Degradation of fuel oxygenates and their main intermediates by *Aquicola tertiaricarbonis* L108

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Growth of *Aquicola tertiaricarbonis* L108 on the fuel oxygenates methyl *tert*-butyl ether (MTBE), ethyl *tert*-butyl ether (ETBE) and *tert*-amyl methyl ether (TAME), as well as on their main metabolites *tert*-butyl alcohol (TBA), *tert*-amyl alcohol (TAA) and 2-hydroxyisobutyrate (2-HIBA) was systematically investigated to characterize the range and rates of oxygenate degradation by this strain. The effective maximum growth rates for MTBE, ETBE and TAME at pH 7 and 30 °C were 0.045 h⁻¹, 0.06 h⁻¹ and 0.055 h⁻¹, respectively, whereas TAA, TBA and 2-HIBA permitted growth at rates up to 0.08 h⁻¹, 0.1 h⁻¹ and 0.17 h⁻¹, respectively. The experimental growth yields with all these substrates were high. Yields of 0.55 g dry mass (dm) (g MTBE)⁻¹, 0.53 g dm (g ETBE)⁻¹, 0.81 g dm (g TAME)⁻¹, 0.48 g dm (g TBA)⁻¹, 0.76 g dm (g TAA)⁻¹ and 0.54 g dm (g 2-HIBA)⁻¹ were obtained. Maximum specific degradation rates were 0.92 mmol MTBE h⁻¹ (g dm)⁻¹, 1.11 mmol ETBE h⁻¹ g⁻¹, 0.66 mmol TAME h⁻¹ g⁻¹, 1.19 mmol TAA h⁻¹ g⁻¹, 2.82 mmol TBA h⁻¹ g⁻¹, and 3.27 mmol 2-HIBA h⁻¹ g⁻¹. The relatively high rates with TBA, TAA and 2-HIBA indicate that the transformations of these metabolites did not limit the metabolism of MTBE and the related ether compounds. Despite the fact that these metabolites still carry a tertiary carbon atom that is commonly suspected to confer recalcitrance to the ether oxygenates, the transformation rates were in the same range as those with succinate and fructose. With MTBE, strain L108 grew at pHs between 5.5 and 8.0 at near-maximal rate, whereas no growth was found below pH 5.0 and above pH 9.0. The optimum growth temperature was 30 °C, but at 5 °C still about 15 % of the maximum rate remained, whereas no growth occurred at 42 °C. This indicates that MTBE metabolites are valuable substrates and that *A. tertiaricarbonis* L108 is a good candidate for bioremediation purposes. The possible origin of its exceptional metabolic capability is discussed in terms of the evolution of enzymic activities involved in the conversion of compounds carrying tertiary butyl groups.

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INTRODUCTION

Methyl *tert*-butyl ether (MTBE) and the related compounds ethyl *tert*-butyl ether (ETBE) and *tert*-amyl methyl ether (TAME) are widely used as oxygenating compounds in gasoline (Kraye von Krauss & Harremoës, 2001), leading to pollution by unnoticed leakages and accidental spills (Baehr *et al.*, 1999; Klinger *et al.*, 2002; Schmidt *et al.*, 2002; Squillace *et al.*, 1996). They threaten water resources by their unpleasant odour and taste and suspected carcinogenicity (McGregor, 2006). Consequently, much

effort is put into studying the environmental fate of these compounds and developing measures against fuel oxygenate pollution. Microbial degradation has been considered in both respects (Deeb *et al.*, 2000; Fayolle *et al.*, 2001; Schmidt *et al.*, 2004). However, MTBE and structurally related compounds were initially found to withstand microbial attack. This was thought to be mainly due to the ether bond in these compounds and the presence of a tertiary carbon atom.

Generally, it has proved difficult to isolate strains from enrichment cultures using MTBE as sole carbon and energy source. Attempts for more than 15 years were of limited success. At present, there are only a few strains capable of growing solely on oxygenates. These include *Methylibium petroleiphilum* PM1 (Nakatsu *et al.*, 2006), *Methylibium* sp. R8 (Rosell *et al.*, 2007), *Hydrogenophaga flava* ENV735 (Hatzinger *et al.*, 2001), *Mycobacterium austroafricanum*

Abbreviations: ETBE, ethyl *tert*-butyl ether; 2-HIBA, 2-hydroxyisobutyric acid; MTBE, methyl *tert*-butyl ether; TAA, *tert*-amyl alcohol; TAME, *tert*-amyl methyl ether; TBA, *tert*-butyl alcohol.

Two supplementary figures showing the influence of temperature and pH on the growth of *A. tertiaricarbonis* on MTBE are available with the online version of this paper.

IFP2012 and IFP2015 (François *et al.*, 2002, 2003; Lopes Ferreira *et al.*, 2006), *Variovorax paradoxus* CL-8 (Zaitsev *et al.*, 2007), and other strains described in little detail (Hernandez-Perez *et al.*, 2001; Lin *et al.*, 2007; Okeke & Frankenberger, 2003; Pruden & Suidan, 2004). In addition, we have recently isolated strain L108 from a polluted site in Germany, which is able to grow on MTBE, ETBE and TAME (Rohwerder *et al.*, 2004, 2006) and, like *Methylibium petroleiphilum* PM1, belongs to the *Ideonella-Leptothrix-Rubrivivax* branch of the β -proteobacteria. Strain L108 was classified as a species of a new genus that was named *Aquicola tertiaricarbonis* (Lechner *et al.*, 2007).

Growth rates on oxygenates are in general low. A mixed culture with *V. paradoxus* CL-8 as the MTBE-degrading entity exhibited a growth rate of 0.012 h^{-1} (Zaitsev *et al.*, 2007); a rate even one magnitude lower was reported for another mixed culture (Lin *et al.*, 2007). *Mycobacterium austroafricanum* IFP2012 (François *et al.*, 2002) and IFP2015 (François *et al.*, 2003), *Hydrogenophaga flava* ENV735 (Hatzinger *et al.*, 2001) and *Methylibium petroleiphilum* PM1 (Hanson *et al.*, 1999) appeared to utilize MTBE in batch degradation experiments, but specific rates were hardly accessible or even impossible to derive from the published data and indicated difficulties in sustaining growth. Here we report on an examination of the potential of strain L108 to grow on MTBE, ETBE and TAME as sole source of carbon and energy under batch conditions. We also investigated productive, i.e. growth-coupled, degradation of the primary metabolites, viz. *tert*-butyl alcohol (TBA), *tert*-amyl alcohol (TAA) and 2-hydroxyisobutyrate (2-HIBA) to identify possible metabolic bottlenecks.

METHODS

Cultivation. Strain L108 was grown in mineral salts solution containing (in mg l^{-1}): NH_4Cl , 760; KH_2PO_4 , 340; K_2HPO_4 , 485; $\text{CaCl}_2 \cdot 6\text{H}_2\text{O}$, 27; $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$, 71.2, and 1 ml l^{-1} of trace element solution; the trace element solution was composed of (in g l^{-1}): $\text{FeSO}_4 \cdot 7\text{H}_2\text{O}$, 4.98; $\text{CuSO}_4 \cdot 5\text{H}_2\text{O}$, 0.785; CoCl_2 , 5; $\text{MnSO}_4 \cdot 4\text{H}_2\text{O}$, 0.81; $\text{ZnSO}_4 \cdot 7\text{H}_2\text{O}$, 0.44; $\text{Na}_2\text{MoO}_4 \cdot 2\text{H}_2\text{O}$, 0.25. The medium was supplemented with a vitamin mixture to final concentrations of (in $\mu\text{g l}^{-1}$): biotin, 20; folic acid, 20; pyridoxine.HCl, 100; thiamine.HCl, 50; riboflavin, 50; nicotinic acid, 50; calcium DL-pantothenate, 50; *p*-aminobenzoic acid, 50; lipoic acid, 50; and cobalamin, 50.

The strain was inoculated into 200 ml mineral salts solution to give an initial biomass concentration of about $25 \text{ mg dry mass l}^{-1}$. Incubation was performed in 600 ml bottles, closed with gas-tight butyl rubber stoppers, on a rotary shaker at 150 r.p.m. at 30°C . The initial pH was 7.0 and was not corrected throughout the experiment. Liquid samples were taken at various times by a sterile syringe puncturing the butyl rubber stopper. Specific growth rates were derived from the linear part of semi-logarithmic plots of the biomass concentration versus time by regression analysis. The kinetic parameters were derived from double-reciprocal plots of rates versus substrate concentrations to obtain the apparent, experimentally relevant values or by nonlinear regression (Haldane equation) to obtain kinetically based parameters according to

$$\mu = \mu_{\max} \times S/[K_s + (1 + S/K_i) \times S] \quad (1)$$

Analytics. Biomass was determined as OD_{700} . OD_{700} multiplied by a factor of 0.54 ± 0.03 was found to give the dry mass in mg ml^{-1} . MTBE, ETBE, TAME, TAA and TBA were determined by head-space gas chromatography as previously described (Rohwerder *et al.*, 2006). 2-HIBA was determined by gas chromatography as the methyl ester according to a procedure given elsewhere (Rohwerder *et al.*, 2006). Fructose, lactate, pyruvate and succinate were measured by HPLC at an oven temperature of 70°C using an Ion 300 OA column (Macherey Nagel). The mobile phase was $0.005 \text{ M H}_2\text{SO}_4$ fed with a rate of 0.6 ml min^{-1} ; detection was by refractive index (RID). The compounds measured by the various methods were detected with 95 % confidence.

MTBE, ETBE, lactate, pyruvate and succinate (sodium salts) and fructose were purchased from Merck; TAME and other chemicals used were obtained from Fluka.

RESULTS

The growth kinetics of strain L108 in batch culture on MTBE, ETBE and TAME and on the major metabolites TBA, TAA and 2-HIBA are shown in Figs 1 and 2, respectively. With MTBE, the maximum specific growth rate, $\mu_{\max}$, was reached at a concentration around 2.5 mM. Data treatment at this concentration range in a double-reciprocal plot revealed a $\mu_{\max}$ of 0.047 h^{-1} and K_s of 0.59 mM. Higher concentrations resulted in inhibition that

Fig. 1. Growth kinetics of *A. tertiaricarbonis* L108 on MTBE, ETBE, and TAME. The flasks were tightly closed with butyl rubber stoppers to avoid loss of the substrates by volatilization. The ratio of liquid to air volume was calculated to guarantee oxygen sufficient for complete aerobic degradation of MTBE. Incubation was performed at 30°C and an initial pH value of 7.0 with shaking at 150 r.p.m. Samples were taken by a syringe via the butyl rubber stoppers. Biomass was measured as OD_{700} immediately after taking the samples. Growth rates were derived by linear regression from semi-logarithmic plots of OD_{700} versus time. Samples for substrate measurement were prepared as indicated by Rohwerder *et al.* (2006). The values on the x-axis indicate the initial substrate concentration in the liquid medium.

Fig. 2. Growth kinetics of *A. tertiaricarbonis* L108 on TBA, TAA and 2-HIBA. For further details see legend to Fig. 1.

levelled off at about 50% of the maximum rate above 6 mM. The above values correspond to the apparent but finally effective kinetic parameters. Inclusion of nonlinear regression analysis by using equation 1 resulted in kinetically more sound parameters. With MTBE as a substrate these amounted to $\mu_{\max}=0.097\text{ h}^{-1}$, $K_s=1.86\text{ mM}$ and $K_i=7.0\text{ mM}$. With ETBE, an effective $\mu_{\max}$ of around 0.06 h^{-1} was obtained, with only slight inhibition appearing at higher concentrations (Fig. 1). Nonlinear regression revealed $\mu_{\max}=0.065\text{ h}^{-1}$, $K_s=0.089\text{ mM}$ and $K_i=35.0\text{ mM}$. Due to the quotient of $\mu_{\max}/K_s$ (specific affinity; Healey 1980), ETBE ($\mu_{\max}/K_s=0.73\text{ h}^{-1}\text{ mM}^{-1}$) was a significantly better substrate for strain L108 than MTBE ($\mu_{\max}/K_s=0.052\text{ h}^{-1}\text{ mM}^{-1}$). TAME was apparently a better growth substrate than MTBE also. The $\mu_{\max}$ of 0.055 h^{-1} was reached already at a TAME concentration of 0.35 mM . The growth rate levelled

off between 5 and 10 mM before slightly decreasing at higher concentrations until approaching zero at 32 mM (data not shown). Due to the higher affinity to TAME, our rate data did not allow calculation of reliable K_s values with this substrate. Because of problems in verifying growth at these low concentrations, experiments were not performed at such substrate levels.

Growth on TAA (Fig. 2) gave a $\mu_{\max}$ of 0.08 h^{-1} at a substrate concentration of 1 mM and this decreased only slightly to 0.07 h^{-1} towards the highest tested concentration of 20 mM . The affinity to TBA was the lowest among all tested growth substrates although exact affinities could not be derived from the present data. A $\mu_{\max}$ of 0.1 h^{-1} was obtained at about 35 mM (Fig. 2). At higher concentrations the activity decreased to about half of the maximum rate at about 75 mM and stopped at 200 mM (not shown). The fastest growth was obtained with 2-HIBA. Double-reciprocal data analysis revealed an apparent $\mu_{\max}$ of 0.19 h^{-1} , obtained at around 5 mM substrate, followed by rather pronounced inhibition to 20% of $\mu_{\max}$ at 29 mM . The apparent K_s for 2-HIBA derived in the same way was 1.05 mM . With nonlinear regression we obtained $\mu_{\max}=0.305\text{ h}^{-1}$, $K_s=1.6\text{ mM}$ and $K_i=7.0\text{ mM}$ by using equation 1.

Table 1 lists all values of $\mu_{\max}$ together with the corresponding yield coefficients, Y , that were derived from biomass formation after substrate exhaustion and maximum specific substrate consumption rates, $q_s=\mu Y^{-1}$. Values obtained with the common substrates pyruvate, succinate, lactate and fructose were included as references for comparison. Yields with the oxygenates and their metabolites were around or above $0.5\text{ g biomass (g substrate)}^{-1}$, with the highest yields of around 0.8 g g^{-1} obtained with TAME and TAA. These values are about twice as high as those obtained with the reference substrates and reflect the more reduced state of the former. The rates of growth and consumption of oxygenates and metabolites were between 2 and 10 times slower than those with the reference substrates. Particularly, growth with

Table 1. Growth parameters and productivity of substrate consumption obtained with *A. tertiaricarbonis* strain L108

Substrate	$\mu_{\max}\text{ (h}^{-1}\text{)}^*$	$Y, \text{ g mol}^{-1}\text{ (g g}^{-1}\text{)}$	$q_s, \text{ mmol g}^{-1}\text{ h}^{-1}\text{ (mg g}^{-1}\text{ h}^{-1}\text{)}$
MTBE	0.045	$48.8 \pm 4.4\text{ (}0.55 \pm 0.05\text{)}$	0.92 (81)
ETBE	0.06	$54.1 \pm 2.0\text{ (}0.53 \pm 0.02\text{)}$	1.11 (113)
TAME	0.055	$83.0 \pm 4.0\text{ (}0.81 \pm 0.05\text{)}$	0.66 (68)
TBA	0.1	$35.5 \pm 2.2\text{ (}0.48 \pm 0.03\text{)}$	2.82 (208)
TAA	0.08	$67.0 \pm 7.0\text{ (}0.76 \pm 0.08\text{)}$	1.19 (105)
2-HIBA	0.17	$52.0 \pm 2.0\text{ (}0.50 \pm 0.02\text{)}$	3.54 (340)
Lactate	0.299	$34.4 \pm 1.0\text{ (}0.38 \pm 0.01\text{)}$	8.69 (790)
Pyruvate	0.314	$25.3 \pm 1.2\text{ (}0.29 \pm 0.01\text{)}$	12.4 (1090)
Succinate	0.318	$38.3 \pm 1.2\text{ (}0.32 \pm 0.02\text{)}$	8.3 (980)
Fructose	0.242	$56.6 \pm 0.8\text{ (}0.31 \pm 0.04\text{)}$	4.28 (770)

*Experimental maximum.

2-HIBA approached the rates obtained with the reference substrates. There was a general trend that the oxygenate metabolites were consumed faster than the mother compounds. This seems to indicate that later stages of the metabolism did not control the rates of oxygenate consumption. Nevertheless, during growth on MTBE and ETBE, TBA concentrations between 3 and 5 μM were always released into the cultivation broth. In addition, similar amounts of the TBA precursor *tert*-butyl formate were detected when strain L108 was cultivated on MTBE. In contrast, the corresponding esters *tert*-butyl acetate and *tert*-amyl formate were not detectable in cultures growing on ETBE and TAME, respectively. In summary, it appears that oxygenate intermediates such as TBA and 2-HIBA, which were previously supposed to be recalcitrant, are substrates almost as good as succinate or fructose for strain L108.

As strain L108 was isolated from an aquifer and hence could be valuable for groundwater remediation, we investigated the influence of temperature and pH on its growth on MTBE. The maximum rate was obtained at 30 °C and dropped to about 50 % at 37 °C, whereas no growth was observed at 42 °C. At 12 °C, only 10 % of the maximum rate remained (see Supplementary Fig. S1, available with the online version of this paper). Surprisingly, the rate rose significantly when the temperature dropped further ($0.012 \pm 0.0035 \text{ h}^{-1}$ at 5 °C vs $0.005 \pm 0.0013 \text{ h}^{-1}$ at 12 °C; $n=3$). Growth continued below 5 °C but was difficult to control; hence the data were not included in Fig. S1. Strain L108 was active over a wide pH range: a plateau of almost constant maximum growth rate extended from pH 5.5 to 8. Beyond this range, there was a rapid decline in growth rate, with no growth at either pH 5 or pH 9 (Supplementary Fig. S2).

DISCUSSION

Batch growth studies over a wide range of substrate concentrations made it evident that *A. tertiaricarbonis* L108 (Rohwerder *et al.*, 2006) is very effective in the productive degradation of all three common fuel oxygenates MTBE, ETBE and TAME. An effective maximum growth rate of about 0.045 h^{-1} with MTBE and up to 20 % higher ones with ETBE and TAME were found. Growth rates on the MTBE and ETBE metabolites TBA and 2-HIBA even amounted to 0.1 h^{-1} and 0.17 h^{-1} , respectively. These high growth rates approach rates obtained with common substrates such as fructose or succinate, indicating that fuel oxygenate metabolism in strain L108 is well established.

Hence, the strain has attained a metabolic capacity that is clearly beyond the level of fortuitous enzyme activity but suggests elevated substrate specificity. The former statement refers to the observation that the conversion of a pollutant is often catalysed by defined enzymes in an unspecific manner, resulting in low rates due to the xenobiotic structure of the compounds (Janssen *et al.*,

2005). Although the oxygenate degradation pathway(s) have not been elucidated completely, three important steps have already been identified in strain L108. The initial monooxygenase reaction attacking both the methyl and ethyl groups of MTBE and ETBE, respectively, is catalysed by a cytochrome P450-type enzyme encoded by the *ethABCD* genes (Breuer *et al.*, 2007). A similar monooxygenase has been previously detected in *Rhodococcus ruber* IFP2001 growing on ETBE (Chauvaux *et al.*, 2001). A different oxygenase system, which has similarity to phthalate dioxygenase, is likely responsible for hydroxylating TBA in strain L108 (Schäfer *et al.*, 2007). The metabolite 2-HIBA is converted by a novel cobalamin-dependent mutase to 3-hydroxybutyrate (Rohwerder *et al.*, 2006). Thus far, this combination of specific enzymes has not been found in other oxygenate-degrading strains. Recently, a monooxygenase of the AlkB type has been described for *Mycobacterium austroafricanum* IFP2012 (Lopes Ferreira *et al.*, 2007), supposed to hydroxylate both MTBE and TBA. Hydroxylation of the latter is inhibited by MTBE, resulting in the accumulation of TBA in strain IFP2012 (François *et al.*, 2002, 2003). For *Methylibium petroleiphilum* PM1, a phthalate dioxygenase-like enzyme and a mutase very similar to the enzymes found in strain L108 have been recently proposed for TBA hydroxylation and 2-HIBA isomerization, respectively (Hristova *et al.*, 2007). However, *ethABCD* is not present in the genome of strain PM1 (Kane *et al.*, 2007). Consequently, at least the initial steps in MTBE degradation deviate in strains PM1 and L108, obviously resulting in different capacities for growth on ether oxygenates such as MTBE and ETBE. The pathway for TAME degradation has not been elucidated so far. Proposing similarity with the pathways for MTBE and ETBE, *tert*-amyl formate and TAA would be intermediates. The latter could be converted to 3-hydroxyisovaleric acid and then split into acetone and acetyl-CoA (Nemecek-Marshall *et al.*, 1999). However, due to the inability of strain L108 to grow on acetone (Lechner *et al.*, 2007), a cobalamin-dependent route via 2-hydroxy-2-methylbutyrate, corresponding to the TBA intermediate 2-HIBA, is more likely.

A comparison of the capacities of various described strains to degrade MTBE and some key intermediates shows that strain L108 is apparently effective (Table 2). Only *Variovorax* sp. strain JV-1 appears to have a similar potential for the degradation of MTBE (Uotila & Zaitsev, 2003). Obviously, this strain is equipped with a set of specific enzymes as efficient as the one found in strain L108. For most of other strains besides those included in the table, reported information did not allow derivation of q_s values for MTBE. Although not always obvious, this may indicate that MTBE degradation was at best weakly coupled to growth in these bacteria, e.g. strains IFP 2012, PM1 and ENV735. With TBA as the substrate, the specific degradation rates of strain L108 were in each case significantly above those found with the other strains (Table 2). This was also true for 2-HIBA except for strain CIP I-2052,

Table 2. Comparison of microbial strains for their specific capacity to degrade MTBE and its main degradation products

Substrate	Strain	q_s (mmol g ⁻¹ h ⁻¹)	Remarks*	Reference
MTBE	<i>A. tertiaricarbonis</i> L108	0.92	sc	This work
	<i>Variovorax</i> sp. JV-1	0.69	22 °C	Uotila & Zaitsev (2003)
	<i>Variovorax paradoxus</i> CL-8	0.39	22 °C	Zaitsev <i>et al.</i> (2007)
TBA	<i>A. tertiaricarbonis</i> L108	2.82	sc	This work
	<i>A. tertiaricarbonis</i> L10	2.02	5 mM†	Rohwerder <i>et al.</i> (2006)
	<i>A. tertiaricarbonis</i> L10	1.65	Co‡	Rohwerder <i>et al.</i> (2006)
	<i>Variovorax</i> sp. CL-3	0.96	28 °C	Uotila & Zaitsev (2003)
	<i>Variovorax paradoxus</i> CL-8	0.94	22 °C	Zaitsev <i>et al.</i> (2007)
	<i>A. tertiaricarbonis</i> CIP I-2052	0.80	Co‡	Piveteau <i>et al.</i> (2001)
	<i>Mycobacterium austroafricanum</i> IFP 2012	0.86	Co‡	François <i>et al.</i> (2002)
	<i>A. tertiaricarbonis</i> L108	3.27	sc	This work
2-HIBA	<i>A. tertiaricarbonis</i> L10	2.93	10 mM	Rohwerder <i>et al.</i> (2006)
	<i>A. tertiaricarbonis</i> L10	1.21	Co‡	Rohwerder <i>et al.</i> (2006)
	<i>Variovorax paradoxus</i> CL-8	0.95	22 °C	Zaitsev <i>et al.</i> (2007)
	<i>A. tertiaricarbonis</i> CIP I-2052	2.53	sc	Rohwerder <i>et al.</i> (2006)
	<i>A. tertiaricarbonis</i> CIP I-2052	1.11	Co‡	Rohwerder <i>et al.</i> (2006)
	<i>Mycobacterium austroafricanum</i> IFP 2012	1.35	sc	François <i>et al.</i> (2002)

*Deviations from standard conditions (sc) are indicated. Standard conditions applied in this study included: incubation at 30 °C and initial pH value of 7.0, as well as the presence of vitamin B12 in addition to Co²⁺.

†Actual substrate concentration (cf. Fig. 2).

‡Incubation in the presence of Co²⁺ but omitting vitamin B12.

which achieved similarly high rates. For strains L10 and CIP I-2052, it has been demonstrated that growth rates on TBA and 2-HIBA significantly decreased when cobalamin was replaced by cobalt ions in the growth medium (Table 2). This phenomenon may be due to the effort in synthesizing cobalamin required for the mutase pathway, converting 2-HIBA into 3-hydroxybutyrate (Rohwerder & Müller, 2007). Although the cobalt dependency of strain IFP2012 for degrading MTBE and its metabolites (François *et al.*, 2002) indicates 2-HIBA mutase activity also in this strain, slower consumption of TBA and 2-HIBA in the presence of cobalamin may be caused by incapacity of taking up the externally added vitamin.

MTBE and ETBE are potent heterotrophic substrates due to their reduced state, as was shown in a theoretical study (Müller *et al.*, 2007). Experimental yields of ≥ 0.5 g g⁻¹ prove the effective use of these substrates for the growth and multiplication of strain L108. A similar yield coefficient of 0.49 g g⁻¹ was obtained with *Variovorax paradoxus* CL-8 (Zaitsev *et al.*, 2007). For *Mycobacterium austroafricanum* IFP2012, yields of 0.44 g g⁻¹ (MTBE), 0.61 g g⁻¹ (TBA) and 0.42 g g⁻¹ (2-HIBA) were reported (François *et al.*, 2002). Biomass synthesis may, however, be nil or even negative at very low rates of substrate consumption (Müller *et al.*, 2007). The enrichment of MTBE-degrading cultures under such conditions indicates that the maintenance requirements are modest in these strains, which may be either an intrinsic property of such strains or an adaptation of productive MTBE degraders. The difficulty of enriching degradative strains in the early

years of MTBE research or the need to provide them with auxiliary substrates such as n-alkanes (Haase *et al.*, 2006; Steffan *et al.*, 1997) may be an indication of the recent acquisition of the regulatory and enzymic capacity to use MTBE as sole carbon and energy source.

Interestingly, the ether-related metabolites, namely TBA, 2-HIBA and TAA, are better substrates than the mother compounds. This raises questions about the evolution of the degradation pathways. MTBE and related ethers have been only recently introduced into nature, since their massive use as fuel additives began only in the late 1980s (Squillace *et al.*, 1997). In contrast, oxygenate-independent sources are known for the tertiary alcohols and 2-HIBA (Fig. 3). 2-HIBA is a by-product of the classical methacrylate synthesis process via 2-hydroxyisobutyronitrile (Rohwerder *et al.*, 2006), which started in the mid-1930s. This may explain why Holowach and coworkers reported already in 1994 the isolation of 2-HIBA-converting bacterial strains from the wastewater of a methacrylate-producing plant (Holowach *et al.*, 1994). In addition, 2-hydroxyisobutyronitrile is a degradation product of the plant cyanoglycoside linamarin (Forslund *et al.*, 2004) and can form 2-HIBA in the presence of nitrilase or nitrile hydratase and amidase activity (Banerjee *et al.*, 2002). A third ether-independent 2-HIBA source could be the conversion of isobutene via the corresponding 1,2-epoxide and 2-hydroxy-2-methylpropanol (Rohwerder & Müller, 2007). Likewise, Hyman and co-workers have recently questioned whether MTBE and ETBE are the only source for TBA (Hyman *et al.*, 2007). Indeed, it has been reported

Fig. 3. Potential substrates and routes involved in the evolution of degradative capabilities for MTBE and related compounds.

several times that the activity of methane monooxygenase and other alkane monooxygenases on isobutane, isopentane and homologous hydrocarbons not only resulted in the corresponding primary and secondary alcohols but also formed the tertiary ones (Dubbels *et al.*, 2007; Imai *et al.*, 1986; Onodera *et al.*, 1990; Patel *et al.*, 1982). Hence, although growth on 2-HIBA and tertiary alcohols was not reported before man started ether oxygenate production, their degradation pathways could have evolved far earlier and totally independent of ether oxygenate contamination (Fig. 3).

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