Microbial degradation of gasoline ether oxygenates: isolation and characterization of a novel MTBE-degrading bacterial strain, *Methylibium* sp. T29

Theses of the PhD Thesis

Zsolt Szabó

Supervisor: Dr. Zoltán Bihari

Lead Researcher

Bay Zoltán Nonprofit Ltd. for Applied Research

Division for Biotechnology (BAY-BIO)

University of Szeged

Faculty of Science and Informatics

Doctoral School of Environmental Sciences

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Introduction

Gasolines have to meet continuously growing demands and rigorous quality standards worldwide. Several additives are incorporated into different types of fuels in order to improve their physical and chemical properties and enable them to satisfy the specific guidelines of environmental and health regulations. Among them are fuel ethers, which, beyond increasing octane ratings, significantly contribute to the more complete combustion of gasolines.

However, particularly due to their chemical structure, these compounds are much more recalcitrant to natural physicochemical and biological degradation processes than other components of gasoline. Hence, because of their excellent water solubility they can generate extensive and persistent pollutions in aquatic environments, posing a serious threat to drinking water supplies. Their most widespread and most abundantly used representative, methyl *tert*-butyl ether (MTBE), was classified as a potential human carcinogen.

According to estimates, the global needs for fuel ethers will be steadily high in the following decades, so the size of impacted areas is expected to be increasing. Accordingly, in Hungary, the number of sites contaminated with MTBE has been growing in recent years and their remediation may start in the near future. For this purpose, bioaugmentation can be the ideal alternative in terms of cost efficiency, but it requires a microbiologically and molecular biologically well-characterized and traceable bacterial strain which is capable of the efficient degradation of the target compound.

Objectives

We aimed to:

1) isolate bacterial consortia capable of the degradation of common fuel oxygenates using inland groundwater samples originating from sites polluted with MTBE-containing gasoline and/or other mixed hydrocarbon compounds.
2) determine the microbial diversity of the isolated consortia and hereby, assess local MTBE-degrading potentials.
3) isolate pure bacterial strains from the most effective enrichments, which are able to utilize MTBE and related fuel ethers as the sole sources of carbon and energy.
4) describe the ideal growth parameters of the individual isolates necessary for the routine lab-scale cultivation and optimum MTBE degradation.
5) carry out the detailed analytical and microbiological investigation of pure strains, and as part of this, determine the rate of MTBE degradation, analyze the metabolic pathway of MTBE utilization and give the range of the metabolizable substrates.
6) identify the key genes involved in the biodegradation of MTBE, which might enable the tracking of the applied strain in the field during upcoming bioremediation processes.

Applied methods

1) Isolation of bacterial consortia being able to degrade MTBE and related fuel oxygenates from groundwater samples by enrichment in liquid media containing MTBE as the sole source of carbon and energy.
2) Microbial diversity analysis of the isolated consortia capable of efficient biodegradation of fuel oxygenates using pyrosequencing.
3) Isolation of MTBE-degrading pure strains from the consortia on solid media containing MTBE or *tert*-butyl alcohol (TBA) as the sole sources of carbon and energy.
4) Identification of the isolated individual bacterial strains by the amplification of their 16S rDNA sequences by colony PCR reactions followed by Sanger sequencing and comparing the data to nucleic acid databases.
5) Detection of the genes previously described to be involved in the degradation of fuel ethers in our consortia and individual isolates by target-specific PCR reactions.
6) Monitoring the rate of MTBE degradation and identifying the possible intermediates of the biodegradation by GC-MS analytics.
7) Comparison of the genomic patterns of the isolates belonging to *Methylibium* genus with pulsed field gel electrophoresis (PFGE) after treated with different restriction enzymes.
8) Detection of the plasmids of *Methylibium* strains and determining their approximate size with S1-PFGE analysis.
9) *De novo* genome sequencing of *Methylibium* sp. T29 and T29-B strains and *de novo* sequencing of plasmids pT29A and pT29B using high throughput Ion Torrent™ next-generation sequencing technology.
10) Analysis of the transformation efficiencies of *Methylibium* strains by preparing electrocompetent cells and transforming them with electroporation.
11) Examination of *mdpA*, one of the key genes in MTBE biodegradation, by creating insertion knockout mutant lines from wild-type *Methylibium* sp. T29 and monitoring their substrate utilization with GC-MS analytics.

Results

1) We succeeded to isolate five MTBE-degrading consortia (5K, 5L, 8K, RL and SC) during laboratory enrichments using diverse groundwater samples from all across Hungary. All of them were able to degrade *tert*-amyl methyl ether (TAME) and TBA as well. Moreover, SC could grow on ethyl *tert*-butyl ether (ETBE), too. All of the consortia were enriched from samples originating from the area of Tiszaújváros and Zalaegerszeg, so in the other locations probably there was no existing endemic MTBE-degrading microbial activity. The microbial diversity of the most efficient MTBE-degrader „RL" and the most versatile „SC" consortia was analyzed with pyrosequencing. From the results we concluded that the predominant phylum and class was *Proteobacteria* and *Alphaproteobacteria*, respectively, in both of our enrichments. Among the identified genera, the proportion of *Methylibium* was remarkably high in both consortia.
2) From the consortia we isolated six individual MTBE-degrading strains (M2, M6, M15, M28, M48 and T29) and the 16S rDNA sequence homology analyses indicated that their closest relative is *Methylibium petroleiphilum* PM1. Out of these isolates, strain T29 proved to be the most effective MTBE-degrader based on preliminary analytical data. None of the genes known to be involved in MTBE degradation could be detected in isolate T29 by gene-specific PCR reactions.
3) The 16S rDNA sequence of strain T29 is identical to PM1's, the cells stained Gram-negative, showed coccobacillus morphology and a fraction of them possessed a single polar flagellum. On ½ × tryptic soy agar (TSA) plates it formed cream-colored colonies and produced a brown extracellular pigment molecule resembling pyomelanin. The strain was deposited as *Methylibium* sp. T29 in the National Collection of Agricultural and Industrial Microorganisms under the accession number NCAIM B.02561.
4) Of the fuel oxygenates tested, similarly to PM1, *Methylibium* sp. T29 could utilize MTBE, TAME and TBA as the sole source of carbon and energy. The most significant metabolic differences between the two strains could be observed regarding the biodegradation of aliphatic and aromatic hydrocarbons. Since T29, unlike PM1, could not grow on *n*-alkanes and of the BTEX components it could only utilize benzene for growth. Antibiotic and heavy metal resistance experiments revealed that T29 was

resistant to ampicillin, tetracycline and mercury (Hg^{2+}), while PM1 was sensitive to all of these compounds.

5) Under the applied experimental conditions, T29 and PM1 had practically identical MTBE-degrading rates. Nevertheless, we observed some transient TBA accumulation during MTBE degradation at PM1 which might suggest that the conversion of TBA is somewhat slower in PM1 than in T29. We could identify TBA and acetone as degradation intermediates in T29, so we assume that the MTBE degradation pathway proceeds through TBA to 2-hydroxyisobutyric acid (2-HIBA), which is then converted into isopropanol by a decarboxylase. Isopropanol is transformed into acetone by a dehydrogenase, followed by oxidation reactions resulting in pyruvate as the end product, which enters the central metabolic pathways.
6) Comparison of the genomes of *Methylibium* sp. T29, *Methylibium* sp. M28 and *Methylibium petroleiphilum* PM1 with pulsed field gel electrophoresis revealed that despite the very high degree of relatedness (≥99% identity in the 16S rDNA sequences) there are major differences among the three isolates at the genomic level. This observation served as a possible explanation for the fail of the earlier PCR-based gene detections in T29.
7) During long-term culturing of *Methylibium* sp. T29 on non-selective media we isolated a spontaneous loss-of-function mutant line, designated as T29-B, which lost its ability to grow on MTBE and TAME, but still grew well on TBA. Moreover, it was resistant to ampicillin and tetracycline, but could not grow in the presence of Hg^{2+}. We also separated a mutant PM1 line (PM1-B) which could grow neither on MTBE and TAME nor on TBA and was sensitive to ampicillin, tetracycline and Hg^{2+}.
8) By S1-PFGE analyses we provided the first experimental evidence for the presence of the 600 kb megaplasmid (pPM1) in PM1. We detected a plasmid both in T29 and in T29-B with a similar size of approximately 50-90 kb and designated them as pT29A and pT29B, respectively, but there was no sign of plasmids in M28 and in PM1-B. Consequently, we proved that T29 does not harbor the PM1-type megaplasmid and thus, we supposed that the genetic arrangement of the two strains was substantially different.
9) The draft genome sequence of *Methylibium* sp. T29 showed an average identity of 97% to the PM1 chromosome and 85% to a small part of the pPM1 megaplasmid. The pT29A plasmid was found to be ~87 kb in size, but contrary to our previous assumptions, we found neither plasmid-bound antibiotic resistance genes nor ones

directly involved in MTBE degradation. We could only identify a number of elements of mercury resistance and a cobalamin synthesis operon, which may have a role in the transformation of 2-HIBA.

10) Based on nucleotide sequence homologies, using the complete genome sequence of PM1 as the reference, we could find all the putative genes involved in the MTBE metabolism of T29 previously described in PM1. However, in contrast to the considerably high similarity of the majority of the two genomes, the genes involved in the MTBE metabolism of PM1 and T29 show significantly lower sequence conservation and different localization (plasmid versus chromosome). By these observations we hypothesize that these genes are located on a mobile genetic element which resides on the megaplasmid in PM1 and on the chromosome in T29. We identified several unique sequences in the genome of T29, missing from PM1's, coding for resistances to different antibiotics and heavy metals.
11) Comparative analysis of the draft genome sequences of *Methylibium* sp.T29 and T29-B indicated that 84 genes are missing from T29-B. During preliminary transcriptomic analyses we could not identify any possible candidate sequences responsible for the loss of MTBE degrading ability in T29-B, so finding these genes requires further investigations.
12) According to our results, T29 can be transformed much more efficiently with both of the tested electroporation protocols than PM1. The achieved maximum transformation efficiency was almost thirty times higher at T29 than at PM1. Furthermore, one of the methods, originally described for the transformation of *Pseudomonas* spp., was successfully adapted to *Methylibium* strains and proved to be more effective at both strains than the one published for the transformation of PM1 before.
13) By creating insertion knockout mutant lines and analyzing their substrate utilization we verified that the *mdpA* gene has an important role in the biodegradation of all tested fuel oxygenates in *Methylibium* sp. T29. Moreover, it is likely that the function and regulation of *mdpA* are considerably different in T29 and in PM1. Using primers designed to amplify the *mdpA* gene of T29 we could successfully detect the strain from environmental samples, so the gene can be used as a molecular marker to track our isolate in the field.
14) Our results establish the future application of strain *Methylibium* sp. T29 during remediation activities at Hungarian sites polluted with MTBE.

Peer-Reviewed Journal Articles

1.[*] **Szabó Z**, Gyula P, Robotka H, Bató E, Gálik B, Pach P, Pekker P, Papp I, Bihari Z: **Draft genome sequence of *Methylibium* sp. strain T29, a novel fuel oxygenate-degrading bacterial isolate from Hungary**. *Stand Genomic Sci* 2015, **doi**:10.1186/s40793-015-0023-z.
IF: 3.167

2. Bihari Z, Szvetnik A, **Szabó Z**, Blastyák A, Zombori Z, Balázs M, Kiss I: **Functional analysis of long-chain *n*-alkane degradation by *Dietzia* spp.** *FEMS Microbiol Lett* 2011, **316**:100-107.
IF: 2.044

3. Szvetnik A, Bihari Z, **Szabó Z**, Kelemen O, Kiss I: **Genetic manipulation tools for *Dietzia* spp.** *J Appl Microbiol* 2010, **109**:1845-1852.
IF: 2.365

4. Bihari Z, **Szabó Z**, Szvetnik A, Balázs M, Bartos P, Tolmacsov P, Zombori Z, Kiss I: **Characterization of a novel long-chain *n*-alkane-degrading strain, *Dietzia* sp. E1**. *Z Naturforsch C* 2010, **65**:693-700.
IF: 0.718

5. Bihari Z, Vidéki D, Mihalik E, Szvetnik A, **Szabó Z**, Balázs M, Kesserű P, Kiss I: **Degradation of native feathers by a novel keratinase-producing, thermophilic isolate, *Brevibacillus thermoruber* T1E**. *Z Naturforsch C* 2010, **65**:134-140.
IF: 0.718

Cumulative impact factor: 9.012

Posters

1. **Szabó Z**, Izing I, László T, Balázs M, Kiss I, Bihari Z: **A case study of the bioremediation of a methyl *tert*-butyl ether-polluted Hungarian aquifer**. *IV International Conference on Environmental, Industrial and Applied Microbiology (BioMicroWorld2011), Torremolinos, Málaga, Spain; 14-16 September 2011*.

[*] Publication served as the basis for the PhD thesis.

2. Bihari Z, Szvetnik A, **Szabó Z**, Blastyák A, Zombori Z, Balázs M, Kiss I: **Functional analysis of *n*-alkane degradation by *Dietzia* spp**. *IV International Conference on Environmental, Industrial and Applied Microbiology (BioMicroWorld2011), Torremolinos, Málaga, Spain; 14-16 September 2011*.