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Enrichment of dissimilatory Fe(III)-reducing bacteria from groundwater of the Siklós BTEX-contaminated site (Hungary)

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Abstract Dissimilatory iron-reducing bacteria are commonly found in microbial communities of aromatic hydrocarbon-contaminated subsurface environments where they often play key role in the degradation of the contaminants. The Siklós benzene, toluene, ethylbenzene, and xylene (BTEX)-contaminated area is one of the best characterized petroleum hydrocarbon-contaminated sites of Hungary. Continuous monitoring of the microbial community in the center of the contaminant plume indicated the presence of an emerging *Geobacter* population and a *Rhodoferax* phylotype highly associated with aromatic hydrocarbon-contaminated subsurface environments. The aim of the present study was to make an initial effort to enrich *Rhodoferax*-related and other dissimilatory iron-reducing bacteria from this environment. Accordingly, four slightly different freshwater media were used to enrich Fe(III) reducers, differing only in the form of nitrogen source (organic, inorganic nitrogen or gaseous headspace nitrogen). Although enrichment of the desired *Rhodoferax* phylotype was not succeeded, *Geobacter*-related bacteria were readily enriched. Moreover, the different nitrogen sources caused the enrichment of different *Geobacter* species. Investigation of the diversity of benzylsuccinate synthase gene both in the enrichments and in the initial groundwater sample indicated that the *Geobacter* population in the center of the contaminant plume may not play a significant role in the anaerobic degradation of toluene.

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Introduction

Dissimilatory Fe(III) reduction is a crucial anaerobic respiratory pathway in which the reduction of Fe(III) is coupled to the terminal decomposition of organic matter. This process occurs in terrestrial and marine aquatic sediments, as well as in natural soils and sediments. Moreover, in the latter two environments, Fe(III) is one of the most abundant electron acceptors available for anaerobic oxidation of organic matter. Iron reducers have a great impact on global biochemical cycles, while in contaminated subsurface environments, they often play key role in the degradation of organic and inorganic contaminants.

In anoxic, hydrocarbon-contaminated subsurface environments, *Geobacter*- and *Rhodoferax*-related bacteria are among the most frequently occurring dissimilatory Fe(III)-reducing microorganisms (Zhuang et al. 2011). Members of the genus *Geobacter* are best known due to their ability to reduce U(VI) (Shelobolina et al. 2008) or to oxidize organic compounds to carbon dioxide while using an electrode as an electron acceptor (Bond et al. 2002; Bond and Lovley 2003). Based on our current knowledge, it is also presumable that members of this genus constitute the majority of iron-reducing bacteria that anaerobically oxidize monoaromatic hydrocarbons (Kunapuli et al. 2010). Much less is known about the ecological role of genus *Rhodoferax*-related bacteria in these environments. This is at least partly due to the fact that this genus contains only four validly described species (Kaden et al. 2014). However, in contaminated subsurface environments (uranium and/or hydrocarbon contamination), the occurrence

of bacteria most closely related to *Rhodoferax ferrireducens* and *Rhodoferax antarcticus* seems to be common (Alfreider and Vogt 2007).

The Siklós benzene, toluene, ethylbenzene, and xylene (BTEX)-contaminated area is among the best characterized petroleum hydrocarbon-contaminated sites in Hungary. The groundwater at this site is shallow, hydrodynamically active, and both oxygen and nitrate limited (Táncsics et al. 2013). Continuous monitoring of the microbial community structure in the center of the contaminant plume indicated the varying dominance of a particular *Rhodoferax* phylotype, which can be clearly linked to aromatic hydrocarbon degradation (Aburto and Peimbert 2011; Táncsics et al. 2013). Moreover, emergence of a *Geobacter* population was observed in the center of the plume from year 2013. Based on all abovementioned data, it was aimed to make an initial effort to enrich *Rhodoferax*-related and other dissimilatory Fe(III)-reducing bacteria from groundwater taken from the center of the contaminant plume of the Siklós BTEX-contaminated site.

Material and methods

Study site and sample collection

Initial groundwater sample was taken at the Siklós BTEX-contaminated site from the ST2 well located at the center of the contaminant plume. Both the contaminated aquifer and the microbial community of the ST2 well groundwater have been deeply characterized in earlier studies (Táncsics et al. 2012, 2013). Briefly, the main contaminant at the site is xylene, followed by benzene, ethylbenzene, and toluene, originated from leakage of a former petrol station (Táncsics et al. 2013). Groundwater samples were collected into sterile 1-L serum bottles (Glasgeratebau Ochs) without headspace, crimp sealed, and transferred to the laboratory while keeping the samples at 15 °C in the dark. Samples were incubated at the same temperature for 2 weeks in the dark to enhance depletion of nitrate prior to enrichments.

Enrichment conditions

Enrichment cultures were set up as a 2 % (*v*/*v*) groundwater in a freshwater mineral medium described by Fahy et al. (2006) with minor modifications: filter-sterilized acetate was added to the medium in 10 mmol/L final concentration, while omitting the addition of NH_4NO_3. Furthermore, Mg was added to the medium in the form of $MgCl_2$, instead of $MgSO_4$. As *Rfx. ferrireducens* and *Rfx. antarcticus* require thiamin and biotin for their growth (Madigan et al. 2000; Finneran et al. 2003), these vitamins were added to the enrichments under sterile circumstances in the following concentrations: 1 mg/L thiamine and 15 μg/L biotin. Additionally, vitamin B_{12} was also added to the medium in 20 μg/L final concentration. To selectively enrich *Rhodoferax*-related bacteria, some general guidelines for the enrichment of purple non-sulfur bacteria were followed. Accordingly, mineral salt medium can be made selective for these bacteria by omitting fix nitrogen forms (NH_4^+) or organic nitrogen sources (e.g., yeast extract) and supplying gaseous headspace of N_2. However, Madigan et al. (2000) observed that trace amount of yeast extract may enhance the growth of *Rhodoferax* spp. Considering all previously mentioned, four slightly different types of enrichment media were prepared with the primary aim to enrich *Rhodoferax*-related bacteria: type I enrichment was amended with 0.05 % (*w*/*v*) yeast extract and NH_4Cl (1 g/L); type II enrichment was amended solely with yeast extract; type III enrichment was amended solely with NH_4Cl, while type IV enrichment lacked both yeast extract and any fix nitrogen form. Enrichments were performed in 100-mL crimp sealed serum bottles with 50-mL medium, which was sparged aseptically with N_2/CO_2 (80:20, *v*/*v*) to ensure anaerobic conditions. As sole electron acceptor, filter-sterilized Fe(III)NTA was added to enrichments at a final concentration of 5 mmol/L under anaerobic circumstances. Enrichments were incubated up to 1 week at 15 °C in the dark without shaking.

DNA isolation

After five consecutive transfers, bacterial cells from the enrichments were harvested by centrifugation, same as for the initial groundwater sample. Total community DNA from initial groundwater was extracted by using the MoBio RNA Power Soil Total RNA isolation Kit with the PowerSoil DNA Elution Accessory Kit (MoBio Laboratories Inc.), while for the enrichments, the UltraClean® Microbial DNA Isolation Kit (MoBio Laboratories Inc.) was used. Quality of nucleic acids was analyzed with gel electrophoresis on 1 % (*w*/*v*) agarose gel stained with ethidium bromide. Quantification of nucleic acids was performed by using Qubit 2.0 Fluorometer (Life Technologies) throughout the study.

Polymerase chain reaction

Amplification of 16S ribosomal RNA (rRNA) genes for cloning purposes was carried out as described earlier (Táncsics et al. 2012) by using the bacterial primers 27F (5′-AGA GTT TGA TCM TGG CTC AG-3′) and 518R (5′-ATT ACC GCG GCT GCT GG-3′), while for terminal restriction fragment length polymorphism (T-RFLP) analysis, the PCR reaction was carried out by using the same PCR primers, with the exception that the forward primer was fluorescently labeled (VIC). Since microaerobic degradation of monoaromatic hydrocarbons via *meta*-cleavage pathway was statistically linked to *Rhodoferax*-related bacteria in our earlier study, PCR

detection of subfamily I.2.C-type catechol 2,3-dioxygenases (C23O) was essayed by using primers XYLE3F (5′-TGY TGG GAY GAR TGG GAY AA-3′) and XYLE3R (5′-TCA SGT RTA SAC ITC SGT RAA-3′) (Táncsics et al. 2013). On the other hand, some *Geobacter* species harbor benzylsuccinate synthase (BSS), key enzyme of anaerobic toluene degradation (Kunapuli et al. 2010). The *bssA* gene, which encodes the α-subunit of the enzyme, is frequently used as a functional biomarker of anaerobic toluene-degrading bacteria (Winderl et al. 2008; von Netzer et al. 2013). Accordingly, amplification of the *bssA* fragments was also attempted by using the primer set of Winderl et al. (2007): 7772f (5′-GAC ATG ACC GAC GCS ATY CT-3′) and 8546r (5′-TC GTC GTC RTT GCC CCA YTT-3′). The PCR mixtures contained 0.2 mmol/L of each of the four dNTPs, 0.3 μmol/L of each primer, 1 U DreamTaq DNA polymerase (Thermo Scientific) with appropriate reaction buffer, 30 ng of template DNA, and molecular-grade water up to 50 μL. Amplifications were performed in a ProFlex PCR System (Life Technologies) with the following cycling conditions: 3-min initial denaturation at 95 °C, 32 cycles of amplification (30 s at 94 °C, 30 s at 52 °C for 16S rRNA fragment and 58 °C for *bssA* fragments, 45 s at 72 °C), and 7 min at 72 °C of terminal extension. Amplification products were checked by electrophoresis on 1 % agarose gels stained with ethidium bromide. Cleanup of the PCR amplicons was done with NucleoSpin Gel and PCR Clean-Up kit (Macherey-Nagel) according to the manufacturer's protocol before further processing.

Terminal restriction fragment length polymorphism

To track changes in the bacterial community composition that occurred during the enrichment experiment, the method of sequence-aided terminal restriction fragment length polymorphism (T-RFLP) was used (Székely et al. 2009). The VIC-labeled 16S ribosomal DNA (rDNA) amplicons generated as described previously were digested with the restriction enzyme *FspB*I (C↓TAG) (Life Technologies) similarly as described earlier (Révész et al. 2006). Fragments were separated on a Model 3130 Genetic Analyzer (Applied Biosystems), while primary evaluation of electropherograms was performed using GeneMapper 4.0 software (Applied Biosystems). Terminal restriction fragment (T-RF) peaks with a peak height below 100 relative fluorescence units or with a peak abundance contribution below 1 % were excluded from further analysis. For consensus profiles, duplicate electropherograms from each sample were aligned with each other using the T-Align program (Smith et al. 2005) using 0.5-bp confidence interval. Subsequently, the consensus profiles were aligned with the T-Align program for all samples. In the resulting data matrix, T-RF peak areas were standardized by dividing the area of each peak by the total peak area of that particular sample. The normalized matrix was used for statistical analysis of T-RFLP data. In order to assess the correlation between the abundance of each T-RF of the enrichment communities and the availability of the applied nitrogen forms, canonical correspondence analysis (CCA) was performed by using the PAST software package. Shannon-Wiener diversity values based on the T-RFLP data were calculated in PAST as $H' = -\ pi \ln pi$, where *pi* is the relative abundance of a single T-RF in a given electropherogram (Winderl et al. 2008).

Bacterial community analysis by sequence-based metagenomics

Total environmental DNA isolated from the initial groundwater sample was used for high-throughput sequencing using an Ion Torrent PGM instrument (Life Technologies). Ion Torrent PGM Fragment libraries of 100 nt were generated according to the appropriate protocols (Ion Torrent PGM, Life Technologies, USA). Total environmental DNA of 1 μg was used for library preparation using Ion Xpress Plus Fragment Library Kit. Adapter ligation and nick translation were performed by Ion Shear Plus Reagents Kit, and size selection was done in 2 % agarose gel. Library amplification was achieved by using Platinum® PCR SuperMix, and quantitation was performed by ION Library TaqMan qPCR. Ion PGM 200 Xpress Template kit was used for emulsion PCR. Sequencing was performed on Ion Torrent PGM™ using Ion 316 chip. High-quality reads (221.267) were generated, and the average read length was 203 nt. Sequences were deposited in the NCBI Short Read Archive (http://www.ncbi.nlm.nih.gov/sra) and are available under the following SRA Study accession number: SRS929832.

The online available *M*etagenomics *R*apid *A*nnotation using *S*ubsystem *T*echnology (MG-RAST) software was used for phylogenetic assessment. An initial quality control (QC) and an automatic normalization of the FASTQ sequence data were performed using selected settings of maximum e-value cutoff of 10^{-5}, minimum percent identity cutoff of 60 %, and minimum alignment length cutoff of 20 nt. Taxonomical assessments were performed after filtering the data, and MG-RAST utilized the M5nr protein database (GO, IMG, KEGG, NCBI RefSeq and GenBank, SEED, UniProt, eggNOG, and PATRIC) and various ribosomal RNA databases (RDP, Silva, and Greengenes), respectively (Meyer et al. 2008).

Cloning, Sanger sequencing, and phylogenetic analysis

Amplicons were cloned and sequenced as described earlier (Táncsics et al. 2012). For the clone libraries, at least 48 clones

were sequenced for each transformation. Sequence reads published in this study were assembled using MEGA6 (Tamura et al. 2013). All sequences were subsequently prescreened for similarities to published sequences by using BLAST; then, phylogenetic classification of 16S rDNA sequences was assigned via the database of type strains of valid prokaryotic names at EzTaxon server 2.1 (Chun et al. 2007). The 16S rDNA sequences were checked for chimeras using the Bellerophon program (Huber et al. 2004), and chimeric sequences were excluded from further analyses. All neighbor-joining (Saitou and Nei 1987) trees published in the study were constructed in MEGA6, performing 1000 bootstrap replicates. In case of functional gene clones, the nucleotide sequences were aligned in MEGA6 and distance matrices were calculated using the Jukes-Cantor method (Jukes and Cantor 1969). Distance matrices were then fed into DOTUR (Schloss and Handelsman 2005), and operational taxonomic units (OTUs) were generated with a distance threshold of 0.02.

Nucleotide accession numbers

The 16S rRNA gene sequences obtained in this study are available under accession numbers KT728574–KT728623, KT728624–KT728675, and KT808885–KT809025. The functional gene sequences obtained in this study are available under accession numbers KT948693–KT948708.

Results and discussion

Bacterial community in the initial groundwater

To get a quick but comprehensive view of the bacterial community composition of the groundwater, a metagenomic approach was used. Taxonomic profiling of metagenomic sequences showed the presence of a complex bacterial community with the predominance of *Betaproteobacteria* by giving more than 47 % of sequences, followed by *Gammaproteobacteria* (20 %), *Deltaproteobacteria* (7 %), *Alphaproteobacteria* (4 %), *Clostridia* (2 %), and *Bacteroidia* (1 %). The most abundant sequences belonged to members of the genus *Dechloromonas* (18 %), followed by *Pseudomonas* (12 %), *Acidovorax* (4 %), *Geobacter* (3 %), *Aromatoleum* (3 %), *Burkholderia* (2 %), *Thauera* (2 %), *Polaromonas* (2 %), *Azotobacter* (2 %), *Rhodoferax* (1 %), and *Azoarcus* (1 %). List of the 30 most abundant eubacterial genera is given in Supplementary Table 1. Since 16S rRNA gene clone libraries were generated in case of each sample analyzed during this study for the purpose of sequence-aided T-RFLP, a comparison could be performed between high-throughput sequencing and traditional Sanger sequencing data. The clone library of the initial groundwater (IG) consisted of 52 clones (Table 1) belonging mainly to phylum *Proteobacteria*. Consistent with the high-throughput sequencing data, majority of the clones belonged to *Betaproteobacteria* (69 %) mostly representing families *Rhodocyclaceae* and *Comamonadaceae*. However, the most abundant clone was affiliated with genus *Quatrionicoccus* and not *Dechloromonas*. This contradiction can be explained by the facts that these genera are the closest relatives of each other in the family *Rhodocylaceae* and that longer read lengths provided by Sanger sequencing enabled a more reliable taxonomic identification. Similarly to the high-throughput sequencing data, the clone library also contained clones affiliated with genus *Geobacter* and *Rhodoferax* as well as *Pseudomonas*, "*Aromatoleum*"/*Azoarcus*, and *Acidovorax*.

Enrichment bacterial communities

The T-RFLP fingerprints clearly showed that composition of the initial bacterial community detailed previously has markedly changed during the enrichments. After five consecutive transfers, a significant decrease was observable in the abundance of facultative anaerobic nitrate reducers in all types of enrichments. The 237-bp T-RF, which was the most dominant in case of the IG and could be linked to clones affiliated with genus *Quatrionicoccus*, completely disappeared during the enrichments. The 236-bp similarly dominant T-RF, which could be linked to some members of the genus *Azoarcus*, showed a significantly decreased abundance in case of type I and II enrichments and was not detectable in type III and IV enrichments. The 111-bp T-RF, which could be linked to members of the genus *Pseudomonas*, also showed a significantly decreased abundance in the enrichments but remained permanently detectable (Fig. 1). Based on the T-RFLP electropherograms, type I and II enrichments showed highly similar community composition, thus clustered closely on the CCA diagram, and yielded similar diversity indices (Figs. 1 and 2). In these enrichments, members of the genera *Citrobacter* (240–241 bp T-RF) and *Clostridium* (186 and 236 bp T-RFs) were similarly abundant. Based on the CCA (Fig. 2), enrichment of these bacteria is highly associated with the presence of yeast extract. Although *Citrobacter* species are fermentative bacteria, some strains are capable of dissimilative Fe(III) or perchlorate reduction (Bardiya and Bae 2004; Liu et al. 2016). Most of the *Clostridium*-like clones were related to *Clostridium tunisiense*, but only at a level of 96.2 % 16S rDNA sequence similarity. This bacterium is an obligate anaerobe with the ability to use acetate as a

Table 1 Relative phylum-level compositions of 16S rRNA gene clone libraries

Phylogenetic affiliation	% of clones in indicated library[b]					T-RF length (bp)[a]	
	IG[c]	Enrichments				Predicted	Measured
		I	II	III	IV		
Betaproteobacteria	*66*	*11*	*7*	*2*	*2*		
Quatrionicoccus rel.	42	–	–	–	–	235	237
Azoarcus related	8	11	7	2	2	235	232/236
Rhodoferax related	4	–	–	–	–	239	239
Acidovorax related	10	–	–	–	–	520	520
Brachymonas related	2	–	–	–	–	524	525
Gammaproteobacteria	*16*	*46*	*48*	*31*	*13*		
Citrobacter related	–	42	46	–	–	241	240/241
Pseudomonas related	16	4	2	31	13	111	111
Deltaproteobacteria	*6*	*10*	–	*67*	*85*		
Geobacter related	4	10		67	85	85/122/252	85/122/254
Desulfobulbus related	2	–	–	–	–	274	275
Negativicutes	*4*	–	–	–	–		
Veillonellaceae rel.	4	–	–	–	–	237	237
Bacilli	*2*	–	–	–	–		
Bacillus related	2	–	–	–	–	257	258
Clostridia	–	*10*	*37*	–	–		
Clostridium related	–	10	37	–	–	185/236	186/236
Cytophagia	*2*	–	–	–	–		
Meniscus related	2			–		29	ND
Bacteroidia	–	*23*	*4*	–	–		
Bacteroides related	–	21	2	–	–	29	ND
Sunxiuqinia related	–	2	*2*	–	–	29	ND
Synergistia	–	–	*2*	–	–		
Cloacibacillus related	–	–	2	–	–	94	94
Others	*4*	–	*2*	–		–	–

ND not determined

[a] Characteristic T-RF lengths (bp) predicted from the sequence data are indicated together with T-RF lengths actually measured in T-RFLP analysis. Values separated by a slash indicate more than one characteristic T-RF for a lineage

[b] Division-level percentages (given in italics) include the genus- or lineage-specific percentages (non-italics)

[c] Initial groundwater

sole source of carbon and energy while reducing elemental sulfur (Thabet et al. 2004). Unfortunately, Fe(III)-reducing ability of this bacterium has not been tested so far. The main difference between bacterial communities of type I and II enrichments was the 85-bp T-RF, which was predominant in type I enrichment but was completely missing from type II enrichment. Based on the 16S clone libraries, this T-RF could be clearly linked to a *Geobacter*-related clone sequence (Table 1). This T-RF and the related clone were also predominant in case of type III enrichment, and its closest relative was *Geobacter lovleyi*, but only at a level of 95 % 16S rDNA sequence similarity. Interestingly, another unique T-RF (122-bp length) in type III enrichment was also associated with a *Geobacter*-like sequence type. The related clones were most closely related to *Geobacter metallireducens* but only at a level of 96.3 % 16S rDNA sequence similarity. Due to the predominance of these unknown *Geobacter*-like bacteria and the complete lack of *Citrobacter*- or *Clostridium*-like clones, bacterial community of type III enrichment clustered separately on the CCA diagram (Fig. 2). It is also presumable that enrichment of these *Geobacter*-like bacteria was due to the presence of the fix nitrogen form (ammonium chloride) (Fig. 2). In case of type IV enrichment only, one predominant T-RF was

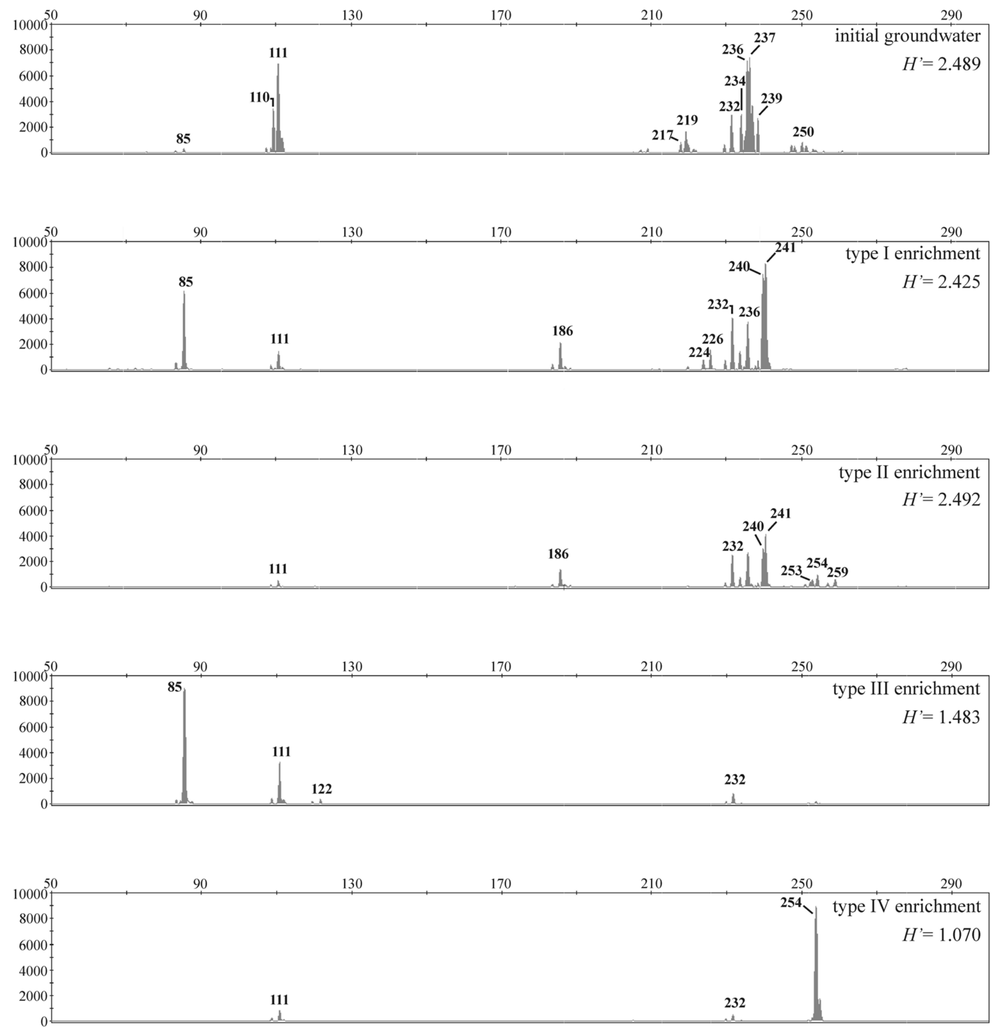

Fig. 1 16S rRNA gene T-RFLP fingerprinting of bacterial community structure in the initial groundwater and the enrichment cultures. *H'* Shannon diversity index. Only T-RFs between 50- and 300-bp length are shown, since no or only marginal T-RF peaks were observable with >300-bp length in case of enrichment cultures

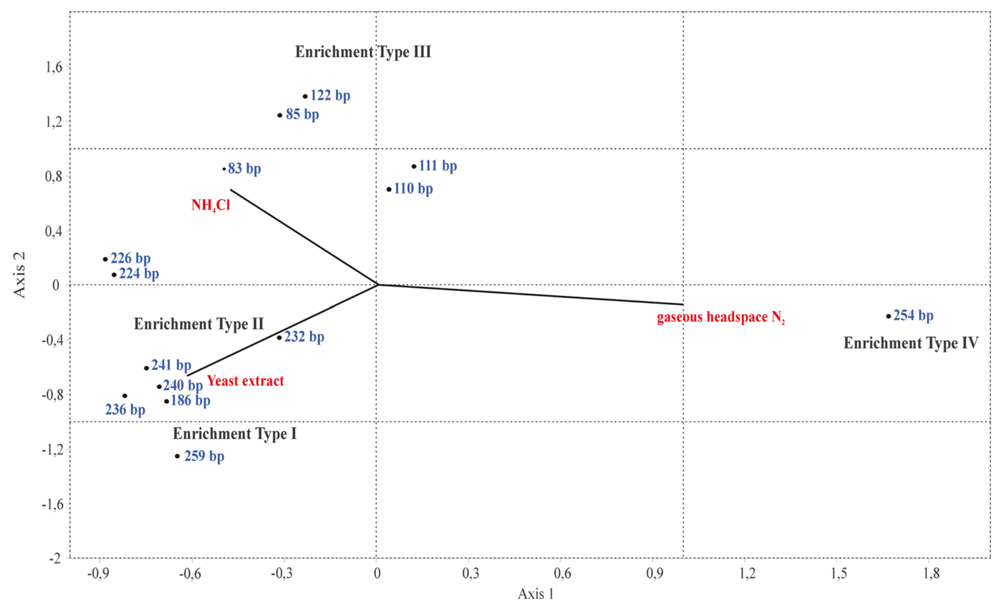

Fig. 2 Canonical correspondence analysis of 16S rDNA T-RFLP fingerprinting data of the enrichments

observable (254-bp T-RF). By analyzing the 16S clone library of this enrichment, it was found that this T-RF is also affiliated with a *Geobacter*-related clone sequence (Table 1). Its closest relative proved to be *Geobacter luticola* at a level of 97.5 % 16S rDNA sequence similarity. According to the CCA result, enrichment of this bacterium may be linked to nitrogen-fixing conditions, since in type IV enrichment, only gaseous headspace N_2 was the only nitrogen source (Fig. 2). It is well known that *Geobacter* species are usually able to fix nitrogen in the absence of ammonium (Zhuang et al. 2011). In the light of this fact, the differential enrichment of *Geobacter* species was an unexpected outcome of the present study. By analyzing the available genome sequences, it is evident that nitrogenase genes are widely distributed among *Geobacter* species. However, presence of nitrogenase genes is not necessarily true for all of them and the recently described *Geobacter soli* GSS01^T is a good example since the genome of this bacterium (Yang et al. 2015) lacks any nitrogenase gene. Therefore, one possible explanation of this differential enrichment of *Geobacter* species is that the dominant *Geobacter*-related bacterium in type I and III enrichments was not able to fix nitrogen. On the other hand, it is still a question why the predominant *Geobacter*-related bacterium of type IV enrichment was missing from the ammonium-amended enrichments. Nevertheless, it is known that minor changes in the environmental conditions may lead to the complete dominance of different organisms (Zhuang et al. 2011). Recently, the genome of *Geobacter bemidjiensis*, a member of the subsurface clade 1 of the genus, was analyzed and compared to genome sequences of non-subsurface *Geobacter* species and significant metabolic and physiological differences were revealed (Aklujkar et al. 2010). Results of the present study may indicate distinct metabolic and physiological differences between more closely related members of the genus as well.

By analyzing bacterial communities of the four enrichments, it became evident that the enrichment conditions applied were not suitable to enrich *Rhodoferax*-related bacteria. On the contrary, *Geobacter* species became to be dominant community members in type I, III, and IV enrichments. This phenomenon is most probably due to the fact that acetate was used as sole source of carbon and energy in the enrichments. Previously, it was observed by Mouser et al. (2009) that addition of acetate in millimolar concentrations to the subsurface at a uranium-contaminated site caused the dominancy of *Geobacter* over other Fe(III)-reducing microbes (e.g., *Rhodoferax*) regardless of ammonium availability. Our results further confirmed this observation. Besides, members of the genus *Pseudomonas* were always detectable (111-bp T-RF on the electropherograms) in the enrichments. Another T-RF with 232-bp length was also commonly detectable. By analyzing the 16S clone libraries, it could be assumed that this latter T-RF can be linked to yet uncultured, distantly *Azoarcus*-related bacteria.

Detection and diversity of subfamily I.2.C-type *C23O* and *bssA* genes

Unfortunately, subfamily I.2.C-type *C23O*s were not detected in any type of the enrichments, although they were present in the IG sample (data not shown) similarly as it was observed earlier (Táncsics et al. 2013). On the contrary, *bssA* genes were detected both in the initial sample and in the enrichments. The largest *bssA* gene diversity was observed in the IG sample. In this case, *bssA* clone sequences could be classified into nine OTUs, most of them affiliating with betaproteobacterial *bssA* gene sequences (Fig. 3). Clearly, *Geobacter*-affiliated *bssA* genes were not detected in the *bssA* clone library of the initial sample. Sequences of OTU3 (19 % of *bssA* clones) showed only low similarity with known *bssA* genes. The most closely related sequence type was an environmental clone (originated from "Testfeld Süd," Germany, 83.3 % sequence similarity) representing a yet unidentified, most probably non-betaproteobacterial *bssA* homologue.

In the enrichment cultures, remarkably decreased *bssA* diversities were found compared to the IG sample. Moreover, *bssA* sequences of enrichments did not match with any sequences found in the initial sample. The dominant *bssA* genotype was the same in all types of enrichments and showed the largest similarity with an environmental clone (originated from Testfeld Süd, Germany, 89.1 % sequence similarity) followed by *bssA* gene of *Azoarcus aromaticum* EbN1 (88.7 % sequence similarity). Consequently, yet unknown possibly dissimilatory Fe(III)-reducing, distantly *Azoarcus*-related bacteria harboring *bssA* gene were possibly present in all of the enrichments. With the exception of type I enrichment, a common minor *bssA* sequence type was also detectable in the enrichments, showing the largest similarity with *bssA* gene of *Thauera* sp. DNT-1, but only at a sequence similarity level of 93.2 %. Although *Geobacter* species were dominant bacteria in type I, III, and IV enrichments, *Geobacter*-affiliated *bssA* genes were not detected in these bacterial communities.

Conclusions

In essence, notable *Geobacter* and *Rhodoferax* populations and high diversity of *bssA* genes were found in the initial bacterial community but without the detection of any *Geobacter*-affiliated *bssA* sequence. The enrichment of

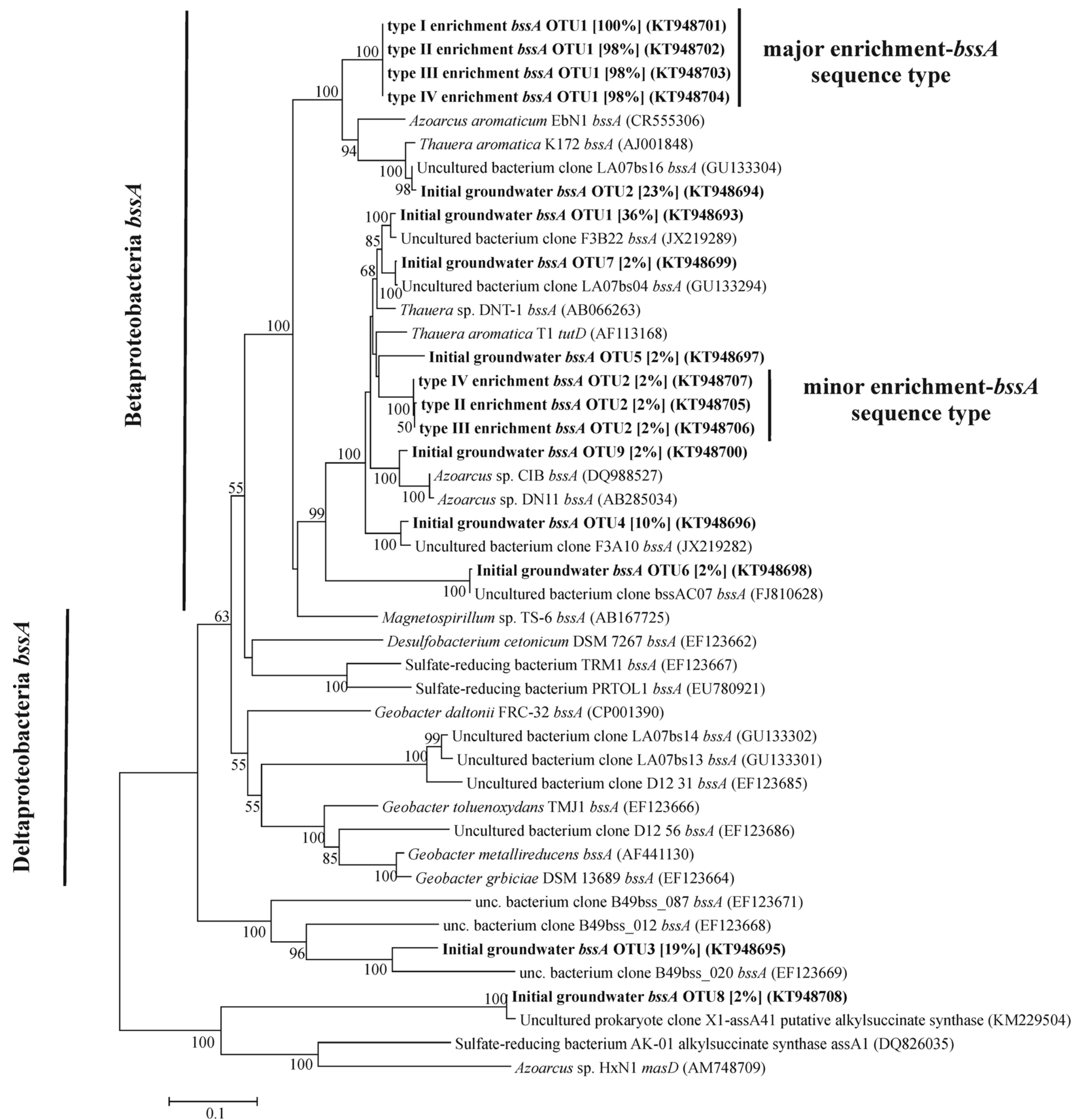

Fig. 3 Phylogenetic tree of *bssA* clone sequences obtained during the study. Clone sequences that are described in the present study are indicated with *boldface type*, while percentages in *squared brackets* represent their abundance in the appropriate clone library. Bootstrap values are shown as percent of 1000 trees, and values below 50 % are omitted. *Scale bar* represents substitutions per nucleotide position

Rhodoferax-related bacteria by using the four slightly different enrichment media was not succeeded, and subfamily I.2.C-type *C23O* genes were also not detectable in the enrichments. On the other hand, *Geobacter*-related bacteria became most dominant in three of the enrichments, and distinct *Geobacter* phylotypes were observable under different nitrogen availability. Nevertheless, only *Betaproteobacteria*-affiliated *bssA* sequences were found even in the *Geobacter*-dominated enrichments. Accordingly, it is highly assumed that although a notable *Geobacter* population can be observed at the Siklós BTEX-contaminated site, these bacteria may not play significant role in the anaerobic degradation of toluene in this environment.

Further, *Rhodoferax* enrichment experiments will be performed with other carbon sources, including malic acid that is universally catabolized by purple non-sulfur bacteria. Besides, the role of *Geobacter* population in the degradation of BTEX-compounds at the Siklós site will be further investigated by revealing the diversity of *bamA* gene, encoding for 6-oxocyclohex-1-ene-1-carboxyl-CoA hydrolase, which enzyme catalyzes the cleavage of the aromatic ring structure under anaerobic conditions.

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