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Ethyl *tert*-butyl ether (ETBE)-degrading microbial communities in enrichments from polluted environments

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HIGHLIGHTS

- The degradation of ETBE was studied in five aerobic enrichment cultures.
- ETBE was degraded in each case with different rates of degradation.
- Several bacterial strains were isolated that could degrade ETBE.
- *Actinobacteria* appeared to play a crucial role in ETBE degradation.

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ABSTRACT

The ethyl *tert*-butyl ether (ETBE) degradation capacity and phylogenetic composition of five aerobic enrichment cultures with ETBE as the sole carbon and energy source were studied. In all cases, ETBE was entirely degraded to biomass and CO_2. Clone libraries of the 16S rRNA gene were prepared from each enrichment. The analyses of the DNA sequences obtained showed different taxonomic compositions with a majority of *Proteobacteria* in three cases. The two other enrichments have different microbiota with an abundance of *Acidobacteria* in one case, whereas the microbiota in the second was more diverse (majority of *Actinobacteria*, *Chlorobi* and *Gemmatimonadetes*). *Actinobacteria* were detected in all five enrichments. Several bacterial strains were isolated from the enrichments and five were capable of degrading ETBE and/or *tert*-butyl alcohol (TBA), a degradation intermediate. The five included three *Rhodococcus* sp. (IFP 2040, IFP 2041, IFP 2043), one *Betaproteobacteria* (IFP 2047) belonging to the *Rubrivivax*/*Leptothrix*/*Ideonella* branch, and one *Pseudonocardia* sp. (IFP 2050). Quantification of these five strains and two other strains, *Rhodococcus* sp. IFP 2042 and *Bradyrhizobium* sp. IFP2049, which had been previously isolated from one of the enrichments was carried out on the different enrichments based on quantitative PCR with specific 16S rRNA gene primers and the results were consistent with the hypothesized role of *Actinobacteria* and *Betaproteobacteria* in the degradation of ETBE and the possible role of *Bradyrhizobium* strains in the degradation of TBA.

1. Introduction

Since the 1970s, fuel oxygenates, methyl *tert*-butyl ether (MTBE), ethyl *tert*-butyl ether (ETBE) or *tert*-amyl methyl ether (TAME), were used to replace tetra-alkyl lead as octane enhancers in gasoline [1]. Due to their high water solubility (MTBE: 48 g L^{-1}; ETBE: 12 g L^{-1}; TAME: 12 g L^{-1}) [2], their release in soil and groundwater when gasoline tanks leak could affect water [3,4]. MTBE pollution in groundwater was well documented, especially in the USA, where MTBE was utilized since the 1980s [5,6]. MTBE was banned in several states in the USA and its use has decreased progressively. However, ETBE is increasingly used to replace MTBE in Europe with a total 2011 ETBE consumption around 3 million metric tons. Despite the increasing amount of ethanol direct blending, the ETBE market shows a positive growth trend (Source: The European Fuel Oxygenates Association) and is one way of using bioethanol (transformed to ETBE). ETBE concentration in gasoline can reach 22% (v/v) (http://www.biofuelstp.eu/etbe.html). ETBE

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has an advantage over ethanol because it produces less CO_2 per unit of energy and lower greenhouse gas (GHG) emissions than ethanol [7]. In Europe, ETBE is produced in France, Germany, the Netherlands, Spain and Poland. Despite the large volume of ETBE produced, the environmental fate of ETBE was not evaluated and the actual level of soil or groundwater contamination is not known except in Dutch drinking water [4] and in France where ETBE contamination of an aquifer was reported [8].

ETBE in water renders it undrinkable due to the low taste detection threshold, 1–2 $\mu g\,L^{-1}$ [4]. Recent studies have reported that at similar concentrations (as with MTBE), ETBE can disrupt multiple organ systems (including the heart and the skeletal system) or water regulation in the organism and significantly alter the mRNA transcript levels of genes required for cellular development in zebra fish embryos [9]. Another study on rats indicated that ETBE has carcinogenic effects [10]. Nevertheless safety assessment studies to evaluate ETBE carcinogenicity in humans are lacking [11].

Studies have described ETBE degradation by several microorganisms, especially from the phylum *Actinobacteria*. *Rhodococcus ruber* IFP 2001 was isolated for its capacity to grow on ETBE with a concomitant accumulation of *tert*-butyl alcohol (TBA) [12]. Other *Rhodococci* utilizing ETBE as the sole carbon source were isolated: *Rhodococcus zopfii* IFP 2005, *Gordonia* sp. IFP 2009 [13], *Rhodococcus wratislaviensis* IFP 2016, and *Rhodococcus aetherivorans* IFP 2017 [14]. Other strains able to grow on MTBE, *Hydrogenophaga flava* ENV 735 [5], *Aquincola tertiaricarbonis* L108 [15], *Variovorax paradoxus* CL-8 [16], *Mycobacterium austroafricanum* IFP 2012 and IFP 2015 [17], were also able to degrade ETBE. From an enrichment culture, US6, degrading ETBE, we isolated two microorganisms working as a consortium, *Rhodococcus* sp. IFP 2042,which transformed ETBE to TBA, and *Bradyrhizobium* sp. IFP 2049, which grew on the TBA produced [18]. In a recent study, the addition of ETBE to soil microbiota resulted in increased microbial respiration. Moreover, phospholipid fatty acids (PLFA) measurements indicated that Gram-positive bacteria became dominant [19].

The aim of our work was to study the ETBE degradation capacities and phylogenetic composition of five ETBE enrichments. This was accomplished by measuring the ETBE degradation, identifying the predominant microbes of the enrichment members (with 16S rRNA gene clone libraries), isolating microorganisms with ETBE and/or TBA degradation capacities and quantifying the relative proportion of each of these isolates in the different enrichments.

2. Materials and methods

2.1. Growth media and culture conditions

Mineral medium (MM) was prepared as previously described [14]. The solid medium used for isolation procedure was trypticase soy agar (TSA) diluted 1/10 (Bacto, Australia). The plates were incubated at 30 °C.

For the cloning step, Luria-Bertani agar (LBA) medium (Duchefa Biochimie, the Netherlands) was used for the growth of the clones. Kanamycin (50 $\mu g\,mL^{-1}$) was added as a selective agent according to the protocol in *E. coli* Top10 cells. The plates were incubated at 37 °C.

2.2. Enrichment procedure

Five environmental samples of groundwater or soil were collected from differently polluted sites in different geographic areas, Belgium (BE1), Germany (GE1), USA (US6) and France (FR3, FR5) (Table 1) and used as inoculum (20%, v/v for the groundwaters and w/v for the soil). The biodegradation capacities of ETBE or MTBE or TBA were evaluated for each sample in 150 mL of MM with ETBE or MTBE or TBA (200 $mg\,L^{-1}$) under constant shaking (100 rpm) at 30 °C and the cultures were incubated as long as necessary to obtain a total biodegradation of ETBE or MTBE or TBA. The cultures were sampled (0.5 mL) weekly during the first month of incubation, then monthly for the following period. In the case of ETBE, when it was completely biodegraded, two other successive ETBE additions (200 $mg\,L^{-1}$) were performed and the biodegradation was monitored to confirm the degradation continued [2]. Then, the cultures were successively transferred twice in MM (20%, v/v) containing ETBE (200 $mg\,L^{-1}$). The oxygenates (ETBE, MTBE) and metabolites (e.g., TBA) were monitored over time on 1 μL-liquid samples after being filtered (0.22 μm) by gas chromatography/flame ionization detection (GC/FID). These ETBE cultures were considered as pre-enrichment cultures, aliquoted and stored at −80 °C. To perform the enrichments for the present study, samples of frozen ETBE pre-enrichments were grown in 150 mL MM containing ETBE (200 $mg\,L^{-1}$) at 30 °C under constant shaking (100 rpm). After ETBE degradation, they were transferred in 3 L flasks (inoculum: 150 mL; MM: 350 mL) with regular additions of ETBE.

2.3. ETBE degradation kinetics

For each enrichment, a pre-culture inoculated with a frozen aliquot was prepared on MM (150 mL) containing ETBE (200 $mg\,L^{-1}$). Then, three sets of experiments were carried out. They were all prepared in parallel, inoculated from the same preculture and cultivated under similar conditions as described below: (i) three flasks (triplicates) were dedicated to measuring ETBE concentration, (ii) two flasks (duplicates) were dedicated to measuring CO_2 and (iii) three flasks (triplicates) were dedicated to biomass determination.

All these experiments were carried out in tightly closed 500 mL Schott flasks equipped with a side arm and a septum and sealed to avoid any substrate losses. Bacterial cells of each pre-culture grown on ETBE were harvested by centrifugation (10,000 × *g* for 10 min), washed with 50 mL of MM, and suspended in 45 mL of MM. Five millilitres of this suspension were used as inoculum in each 500 mL Schott flasks containing 150 mL MM containing ETBE (150 $mg\,L^{-1}$). The volume of headspace was sufficient to prevent any O_2 limitation. After inoculation and ETBE addition, cultures were incubated at 30 °C under shaking (120 rpm). Abiotic controls consisted of flasks containing ETBE-supplemented MM and incubated similarly. ETBE and TBA were regularly quantified by GC/FID. CO_2 in the gas phase was measured by gas chromatography/thermal conductivity detection (GC/TCD). Biomass production was evaluated by measuring the dry weight at the end of the incubation.

2.4. Isolation procedure

Samples from each enrichment were serially diluted and plated onto TSA (diluted 1/10). The plates were incubated at 30 °C as long as necessary to allow the growth of colonies. Individual colonies were picked and streaked onto TSA (diluted 1/10) for isolation. Each isolate was checked for purity by re-streaking and then tested for ETBE and TBA biodegradation capacities in liquid MM containing ETBE or TBA. DNA was extracted and the 16S rRNA gene (*rrs*) was sequenced.

2.5. Strains and preservation

Stock cultures of the enrichments and of newly isolated strains (IFP 2040, IFP 2041, IFP 2043, IFP 2047, IFP 2050) as well as previously isolated strains *Rhodococcus* sp. IFP 2042 and *Bradyrhizobium* sp. IFP 2049 were kept frozen at −80 °C in MM containing 20% glycerol (v/v). Strain IFP 2050 was deposited at the CNCM (Pasteur Institute, France) under number I-4656. Strains IFP 2042 and