *Burkholderia*, *Thauera*, *Polaromonas*, *Azotobacter* and *Rhodoferax*. Diversity of this community was markedly decreased during enrichments. Interestingly community

structure of enrichments amended with yeast extract showed high degree of similarity and considerably differed from the two enrichments lacking yeast extract. Furthermore, microbial community structure of these latter two enrichments also showed high degree of similarity with each other. In the microbial communities of type I and II enrichments the most dominant community members belonged to the genera *Citrobacter* (class of Gammaproteobacteria, order of Enterobacteriales), *Bacteroides* (class of Bacteroidetes) and *Clostridium* (class of Clostridia). Members of these genera are usually strict anaerobes, except *Citrobacter* species, which are facultative anaerobes but can use Fe(III) as electron acceptor in the absence of oxygen. The presence and dominancy of *Citrobacter* and *Bacteroides* genera related bacteria was most probably caused by the presence of yeast extract in the enrichment medium, since these microbes were completely missing from enrichment lacking yeast extract. Although microbial communities of type I and II enrichments showed high degree of similarity, there was at least one large difference between them: *Geobacter*-related bacteria were dominant in type I enrichment, but were missing from type II enrichment, possibly due to the lack of fix inorganic nitrogen source (ammonium-chloride). Some members of the genus *Geobacter* are well known anaerobic (iron-reducing) toluene degraders, harboring benzylsuccinate synthase (bssA) gene, key gene of anaerobic degradation of toluene. The detected *Geobacter* 16S clone sequences in type I enrichment showed the largest similarity with that of *Geobacter lovleyi*, but only at a level of ~95%. In the microbial community of type III enrichment only members of two genera were detected: *Geobacter* and *Pseudomonas*, with the overwhelming dominance of *Geobacter*. The majority of *Geobacter* clone sequences were identical with that of were found in type I enrichment showing 95% similarity with *G. lovleyi*, while the remaining *Geobacter* clone sequences showed the largest similarity with *G. metallireducens* at a level of 96%. As a consequence, both *Geobacter* related bacteria represented yet unknown lineages of the genus. *Pseudomonas* related clone sequences were most closely related to *P. stutzeri* (~99%). In the microbial community of type IV enrichment only one genus was predominant: the *Geobacter*. However, clone sequences showed, that in this enrichment *Geobacter*-related 16S rDNA sequences showed the largest similarity (98%) with that of G. luticola and not with *G. lovleyi*. Consequently, the lack of fix inorganic nitrogen source (ammonium-chloride) caused the enrichment of a completely different *Geobacter*–related bacterium, which assumes distinct differences in the physiology of *Geobacter* species.

Regarding functional genes (C23O and bssA) the subfamily I.2.C related C23O genes (along with *Rhodoferax* related bacteria) were only detected in the type I enrichment but only after the first transfer then later they relatively quickly perished, and were not detectable at all

after the third transfer. Subsequently, yeast extract seems to be necessary for the enrichment and isolation of *Rhodoferax* related bacteria of the Siklós groundwater. However, it is also evident that under the enrichment conditions used they are easily outcompeted by other iron-reducing bacteria. On the other hand bssA genes were permanently detectable in every type of enrichment. In the bacterial community of the groundwater used to initiate the enrichments at least 7 bssA genotypes were detected, mostly (5 genotypes) affiliated to Azoarcus/Thauera related bssA genes. In all type of enrichments one and the samme predominant bssA genotype was found at all. However, this genotype was not detected in the initial groundwater sample. This bssA genotype is most probably not affiliated with any member of the genus *Geobacter* as different Geobacter-related bacteria were enriched in type III and IV enrichments, which would assume different bssA genotypes in these enrichments. Results of a qPCR approach also showed that this bssA gene should be affiliated to a minor community member. This minor community member can be a yet uncultured Fe(III)-reducing Rhodocyclaceae bacterium, most closely related to *Azoarcus* species (at a level of 92% 16S rDNA similarity). This bacterium was the only one which was detectable in all type of enrichments. Results obtained were published as an invited oral presentation at the 17th International Congress of the Hungarian Society for Microbiology, while a manuscript is under preparation and planned to be submitted to a journal in the field of microbial ecology.

IV. Isolation approaches

Another cardinal task of our research was to isolate bacterial strains from microcosms, enrichments or from other BTEX-contaminated environments and to look for subfamily I.2.C C23O genes in their genome. The first isolation attempts were made by using samples from anaerobic microcosms. Microcosm samples were diluted serially and plated directly on R2A agar, and parallel with this undiluted samples were used to inoculate enrichment medium. Enrichments were made aerobically in defined freshwater medium supplemented with trace element and vitamin solutions. The sole source of carbon and energy was acetate, since members of the targeted genera (*Rhodoferax*, *Albidiferax*, *Azoarcus* of the class Betaproteobacteria) are known to be able to utilize this carbon source. Unfortunately, nor direct plating nor the enrichment approach resulted *Rhodoferax*, *Albidiferax* or *Azoarcus* related strains. Moreover, the acetate enrichment caused a dramatic decrease in the initial diversity; consequently mainly members of the genus *Pseudomonas* and *Aeromonas* (both belong to the class of Gammaproteobacteria) were detectable and isolable. These results were

published as a conference poster at the annual symposium of the Hungarian Society for Microbiology in 2014 by my PhD student Milán Farkas.

Although isolation attempts from the microcosms yielded no betaproteobacterial strain with subfamily 1.2.C C23O gene, we managed to isolate a bacterium, designated as strain Buc^T which possess the gene in question in its genome. The 16S rRNA gene sequence of strain Buc^T is available in GenBank under accession number KF667502. This strain was isolated from biofilm developed on the surface of a biofilter made for petroleum hydrocarbon removal. Phylogenetic analysis based on 16S rRNA gene sequences indicated that strain Buc^T formed a distinct phyletic lineage within the genus *Zoogloea* (class Betaproteobacteria). Comparative 16S rRNA gene sequence analysis revealed that strain Buc^T was most closely related to *Z. caeni* EMB43^T with 16S rRNA gene similarity of 97.2%, followed by *Z. oryzae* A-7^T, *Z. ramigera* ATCC 19544^T, and *Z. resiniphila* DhA-35^T with 16S rRNA gene similarities of 95.9, 95.5, and 95.4% respectively. Level of DNA-DNA relatedness between strains Buc^T and *Z. caeni* EMB43^T was 31.6%, clearly below the cutoff-point recommended for the delineation of bacterial species. It was also observed that strain Buc^T is able to use gasoline-crude oil mixture as sole source of carbon and energy. All of the physiological, biochemical and phylogenetic data suggested that strain Buc^T represented a single novel species of the genus *Zoogloea*, for which the name *Zoogloea oleivorans* sp. nov. was proposed by us. The strain was deposited in two international collections – in the German Collection of Microorganisms and Cell Cultures (DSMZ) and in the National Collection of Agricultural and Industrial Microorganisms of Hungary (NCAIM) under accession numbers DSM 28378T and NCAIM B 02570T, respectively. Finally, description of the new species was validly published in the *International Journal of Systematic and Evolutionary Microbiology* (Farkas et al., 2015).

V. Degradation of toluene under microaerobic conditions by pure strains

One of the last tasks of our research was to investigate the microaerobic degradation of toluene by pure strains harboring subfamily I.2.C C23O gene to test the hypothesis that the presence of this gene helps the host strain to degrade monoaromatic hydrocarbons under microaerobic conditions. Our most promising strain was strain ST2-6 formerly isolated from the Siklós BTEX-contaminated site. This bacterium was most closely related to Pseudomonas extremaustralis (>99% 16S rDNA similarity) and proved to harbor two types of C23O genes: a I.2.A and a I.2.C type. Whole genome analysis of this strain was performed and it turned out

that its genome is 6.2 Mbp large and contains a ~300 kbp large megaplasmid where both C23O genes are located. We investigated the BTX degradation ability of this strain and it was found that benzene, toluene, para- and meta xylenes were readily and rapidly degradable under fully aerobic conditions by strain ST2-6. Toluene degradation was tested under microaerobic conditions as well. These experiments were conducted in 100 mL serum bottles with crimp sealing, by using 50 mL defined freshwater medium with 5 mg/L toluene as sole source of carbon and energy. Prior to the addition of toluene the bottles were sparged with nitrogen and hypoxic conditions (0.5 mg/L DO) were created by the addition of known amount of sterile air. In parallel, another *Pseudomonas* strain of our strain collection (strain PS2, most closely related to *P. putida*) was used as control strain, since this bacterium harbored only I.2.A-type C23O gene. In case of both strains cell numbers were equal in all of the serum bottles (10^5 CFU/mL). Fully aerobic controls in case of both strains were also investigated. It was found that strain ST2-6 could rapidly degrade toluene even under microaerobic conditions without lag phase, while strain PS2 started to slowly degrade toluene just after a 48h long lag phase. Strain ST2-6 degraded almost 100% of toluene after 24h under both aerobic and microaerobic conditions. On the other hand, after 6 days of incubation bottles inoculated with strain PS2 still contained 2.8±0.45 mg/L toluene under microaerobic conditions while 3.01±0.1 mg/L toluene was present in the fully aerobic bottles. Consequently, results confirmed that strain ST2-6 is an excellent microaerobic toluene degrader. Gene expression analyses are still in progress to figure out the role of the two different C23O genes of strain ST2-6 in the hypoxic degradation of toluene. We also planned to investigate *Hydrogenophaga pseudoflava* strain HB3 in the last stage our research. This strain was initially isolated from a benzene degrading enrichment culture and was shown to harbor a I.2.C-type C23O gene and after isolation it was stored at -80°C. Unfortunately, the strain lost its viability after long storage under -80°C, but we are still trying to revitalize this strain by testing all vials stored in the ultra-low deep freezer. Results of this research period has not been published, as they can potentially establish a patent.

VI. References

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