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ORIGINAL PAPER

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Biodegradation of ethyl *t*-butyl ether (ETBE), methyl *t*-butyl ether (MTBE) and *t*-amyl methyl ether (TAME) by *Gordonia terrae*

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Abstract *Gordonia terrae* strain IFP 2001 was selected from activated sludge for its capacity to grow on ethyl *t*-butyl ether (ETBE) as sole carbon and energy source. ETBE was stoichiometrically degraded to *t*-butyl alcohol (TBA) and the activity was inducible. A constitutive strain, *G. terrae* IFP 2007, derived from strain IFP 2001, was also selected. Methyl *t*-butyl ether (MTBE) and *t*-amyl methyl ether (TAME) were not used as carbon and energy sources by the two strains, but cometabolic degradation of MTBE and TAME was demonstrated, to TBA and *t*-amyl alcohol (TAA) respectively, in the presence of a carbon source such as ethanol. No two-carbon compound was detected during growth on ETBE, but formate was produced during cometabolic degradation of MTBE or TAME. A monooxygenase was involved in the degradation of ethers, because no degradation of ETBE was observed under anaerobic conditions and the presence of a cytochrome P-450 was demonstrated in *G. terrae* IFP 2001 after induction by cultivation on ETBE.

Introduction

Oxygenated compounds, in particular ethers such as methyl *t*-butyl ether (MTBE), ethyl *t*-butyl ether (ETBE) and *t*-amyl methyl ether (TAME), are used to enhance the octane index of gasoline. MTBE is the most common and its widespread accidental release has been reported (Andrews 1998).

The extensive dispersion and poor natural attenuation of MTBE in soils and aquifers are due to the resistance of this product to biodegradation, its high water solubility and its low reactivity with organic matter.

Evidence from laboratory experimentation for aerobic processes of ether degradation is building up. Mixed cultures (Salanitro et al. 1994) and pure cultures (Hanson et al. 1999; Mo et al. 1997) have been reported to mineralize MTBE. Several recent studies have clearly shown the degradation of MTBE into *t*-butyl alcohol (TBA) by pure strains in processes of cometabolism linked in particular to the degradation of hydrocarbons (Garnier et al. 1999; Hardison et al. 1997; Hyman et al. 1998; Steffan et al. 1997). Such studies are important for a better understanding of the fate of ethers in the environment and also for the development of processes of bioremediation of contaminated aquifers (Salanitro et al. 1999).

Few data are available concerning ETBE biodegradation (Steffan et al. 1997; Yeh 1992). We isolated a strain of *Gordonia terrae* (*G. terrae* IFP 2001) capable of growing rapidly on ETBE and converting it into TBA (Fayolle et al. 1998). The conditions and mechanisms of biodegradation of ETBE, MTBE and TAME by the wild-type strain and by a constitutive variant, strain IFP 2007, are reported here.

Materials and methods

Bacterial strains

An aerobic Gram-positive ETBE-degrading strain, *G. terrae* IFP 2001, was isolated from activated sludge sampled at an urban waste water treatment plant (Fayolle et al. 1998) and registered at the Collection Nationale de Cultures de Microorganismes (CNCM), Paris, France under number CIP I-1889.

G. terrae IFP 2007 was isolated by subculturing *G. terrae* IFP 2001 on ETBE-supplemented mineral medium; and it expressed constitutively the ETBE degradation activity. It was also deposited at the CNCM, under number CIP I-2194. Strains were preserved in mineral medium (20% glycerol) at −80 °C.

Culture conditions

The culture media used were Luria-Bertani (LB) broth and a vitamin-supplemented mineral (MM) medium (Fayolle et al. 1998).

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Cultivation was carried out under aerobic conditions on MM medium using sealed flasks to prevent substrate volatilization. Substrate (ETBE, MTBE, TAME or ethanol) was added after inoculation (10%). Incubation was carried out at 30 °C with orbital shaking. At various times, 1.5-ml samples of the culture were filtered (0.22-μm FP Point 2 filter; Schleicher & Schuell) for substrate and product determination in the aqueous phase.

Degradation assay with resting cells

G. terrae IFP 2001 was cultivated on ETBE-supplemented MM medium and *G. terrae* IFP 2007 on LB broth. After 24 h incubation, bacteria were harvested by centrifugation (20,000 *g* for 20 min), washed twice in 100 mM Tris-HCl buffer at pH 7.0 and resuspended in Tris-HCl. Ether was added to 20-ml cell suspensions in 125-ml sealed flasks which were then incubated at 30 °C with orbital shaking. Filtered samples were analyzed. Specific activities (μmol ether degraded g^{-1} protein min^{-1}) were calculated from the maximal biodegradation rates.

Oxygen consumption experiments

Oxygen consumption by resting cells in the presence of ETBE or ethanol was monitored using an Ingold oxygen electrode (Mettler-Toledo, France) in a 7-ml glass reactor at 30 °C which was filled with a cell suspension prepared as described above in oxygen-saturated Tris-HCl buffer. The reaction was started by substrate addition (100 mg l^{-1} final concentration).

Spectrophotometric analysis of cell extracts

G. terrae IFP 2001 was cultivated either under inducing conditions (MM medium containing ETBE) or not (LB broth). Cells of both cultures were harvested, washed twice and re-suspended in 100 mM phosphate buffer (pH 7.0) containing 1 mM phenylmethylsulfonyl fluoride and 5 mM mercaptoethanol. They were then broken using a French press (three times at 10.35 kPa) and harvested (35,000 *g* for 20 min). The supernatants used as crude extracts were reduced with solid sodium dithionite ($Na_2S_2O_4$) and the presence of cytochrome P-450 was determined according to Omura and Sato (1964).

Analytical procedures

ETBE, MTBE, TAME, other ethers, TBA and *t*-amyl alcohol (TAA) were quantified as previously described (Fayolle et al. 1998). Filtered samples of the aqueous phase were directly injected into the column.

Formic and acetic acids were quantified using enzymatic kits (Boehringer-Mannheim, Meylan, France).

Formaldehyde was assayed by the chromotropic acid method (Steffan et al. 1997).

Table 1 Growth characteristics of *Gordonia terrae* IFP 2001 and IFP 2007 on ethyl *t*-butyl ether (ETBE). Growth took place in sealed flasks on minimal medium supplemented with 10 mmol ETBE l^{-1}. Inoculation (initial OD_{600} of about 0.08 for both strains) was done with washed cells of a preculture on Luria-Bertani broth. The growth phase took place over a period of about 50 h. The biomass yield was measured in g biomass g^{-1} ETBE degraded, based on final determinations

Strain	*G. terrae* IFP 2001	*G. terrae* IFP 2007
μ_{max} (h^{-1})	0.042 ± 0.002	0.040 ± 0.002
Biomass yield	0.20 ± 0.02	0.24 ± 0.05
Lag time (h)	≥20	0–0.5

Cell growth was monitored by measuring the optical density at 600 nm; and biomass concentrations [g cells (dry weight) l^{-1}] were obtained from direct determinations.

Protein concentrations were estimated using a kit (Bio-Rad, Germany).

Chemicals

MTBE (98%), TAME (97%), ETBE (99%) and TBA (99.5%) were purchased from Aldrich (Steinheim/Albuch, Germany).

Results

Growth of *G. terrae* on ethers

The capacity of *G. terrae* IFP 2001 to grow in aerobic conditions on ETBE, MTBE or TAME supplied as the sole carbon and energy sources was first tested in sealed flasks containing MM medium. Only ETBE was used as a growth substrate; and a quantitative study of growth on ETBE was carried out. The results, summarized in Table 1, show first with strain IFP 2001 that substantial growth took place on ETBE with an appreciable exponential growth rate. Concomitant accumulation of TBA occurred in stoichiometric amounts with respect to ETBE degraded. However, a long lag phase was observed before the onset of growth (but not when the inoculum was grown on ETBE-supplemented medium), suggesting that biodegradation activity had to be induced. Accordingly, a strain constitutively expressing ETBE degradation activity, *G. terrae* IFP 2007, was isolated after repeated transfers of *G. terrae* IFP 2001 cultures on ETBE-supplemented MM medium. Using washed cells of *G. terrae* IFP 2007 grown on LB broth as inoculum, ETBE was consumed without a lag period, in accordance with the constitutive character of ETBE degradation activity in this strain. Moreover growth and TBA production profiles of strain 2007 on this medium were found to be similar to those of strain IFP 2001 (Table 1). Like strain IFP 2001, strain IFP 2007 did not grow on MTBE or TAME under these conditions (data not shown).

Degradation of ethers by resting cells of *G. terrae*

The capacity of *G. terrae* to degrade MTBE, TAME and other ethers was investigated with resting cells after growth on ETBE. The induced resting cells of strain 2001 degraded the three ethers tested (Table 2). TBA was produced from ETBE and from MTBE and TAA was produced from TAME without an initial lag phase (data not shown), indicating that ETBE-induced cells also possessed the capacity to degrade MTBE and TAME. Neither TBA nor TAA were transformed during extended incubation periods in contrast to the case of propane-oxidizing bacteria (Hyman et al. 1998; Steffan et al. 1997). Accordingly, both alcohols were

Table 2 ETBE, methyl *t*-butyl ether (MTBE) and *t*-amyl methyl ether (TAME) degradation by induced resting cells of *G. terrae* IFP 2001. Values were obtained from time course experiments of degradation, sampled at least every 2 h, using about 0.5 g cells l^{-1}, each ether (1–1.2 mmol l^{-1}) being tested separately. *TAA* *t*-amyl alcohol, *TBA* *t*-butyl alcohol

Ether tested	Product of degradation	Ratio[a] (mol alcohol produced/mol ether degraded)	Specific activity (μmol ether g^{-1} protein min^{-1})
ETBE	TBA	1.06 ± 0.15	59 ± 4
MTBE	TBA	0.90 ± 0.20	14 ± 0.5
TAME	TAA	0.91 ± 0.20	45 ± 1.5

[a] Ratio of final alcohol to initial ether concentrations

produced approximately in stoichiometric amounts with respect to the ether degraded.

The capacity of ETBE-induced resting cells to degrade other ethers was tested using a series of different ether structures as possible substrates for the degradation system. The results presented in Table 3 showed that these ethers were degraded to different extents. These data demonstrated the wide spectrum of activity of the ETBE-degradation system. Resting cells of strain 2007 also degraded MTBE and TAME. As their activity did not require induction by ETBE, they were more suitable for further studies of the mechanisms of ether degradation. As shown in Fig. 1, ETBE, MTBE and TAME were degraded without a lag period by resting cells of strain 2007 grown on LB medium. However, whereas ETBE was completely consumed in 10 h (Fig. 1a), degradation of MTBE (Fig. 1b) and TAME (Fig. 1c), into TBA and TAA respectively, was not complete and stopped much before ether exhaustion. Because of the partial degradation of MTBE and TAME, since these compounds were not used as growth substrates by *G. terrae*, ethanol (a good growth substrate) was added after degradation of MTBE and of TAME degradation had stopped (Fig. 1b, c). In both cases, ether degradation resumed, showing that cometabolism was involved in the degradation of MTBE and TAME in the presence of ethanol by the ether-degrading system.

Product formation during MTBE and TAME degradation

No two-carbon compounds (acetaldehyde, acetate) could be detected during ETBE degradation by strains 2001 and 2007, probably because they were rapidly used as carbon substrates. Because MTBE and TAME appeared to be used by cometabolism, the identification of their degradation products could provide useful information on ether metabolism. Resting cells of strain 2007 were incubated in MTBE-supplemented MM medium in sealed flasks. The cell suspensions were examined for degradation products. In addition to TBA, formate was produced in the culture medium but no formaldehyde was detected. However, whereas the accumulation of TBA was nearly stoichiometric with the consumption of MTBE, much lower amounts of formate were determined (0.3 mol formate mol^{-1} MTBE), suggesting the formation of other undetected products (data not shown).

Involvement of a cytochrome P-450 oxygenase in ether bond cleavage

Resting cells of *G. terrae* were used to test the degradation of ETBE under aerobic and anaerobic conditions. As illustrated in Fig. 2, aerobic conditions were required for ETBE degradation (Fig. 2a). Furthermore, oxygen consumption was much higher when resting cells were incubated with ETBE than with a related growth substrate, ethanol, or without substrate (Fig. 2b). This suggested that the measured rate of oxygen consumption with ETBE was essentially due to the initial step of oxidation. Accordingly, in these experiments, the rate of oxygen consumption with ETBE (1.67 ± 0.08 mmol oxygen g^{-1} h^{-1}) was similar to the rate of ETBE consumption (1.7 ± 0.1 mmol ETBE g^{-1} h^{-1}). These data suggested that the enzymatic system was an oxygenase with an oxygen:ETBE stoichiometry of 1:1.

Table 3 Degradation of various ethers by ETBE-induced resting cells of *G. terrae* IFP 2001. Cell concentration was 0.5 g l^{-i}. Each ether was tested at a final concentration of about 100 mg l^{-1}. The flasks were incubated for 24 h. Samples were analyzed at 0 h and 24 h

Substrate tested	Developed formula	Degradation (%)
Ethyl *t* -butyl ether	CH_3-CH_2-O-C-$(CH_3)_3$	100
Butyl ethyl ether	CH_3-CH_2-O-$(CH_2)_3$-CH_3	100
Ethylene glycol di-ethyl ether	CH_3-CH_2-O-$(CH_2)_2$-O-CH_2-CH_3	100
Di-butyl ether	CH_3-$(CH_2)_3$-O-$(CH_2)_3$-CH_3	100
Propylene glycol butyl ether	CH_3-$(CH_2)_3$-O-$(CH_2)_3$-OH	83
Di-isopropyl ether	$(CH_3)_2$-CH-O-CH-$(CH_3)_2$	78
t-Butoxy ethylene glycol ethyl ether	$(CH_3)_3$-C-O-$(CH_2)_2$-O-CH_2-CH_3	72
t-Butoxy ethylene glycol methyl ether	$(CH_3)_3$-C-O-$(CH_2)_2$-O-CH_3	63
Propyleneglycol propyl ether	CH_3-$(CH_2)_2$-O-$(CH_2)_3$-OH	55
Ethyleneglycol di-methyl ether	CH_3-O-$(CH_2)_2$-O-CH_3	29

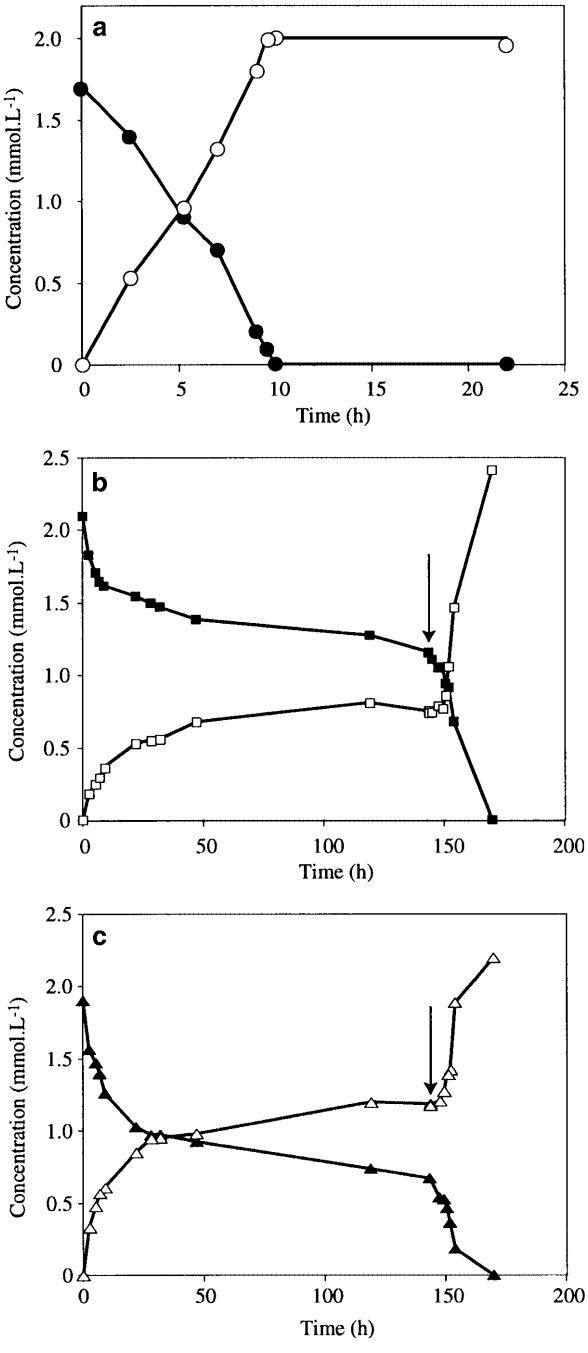

Fig. 1a–c Degradation of ethyl *t*-butyl ether (ETBE), methyl *t*-butyl ether (MTBE) and *t*-amyl methyl ether (TAME) by *Gordonia terrae* constitutive strain IFP 2007. Degradation kinetics were determined with resting cells at a concentration of 0.11 g l^{-1}, incubated in the presence of ETBE (**a**), MTBE (**b**) and TAME (**c**). Data-points represent residual concentrations of ETBE (●), MTBE (■), TAME (▲) and the concentration of *t*-butyl alcohol (TBA) formed during ETBE degradation (○) or during MTBE degradation (□) and the concentration of *t*-amyl alcohol (TAA) formed during TAME degradation (△). *Arrows* indicate addition of ethanol (2 mmol l^{-1}) after 140 h incubation in (**b**) and (**c**). Because ethers were in part present in the gaseous phase, determination in the aqueous phase led to an underestimation of 10–15% of their total concentrations

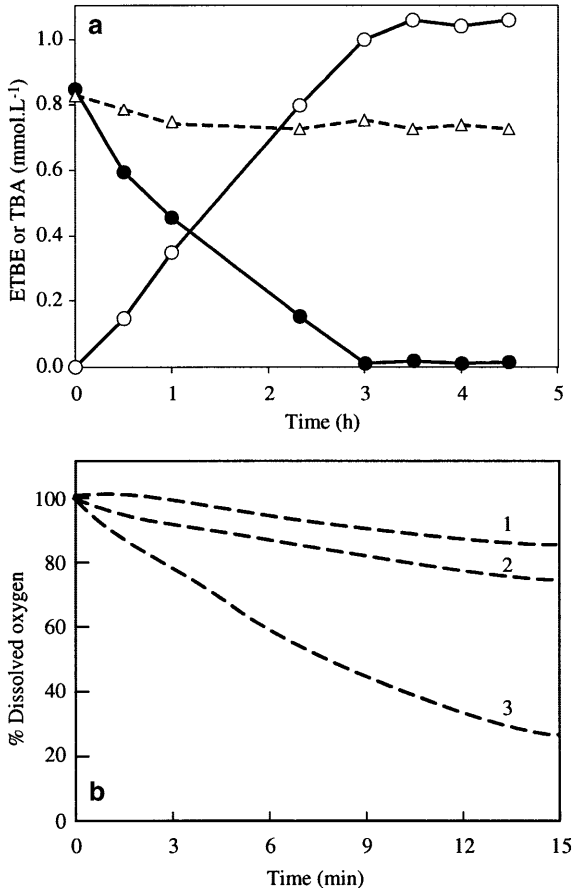

Fig. 2a, b Oxygen utilization during ETBE degradation by *G. terrae* IFP 2007. Degradation kinetics were determined with resting cells at a concentration of 0.36 g l^{-1}. **a** Oxygen requirement. Residual ETBE (●) and TBA formed (○) during ETBE degradation under aerobic conditions; and ETBE under anaerobic conditions in sealed flasks containing nitrogen-flushed Tris HCl in anaerobic chamber (△). **b** Oxygen consumption. Dissolved oxygen consumption by resting cells incubated without substrate (*curve 1*), with 2 mmol ethanol l^{-1} (*curve 2*) and with 1 mmol ETBE l^{-1} (*curve 3*)

Cytochrome P-450 is an oxygenase involved in the degradation of hydrocarbons and xenobiotics; and its possible implication in the *G. terrae* system was tested. In cell-free extracts from ETBE-induced cells of *G. terrae* 2001, the differential absorption spectra presented in Fig. 3 showed the presence of a peak close to 447 nm and corresponding to a cytochrome P-450 content of about 400 pmol mg^{-1} protein. This peak was not observed in the extracts from non-induced cells. The results thus showed that a cytochrome P-450 oxygenase was involved in ether degradation by *G. terrae*.

Discussion

Literature on the growth characteristics of pure cultures on ether oxygenates is limited and concerns only MTBE. (Hanson et al. 1999; Mo et al. 1997; Steffan et al. 1997). The available data indicate that both the growth rates and the substrate yields on this compound are low. Here,

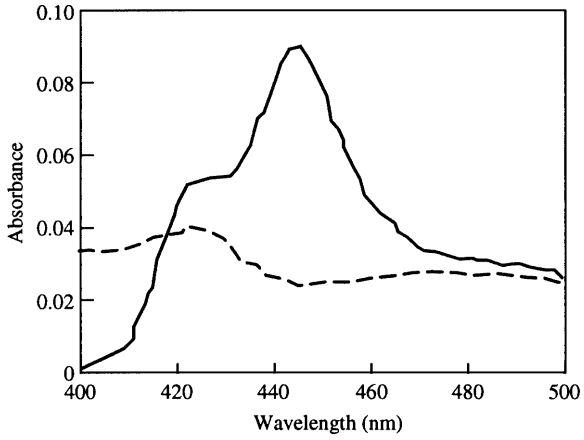

Fig. 3 Differential absorption spectra of reduced extracts of *G. terrae* IFP 2001 with and without carbon monoxide: ETBE-induced (—) and non-induced (– – –) cell extracts. Protein concentrations were 1.9 g l^{-1} for both extracts. An extinction coefficient of 91 mM^{-1} cm^{-1} (Miles et al. 1992) was used to calculate the content of cytochrome P-450 in the extracts

good growth of *G. terrae* on ETBE was observed, an important point with respect to the intrinsic biodegradability of this oxygenate. Studies with *G. terrae* allowed us to document the little known aspect of the regulation of ether degradation. Comparison of the wild-type strain (IFP 2001) to its constitutive variant (IFP 2007) showed that ETBE and other ethers were degraded by an enzymatic system with a wide specificity that could be induced in the wild-type strain by ETBE. TBA was produced from MTBE and ETBE and TAA from TAME. Accumulation of the corresponding tertiary alcohol from MTBE or TAME provided evidence that their attack by *G. terrae* occurred through the same mechanism as that observed for ETBE. No intermediates were detected during growth on ETBE but formate was produced during MTBE degradation. The formation of *t*-butyl formate has been reported during the degradation of MTBE by cometabolism in the presence of butane (Hardison et al. 1997) but was not observed here. The results also suggested that neither TAME nor MTBE were used by *G. terrae* for growth, because neither TBA nor one-carbon compounds could be used as carbon and energy sources by this bacterium, in contrast to the two-carbon compounds derived from ETBE. The results thus pointed out the structural aspects that make microorganisms growing on MTBE rare since, besides completing the initial oxygenation step, they must be methylotrophic or capable of growing on TBA, a recalcitrant compound.

MTBE and TAME degradation by *G. terrae* involved cometabolism in the presence of ethanol, which could be used as a carbon and energy source by the bacterium. Ethanol was also used as the electron source for the initial MTBE oxidation, as this step was shown to involve a cytochrome P-450 monooxygenase, a point implying the participation of a reduced co-substrate (usually NADH or NADPH). The implication of cytochrome P-450 in the degradation of MTBE has been reported in microorganisms growing on short chain alkanes (Hardison et al. 1997; Hyman et al. 1998; Steffan et al. 1997). However, *G. terrae* IFP 2001 did not grow on short chain alkanes such as propane or butane and this makes the physiological role of the *G. terrae* cytochrome P-450 system unclear at present. Also, this point indicates that the system involved is new among the MTBE/ETBE degraders reported to-date.

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