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Complete Degradation of Ethyl *tert*-Butyl Ether by Co-culture of *Rhodococcus erythropolis* ET10 and *Pseudonocardia benzenivorans* No. 8

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Rhodococcus erythropolis ET10 isolated from soil degraded ethyl *tert*-butyl ether (ETBE) and *tert*-butyl acetate (TBAc) and produced *tert*-butyl alcohol (TBA). However, metabolite TBA was not further degraded. We also isolated a TBA-degrading bacterium from andosol, which was identified as *Pseudonocardia benzenivorans* No. 8 (strain No. 8). Strain No. 8 degraded 20 mM TBA. Organic substances such as glucose, glycerol and sucrose enhanced the specific TBA degradation rates of strain No. 8. Glucose showed the highest TBA degradation effect. The co-culture of strains ET10 and No. 8 was effective for complete ETBE degradation.

Key words: biodegradation, ETBE, TBA, *Rhodococcus erythropolis*, *Pseudonocardia benzenivorans*

1. Introduction

For prevention of global warming, ETBE synthesized from a mixture of ethanol and isobutene is added to automotive gasoline to enhance its octane number [5,7]. This ETBE mixture gasoline is sold as a biological petroleum. ETBE-associated hepatotoxicity in mice is well known [20]. Moreover, ETBE contamination of water resource in the Netherlands and groundwater in Germany has been reported [28,34]. ETBE exhibits low bio-degradability [1,5,6,9,17,26]. TBA is also found at many sites around the world as a metabolite of ETBE. Therefore, cleanup of ETBE-contaminated soil and groundwater is difficult. The use of ETBE-degrading bacteria is expected to remove ETBE from polluted sites [1,26]. There is concern that TBA increases the incidence of renal tubule adenoma in male rats [2,19].

Previously, we isolated an ETBE-degrading bacterial strain from soil in Ibaraki Prefecture, Japan. The soil was placed into a vial containing mineral medium supplemented with ETBE. ETBE-degraded cultures were streaked onto mineral agar plates with ETBE. Growing colony was selected, and was purified. The strain was identified as *R. erythropolis* by morphological, physiological and 16S rRNA gene analyses and named as *R. erythropolis* ET10 (strain ET10) [24].

In the present study, we isolated a TBA-degrading bacterium identified as *Pseudonocardia benzenivorans* No. 8 (strain No. 8). The characteristics of TBA degradation by strain No. 8 were examined. Moreover, we studied the complete degradation of ETBE by co-cultures of strain No. 8 and *Rhodococcus erythropolis* ET10 (strain No. 10), which was able to partially degrade ETBE.

2. Materials and methods

2.1 Medium and chemicals

MM liquid medium contained the following components (in mg L^{-1}): NH$_4$Cl, 2,140; K$_2$HPO$_4$, 1,170; KH$_2$PO$_4$, 450; MgSO$_4$·7H$_2$O, 120; FeSO$_4$·7H$_2$O, 28; Ca(NO$_3$)$_2$·4H$_2$O, 4.8; CaCO$_3$, 100; MnSO$_4$·4-6H$_2$O, 0.6; H$_3$BO$_3$, 0.05; ZnSO$_4$·7H$_2$O, 0.1; (NH$_4$)$_6$Mo$_7$O$_{24}$·4H$_2$O, 0.01; Co(NO$_3$)$_2$·6H$_2$O, 0.6; NiSO$_4$·7H$_2$O, 0.06; and CuSO$_4$·5H$_2$O, 0.06; in Milli-Q water. The medium was sterilized by autoclaving at 121°C for 20 min. MM agar plates were made by adding 3% agar to MM liquid medium.

ETBE and TBAc were purchased from Tokyo Chemical Industry Co., Ltd. (Tokyo, Japan). TBA and lactose were purchased from Junsei Chemical Co., Ltd. (Tokyo, Japan). Reagent-grade agar, starch, glycerol, and glucose were purchased from Wako Pure Chemical Industries, Ltd. (Osaka, Japan).

2.2 Measurement of ETBE, TBA and TBA$_C$

ETBE and TBA were evaluated by the headspace gas method using a gas chromatography-mass spectrometry (GCMS) system (GC-2010/GCMS-QP2010 Ultra; Shimadzu Co., Kyoto, Japan) equipped with an Rtx®-5MS capillary column (0.25 mm i.d.×30 m; Restek Co., PA). Before injection of the sample to the GCMS, the sample vial was kept at 25°C. For measurement of ETBE and TBA concentrations, 50 μL of headspace gas was injected into the GCMS in all experiments. ETBE and TBA concentrations were calculated using a calibration curve as the amount per bottle.

2.3 Isolation of TBA-degrading bacteria

A total of 9.3 g of andosol (Kanuma City, Tochigi Prefecture, Japan) was suspended in 10 mL of axenic Milli-Q water, and 1 μL of the suspension was streaked onto an MM agar plate. The plate, with 200 μL of TBA, was sealed with Parafilm M® film (Structure Probe, Inc. and SPI Supplies, PA) and incubated statically for 7 day at 30°C. Growing colonies were collected by using a 1-μL culture loop and inoculated into 60-mL vials containing 10 mL of MM liquid medium supplemented with 10 μmol of TBA (10 μmol TBA bottle^{-1}). These vials were sealed with butyl rubber caps and crimped with aluminum rings and then incubated at 30°C with shaking at 120 rpm for 16 days. TBA degradation was evaluated using the GCMS headspace gas method. A TBA-degrading culture was selected. The culture was streaked on an MM ager plate with 200 μL of TBA and cultured at 30°C for 5 days. A white-colored colony (No. 8) was isolated. To confirm TBA degradation, 0.03 mg of strain No. 8 was inoculated into a 60-mL vial containing 10 mL of MM medium with 10 μmol of TBA. The culture was incubated at 30°C with shaking at 120 rpm for 16 days. The TBA-degradation ability of strain No. 8 was determined by the headspace GCMS method.

2.4 ETBE, TBAc and TBA degradation

The growth of strain ET10 was measured turbidometrically at 660 nm (OD$_{660}$) using a spectrophotometer (UVmini-1240, Shimadzu Co., Kyoto, Japan) and converted to dry weight (mg L^{-1}). Approximately 0.4 mg of strain ET10 was inoculated into 60-mL vials containing 10 mL of MM medium with 10 to 100 μmol of ETBE (10–100 μmol ETBE bottle^{-1}). The vials were sealed with butyl rubber stoppers and crimped with aluminum caps. These cultures were incubated at 30°C with shaking at 120 rpm for 32 days. The concentrations of ETBE, TBA, *tert*-butyl formate (TBF) and TBAc were measured by the headspace GCMS method.

2.5 TBAc degradation and TBA production in MM medium

Approximately 0.2 mg of strain ET10 was inoculated into 60-mL vials containing 10 mL of MM medium with 10 or 100 μmol of TBAc (10 or 100 μmol TBAc bottle^{-1}). These cultures were incubated at 30°C with shaking at 120 rpm for 26 days. TBAc degradation and TBA production were confirmed by GCMS using the headspace gas method.

2.6 Identification of TBA-degrading bacteria

Identification of strain No. 8 was performed biochemically using the oxidation-fermentation (OF) test, oxidase test, catalase test, assessment of motility and spore formation, Gram staining, morphological colony type, and 16S rRNA gene analyses.

Genomic DNA from an isolated bacterium was extracted using a FastDNA® for soil kit (Q-biogene) according to the manufacturer's protocol. The nearly full-length 16S rRNA gene of the isolated bacterium was PCR amplified with primers 27f (5'-AGAGTTTGATCMTGGCTCAG-3') and 1492r (5'-TACGGYTACCTTGTTACGACTT-3') using a Takara Thermal Cycler Dice Gradient (Takara Bio, Otsu, Japan). The PCR mixture consisted of PCR buffer for KOD -plus-, 0.2 U KOD -plus- DNA polymerase (TOYOBO, Osaka, Japan), 1.0 mM MgSO$_4$, 0.2 mM of each dNTP, 0.3 μM of each primer, 2 μL of template DNA, and nuclease-free water to a final volume of 10 μL. The PCR conditions were as follows: initial denaturation at 95°C for 5 min, followed by 30 cycles of denaturation at 94°C for 30 s, annealing at 55°C for 30 s, extension at 68°C for 90 s, and then final extension at 68°C 10 min. Due to TA cloning, deoxyadenine triphosphate was attached to the 3' end of the amplicons using 10× A-attachment mix (TOYOBO) according to the manufacturer's protocol. The "a"-attached amplicons were cloned into the pMD20-T vector using a Mighty TA cloning kit (Takara Bio) according to the manufacturer's protocol. The constructed vectors were transformed into *Escherichia coli* JM109 competent cells (Takara Bio). Transformed *E. coli* JM109 was cultured on Luria-Bertani plates containing 100 μg mL^{-1} ampicillin, 5-bromo-4-chloro-3-indolyl-β-d-galactopyranoside (X-gal) and isopropyl-β-d-thiogalactopyranoside (IPTG) at 37°C overnight and then characterized by blue-white selection. The white colonies were checked using direct PCR with the vector primers M13 primer M4 (5'-GTATTTCCCAGTCCGAC-3') and M13 primer RV (5'-CAGGAAACAGCTATGAC-3') and Quick *Taq* HS DyeMix (TOYOBO, Osaka, Japan) to determine whether they had an insert fragment of the correct size. Twelve *E. coli* JM109 colonies with a PCR fragment of the correct size were picked and used for further sequencing analysis. The positive fragments were sequenced using a BigDye Terminator kit v.3.1 (Applied Biosystems), with selected primers, on an Applied Biosystems 3730 DNA Analyzer (Applied Biosystems). The selected primers used for sequencing were M13 primer M4, 8f (5'-AGAGTTTGATCCTGGCTCAG-3'), 533f (5'-CAGCAGCCGCGGTAA-3'), 1114f (5'-GCAACGAGCGCAACCC-3'), 529r (5'-CGCGGCTGCTGGCAC-3'), 907r (5'-CCGTCAATTCMTTTRAGTTT-3'), 1392r (5'-ACGGGCGGTGTGT-3') and M13 primer RV. The obtained sequence fragments were assembled using ATGC sequence assembly software, ver. 7 (GENETYX, Tokyo,

Japan).

The determined 16S rRNA gene sequence of strain No. 8 was compared with reference sequences using BLASTN, and closely related sequences were obtained from the GenBank databases. Multiple alignments and the calculation of distance matrices for the aligned sequences were carried out using ClustalW, and a phylogenetic tree was constructed using the neighbor-joining method with the MEGA6 software package [33]. Bootstrap re-sampling analysis (1000 replicates) was carried out to estimate the confidence of tree topologies (Fig. 4).

2.7 Effect of TBA concentration on TBA degradation

To examine the effect of TBA concentration on TBA degradation by strain No. 8, *ca.* 0.5 to 20 μmol bottle^{-1} of TBA was added to 10 mL of MM medium with or without 0.1 mg of strain No. 8 in 60-mL vials. These cultures were incubated at 30°C for 20 days on a rotary shaker at 120 rpm. TBA degradation was measured by the headspace GCMS method.

2.8 Effect of co-culture of *R. erythropolis* ET10 and *P. benzenivorans* No. 8

The effect of co-culture of strains ET10 and No. 8 on ETBE

degradation and TBA production and degradation was also examined. Approximately 0.2 mg of strain No. 8 and 0.4 mg of strain ET10 were inoculated into 60-mL vials containing 10 mL of MM medium with 10 μmol ETBE bottle^{-1}. These cultures were maintained at 30°C with shaking at 120 rpm. The concentrations of ETBE and TBA were measured by the headspace GCMS method.

2.9 Effect of organic substances on TBA degradation by *P. benzenivorans* No. 8

The effect of organic substances on TBA degradation by strain No. 8 was determined. A total of 5 mg of glucose, lactose, sucrose, succinic acid, glycerol or acetic acid was added to 60-mL vials containing 10 mL of MM liquid medium with 10 μmol bottle^{-1} of TBA. Cultures were incubated at 30°C with shaking at 120 rpm for 21 days. TBA degradation was measured by the headspace GCMS method.

The degradation rate constant K (day^{-1}) was calculated in the logarithmic phase using the following equation (1):

$$K = (1/t - t_0) \cdot \ln (C_0/C) \tag{1},$$

C: Concentration of TBA at time t (μmol·10 mL^{-1});
C_0: Concentration of TBA at time t_0 (μmol·10 mL^{-1});
t: Cultivation time (days);
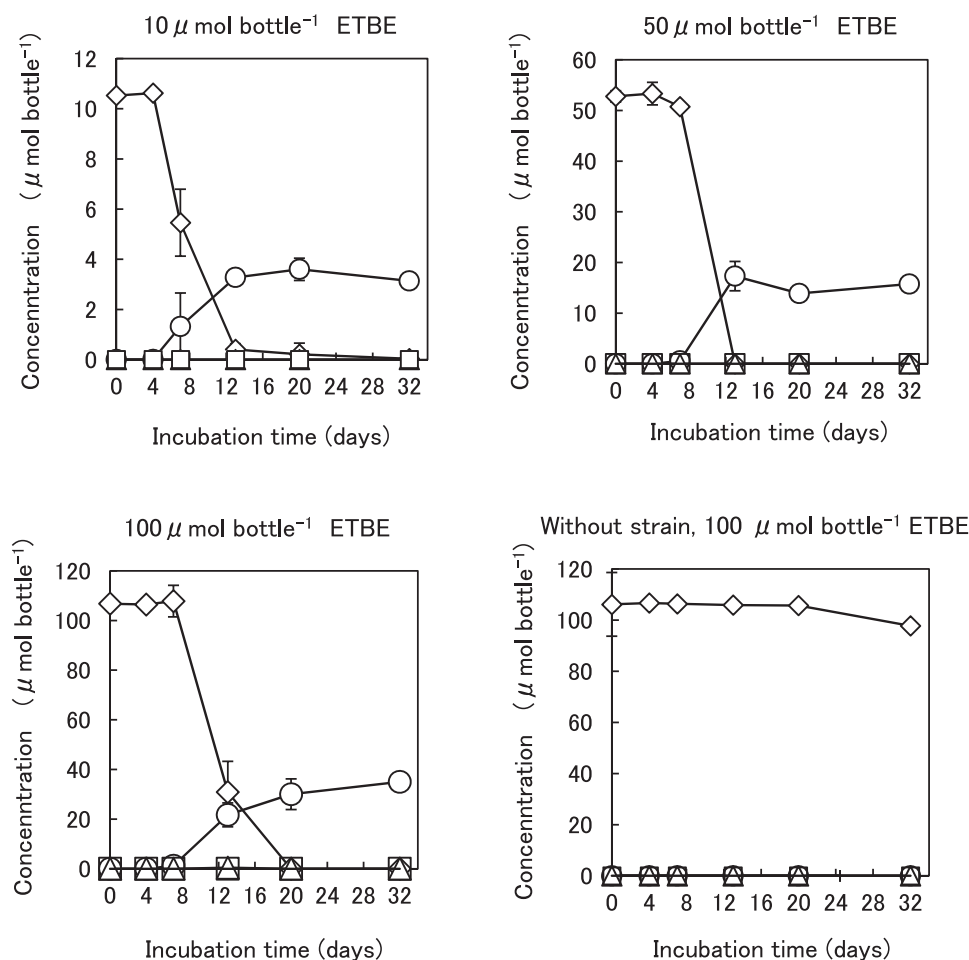t_0: Starting time of logarithmic phase (day).

Fig. 1. Effect of concentration on ETBE degradation and TBA production by strain ET10 in MM medium. Values are means (n=3), and error bars represent standard deviation. ◇, ETBE; ○, TBA; △, TBF; □, TBAc

3. Results

3.1 ETBE degradation and TBA and TBAc production by strain ET10

ETBE degradation and TBAc and TBA production by strain ET10 were investigated. ETBE was degraded over 12 to 32 days at 10 to 100 μmol bottle⁻¹ of ETBE addition. TBAc was not detected, but TBA was produced in conjunction with ETBE degradation. The concentration of TBA reached a maximum at 20 days. TBAc was not detected over 30 days of incubation (Fig. 1).

3.2 Degradation of TBAc by strain ET10

It is known that the metabolism of ETBE (in *Rhodococcus ruber* IFP 2001) proceeds from ETBE to TBA via TBAc [9]. We could not detect TBAc as a metabolite from ETBE in strain ET10 culture. Therefore, we examined the production of TBA from TBAc by strain ET10 at high and low concentrations. TBAc was completely degraded in 5 days at the low concentration, whereas 80% of TBAc was converted to TBA in 14 days at the high concentration. Removed TBAc was stoichiometrically changed to TBA (Fig. 2). From these results, we suggested that strain ET10 expressed esterase which transformed TBAc to TBA shown in Fig.6.

3.3 Isolation and characterization of a TBA-degrading bacterium

Three TBA-degrading bacteria, strain No. 8, strain No. 3S and strain No. 3M were isolated from andosol. Degradation activities of strain No. 3S and strain No. 3M were relatively low. Therefore, we adopted the strain No. 8 as an experimental strain.

Strain No. 8 was gram positive, non-motile, non–spore forming, and rod shaped. Formed colonies were smooth, and their color was pale white. Strain No. 8 was non-fermenting in the OF test. Oxidase test was negative, and catalase test was positive. A phylogenetic tree was constructed based on the 16S rRNA sequences of strain No. 8 and the most closely related bacterial species. Strain No. 8 displayed the highest homology to *Pseudonocardia benzenivorans* B5, 99.8%; *P. dioxanivorans* CB1190, 99.4%; *P. hydrocarbonoxydans* IMSNU22140, 99.1%; *P. sulfidoxydans* DSM44248, 99.1%; *P. tetrahydrofuranoxydans* K1, 98.5%. From these characteristics, it was determined that strain No. 8 was *P. benzenivorans* (Fig. 3).

3.4 Effect of concentration on TBA degradation by *P. benzenivorans* No. 8

TBA degradation was initiated at 0.5 to 5.0 μmol bottle⁻¹. TBA was completely degraded in 10 days at less than 1 μmol bottle⁻¹. At the high concentration of 20 μmol bottle⁻¹, TBA was degraded in 20 days. The high concentration thus showed a longer lag degradation time (Fig. 4).

3.5 Effect of co-culture of strains ET10 and No. 8 on ETBE and TBA degradation

ETBE degradation was determined by the co-culture of strains ET10 and No. 8. Strain ET10 degraded ETBE and produced TBA. The produced TBA was not degraded by strain ET10. The co-culture of strains ET10 and No. 8 resulted in complete degradation of ETBE in 25 days, and TBA was not detected (Fig. 5).

3.6 Effect of organic substances on the degradation of TBA by strain No. 8

Table 1 shows the effect of organic substances on the degradation rate constant (day⁻¹) of TBA for strain No. 8. The highest degradation rate constant was 0.33 day⁻¹ in
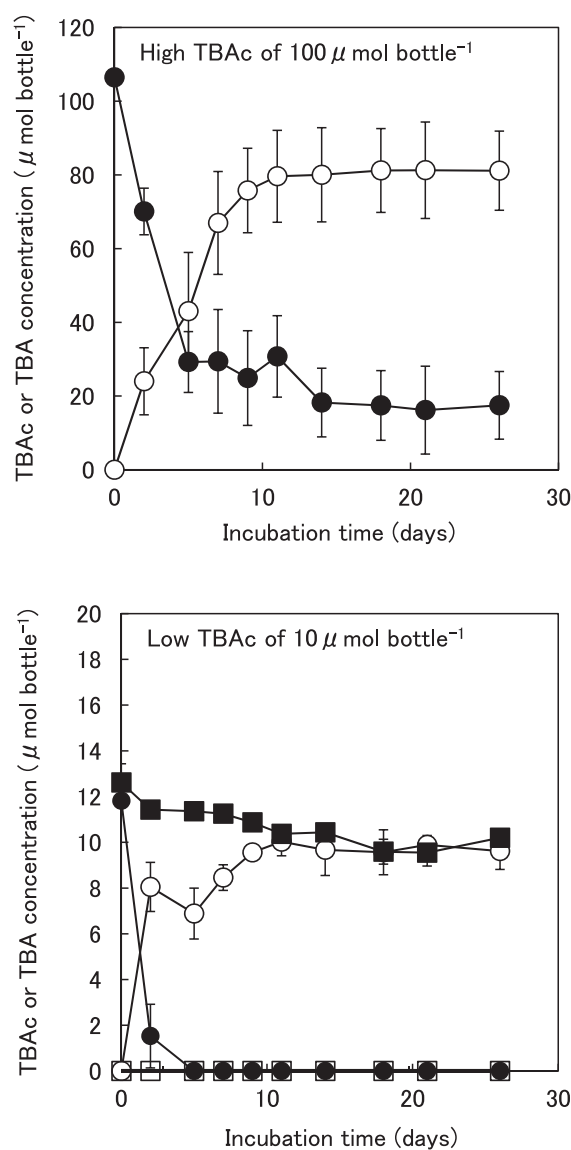

Fig. 2. Effect of TBAc concentration on TBA production in MM medium by strain ET10.
Values are means (n=3), and error bars represent standard deviation.
● ; TBAc, ○ ; TBA, ■ ; TBAc without strain ET10, □ ; TBA without strain ET10
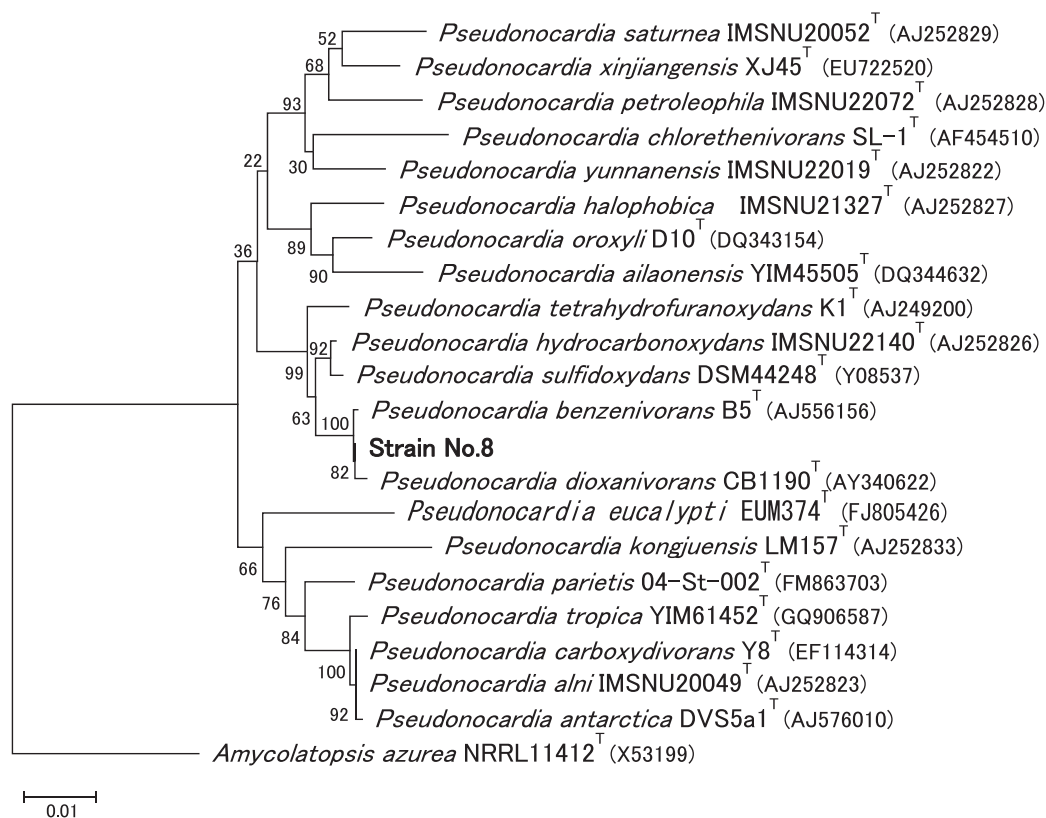
Fig. 3. Neighbour-joining tree derived from 16S rRNA gene sequences showing the relationships between strain No. 8 and related *Pseudonocardia* species.

Bootstrap percentages (based on 1000 replications) are given at branching points.

Bar, 0.01 substitutions per nucleotide position. *Amycolatopsis azurea* NRRL11412[T] was used as the outgroup to root the tree. Accession numbers are given in parentheses. [T]; type strain.
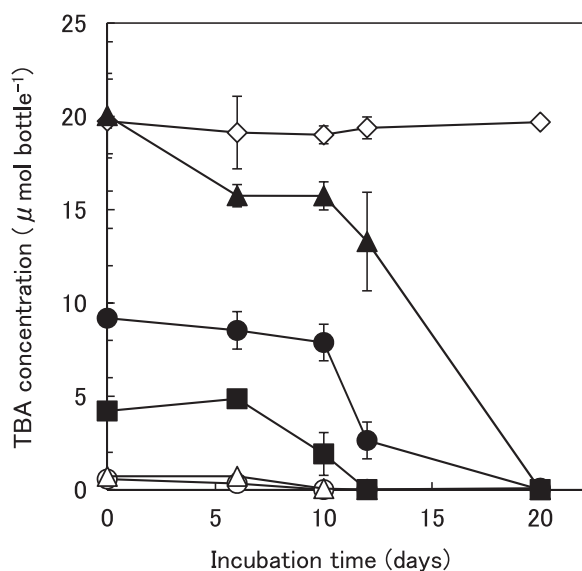

Fig. 4. Effect of concentration on TBA degradation by strain No. 8 in MM medium.

◇ ; 20 µmol bottle⁻¹ TBA without strain, ◯ ; 0.5 µmol bottle⁻¹ TBA, △ ; 1 µmol bottle⁻¹ TBA, ■ ; 5 µmol bottle⁻¹ TBA, ● ; 10 µmol bottle⁻¹ TBA, ▲ ; 20 µmol bottle⁻¹ TBA
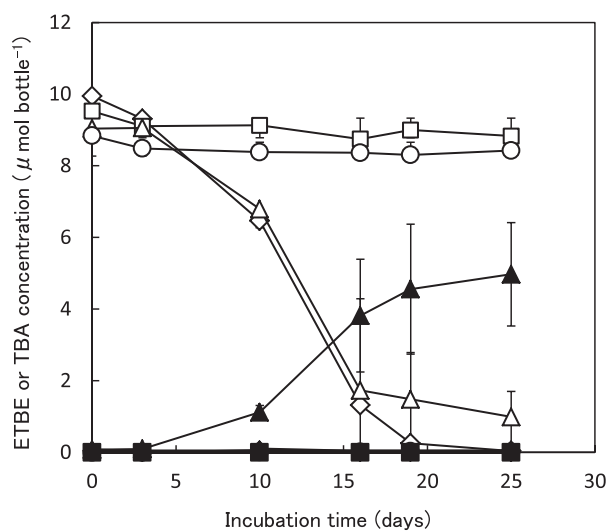

Fig. 5. Effect of ETBE degradation, TBA production and TBA degradation by combination of strain ET10 and strain No. 8 in MM medium.

□ ; ETBE, with strain No. 8, △ ; ETBE, with strain ET10, ◇ ; ETBE, with strain ET10 and strain No. 8, ◯ ; ETBE without strain, ■ ; TBA production, strain No. 8, ▲ ; TBA production, strain ET10, ◆ ; TBA production, with strain ET10 and strain No. 8, ● ; TBA production, without strain.

culture supplemented with glucose. The addition of other organic substances slightly increased the degradation rate constant compared with no addition (Table 1).

Table 1. Effect of organic substances on the specific TBA degradation rate by strain No. 8 in MM medium with 10 μmol bottle^{-1} of TBA

Organic substance	Degradation rate constant (day^{-1})
No organic substance	0.04
Glucose	0.33
Glycerol	0.14
Lactose	0.08
Sucrose	0.10
Succinic acid	0.09
Acetic acid	0.06

Complete degradation was observed in 13 days.
Lag time is three days. Each organics are 5 mg bottle^{-1}.

4. Discussion

Table 2 summarizes the ETBE-degrading *Rhodococcus* species. Various *Rhodococcus* spp. and *R. erythropolis* ET10 are able to degrade ETBE but not TBA. A mixed culture of *R. wratislaviensis* IFP 2016 and *R. aetherivorans* IFP 2017 completely degraded 13 different compounds. However, TBA was not degraded [1]. A mixed culture of *R. erythropolis* ET10 and *P. benzenivorans* No. 8 degraded ETBE, and metabolite TBA was completely degraded, as shown in Figure 5.

It is known that TBA is not easily degraded by micro-organisms. Table 3 summarizes current data regarding TBA-degrading microorganisms and the degradation rates of various bacteria. Some of these bacteria, including *Aquincola tertiaricarbonis* L108 [21,29], *Comamonas testosteroni* E1 [17], *Pseudonocardia* sp. [3], *Pseudonocardia benzenivorans* No. 8, *Methylibium petroleiphilum* PM1 [25,29,30], *Bradyrhizobium* sp. IFP 2049 [4], *Mycobacterium austroafricanum* IFP 2015 [10]

Table 2. Summary of ETBE degrading *Rhodococcus* spp.

Organisms	Degradation of substrate			Reference
	ETBE	MTBE	TBA	
Rhodococcus erythropolis ET10	+	ND	–	This study 24)
Rhodococcus ruber IFP 2001	+	–	–	6, 9)
Rhodococcus ruber E10	+	+	ND	26)
Rhodococcus equi IFP 2002	+	–	–	6)
Rhodococcus aetsherivora IFP 2017	+	+	–	1)
Rhodococcus wratislaviensis IFP 2016	+	+	–	1)
Rhodococcus sp. IFP 2042	+	ND	–	4)

+; degradation, –; no degradation, ND; not determined,

Table 3. Summary of TBA degradation rates by various bacteria

Organisms	Type of metabolism	Metabolic or cometabolic carbon sources	The highest TBA concentration in the experiments	TBA degradation rate	Degradation		Reference
					MTBE	ETBE	
Aquincola tertiaricarbonis L108	Assimilation	TBA	5700 mg l^{-1}	2.82 mmol g biomass^{-1} h^{-1} (208 mg g biomass^{-1} h^{-1})	+	+	21, 29)
Comamonas testosteroni E1	Assimilation	TBA	200 mg l^{-1}	0.017 mmol l^{-1} h^{-1}	+	+	17)
Pseudonocardia sp.	Assimilation	TBA	29.6 mg l^{-1}	0.008 mmol l^{-1} h^{-1}	+	+	3)
Pseudonocardia benzenivorans No. 8	Assimilation	TBA	148 mg l^{-1}	0.008 mmol l^{-1} h^{-1}	ND	–	This study
Methylibium petroleiphilum PM1	Assimilation	TBA	45 mgl^{-1}	14.4 mmol l^{-1} g protein^{-1} h^{-1}	+	–	29, 30, 25)
Bradyrhizobium sp. IFP 2049	Assimilation	TBA	198 mg l^{-1}	0.022 mmol l^{-1} h^{-1}	ND	–	4)
Mycobacterium austroafricanum IFP 2015	Assimilation	TBA	316 mg l^{-1}	0.81 mmol g biomass^{-1} h^{-1}	+	+	10)
Mycobacterium austroafricanum IFP 2012	Assimilation	TBA	10.4 mg l^{-1}	0.003 mmol l^{-1} h^{-1}	+	ND	12)
Mycobacterium austroafricanum IFP 2012	Cometabolism	Hexane Propane, Hexadecane	200 mg l^{-1}	0.16, 0.10, 0.095 mmol g biomass^{-1} h^{-1}	+	ND	11)
Mycobacterium vaccae JOB5	Cometabolism	Propane	1480 mg l^{-1}	0.62 mmol g protein^{-1} h^{-1}	+	ND	15, 31)
Mycobacterium austroafricanus JOB5	Cometabolism	2-Methylbutane	7.4 mg l^{-1}	2.52 mmol g protein^{-1} h^{-1}	+	ND	14)
Mycobacterium duvalii TA5	Cometabolism	Glucose	11.1 mg l^{-1}	0.005 mmol l^{-1} h^{-1}	+	ND	23)
Methylibium sp. R8	Cometabolism	MTBE	500 mg l^{-1}	1.08 mmol g protein^{-1} h^{-1}	+	–	29)

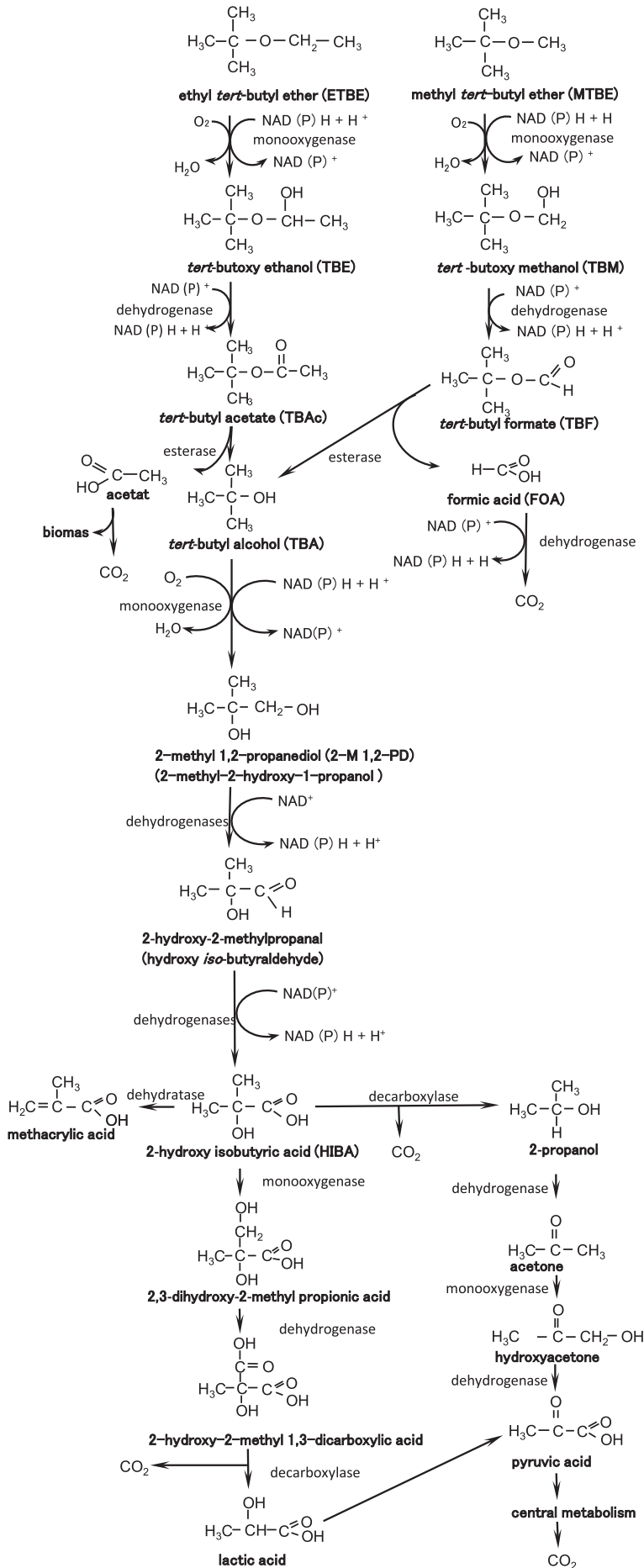+; Degradation, –; No degradation, ND; not determined,

Fig. 6. Proposed pathways for ETBE or MTBE degradation by microorganisms.
This pathway was modified from following authors [5,7–9,13,22,23,27,32].

and *Mycobacterium austroafricanum* IFP 2012 [12] are able to assimilate TBA. Most of the TBA-degrading bacteria are able to degrade methyl *tert*-butyl ether (MTBE). However, some of them are not able to degrade ETBE.

The highest degradation rate was 14.4 mmol g protein^{-1} h^{-1} by *M. petroleiphilum* PM1 for the protein base [30]. *Aquincola tertiaricarbonis* L108 showed the highest TBA degradation rate of 2.82 mmol g biomass^{-1} h^{-1} for the biomass base [21]. The degradation rates were very low for L^{-1} h^{-1} base. The highest degradation rate was by *Bradyrhizobium* sp. IFP 2049 at 0.022 mmol L^{-1} h^{-1} for L^{-1} h^{-1} base. *Pseudonocardia benzenivorans* No. 8 (present study) and *Pseudonocardia* sp [3] showed the lowest TBA degradation rate of 0.008 mmol L^{-1} h^{-1}. The reason for the low degradation rates was based on the low cell concentration of L^{-1}.

Mycobacterium co-metabolizes TBA. The degradation rate of *M. austroafricanum* IFP 2012 is significantly increased by the addition of hexane, propane and hexadecane. The degradation rate increased more than 50-fold with hexane. The TBA degradation rate increased with 2-methylbutane and glucose as a co-metabolic substrate by *M. vaccae* JOB5 (*M. austroafricanum* ATCC 29678) and *M. duvalii*.

Figure 6 shows the proposed pathways for microbial ETBE and MTBE degradation. ETBE is degraded through *tert*-butoxy-ethanol (TBE), *tert*-butyl acetate (TBAc), TBA [9,27], 2-methy-1,2-propanediol (2-M-1, 2-PD), 2-hydroxy-2-methylprop anal, 2-hydroxy isobutyric acid and pyruvic acid [5,7-9,13,22,23,27,32].

Three different enzymes are necessary to degrade ETBE and MTBE to TBA: oxygenase, dehydrogenase and esterase. *Rhodococcus ruber* IFP 2001 [9], *Comamonas testosteroni* E1 [17], *M. vaccae* JOB5 [31] and *M. austroafricanum* IFP 2012 use monooxygenase for the degradation of MTBE and ETBE [12]. Ferreira *et al.* (2006) reported that TBAc was produced from TBE by dehydrogenase and decomposed to TBA and acetate by esterase [9].

Produced TBA is decomposed by a monooxygenase and dehydrogenase. TBA is converted to 2-M 1,2-PD by a monooxygenase [22]. *M. austroafricanus* JOB5 degrades TBA using an alkane-inducible monooxygenase with n-nonane, 2-methylbutane and *p*-xylene as cometabolic substrates [14]. *M. vaccae* JOB5 degrades TBA with propane as a cometabolic substrate using a short-chain alkane monooxygenase [31].

It is reported that bacteria of the genus *Rhodococcus* are able to degrade ETBE but not TBA [1,4,6]. These results suggest that the monooxygenase of *Rhodococcus* species cannot degrade TBA. Mixed cultures of *Rhodococcus* sp. IFP 2042 and *Bradyrhizobium* sp. IFP 2049 degrade ETBE to CO$_2$ [4]. *Pseudonocardia benzenivorans* and *P. dioxanivorans* mineralize benzene and 1,4-dioxane, respectively [16,18]. Digabel and Guichard (2014) [3] reported that *Pseudonocardia* sp. degrade ETBE and TBA. In our research, *P. benzenivorans* No. 8 assimilated TBA. Therefore, it was hypothesized that these *Pseudonocardia* species are promising for the degradation of recalcitrant organic substances such as aromatic hydrocarbons, ether, ETBE and TBA.

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