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n-Alkane assimilation and *tert*-butyl alcohol (TBA) oxidation capacity in *Mycobacterium austroafricanum* strains

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Abstract *Mycobacterium austroafricanum* IFP 2012, which grows on methyl *tert*-butyl ether (MTBE) and on *tert*-butyl alcohol (TBA), the main intermediate of MTBE degradation, also grows on a broad range of *n*-alkanes (C_2 to C_{16}). A single *alkB* gene copy, encoding a non-heme alkane monooxygenase, was partially amplified from the genome of this bacterium. Its expression was induced after growth on *n*-propane, *n*-hexane, *n*-hexadecane and on TBA but not after growth on LB. The capacity of other fast-growing mycobacteria to grow on *n*-alkanes (C_1 to C_{16}) and to degrade TBA after growth on *n*-alkanes was compared to that of *M. austroafricanum* IFP 2012. We studied *M. austroafricanum* IFP 2012 and IFP 2015 able to grow on MTBE, *M. austroafricanum* IFP 2173 able to grow on isooctane, *Mycobacterium* sp. IFP 2009 able to grow on ethyl *tert*-butyl ether (ETBE), *M. vaccae* JOB5 (*M. austroaafricanum* ATCC 29678) able to degrade MTBE and TBA and *M. smegmatis* mc2 155 with no known degradation capacity towards fuel oxygenates. The *M. austroafricanum* strains grew on a broad range of *n*-alkanes and three were able to degrade TBA after growth on propane, hexane and hexadecane. An *alkB* gene was partially amplified from the genome of all mycobacteria and a sequence comparison demonstrated a close relationship among the *M. austroafricanum* strains. This is the first report suggesting the involvement of an alkane hydroxylase in TBA oxidation, a key step during MTBE metabolism.

Keywords *Mycobacterium austroafricanum* · Alkane hydroxylase · *tert*-Butyl alcohol (TBA) Oxidation · *n*-Alkanes · *alkB* Gene expression · MTBE · Metabolism

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Introduction

The incorporation of MTBE in gasoline over the last 25 years to boost the octane index has resulted in the frequent identification of MTBE as a groundwater pollutant (Johnson et al. 2000; Schmidt et al. 2003). MTBE is not easily biodegradable and to date, few data are available regarding the monooxygenase involved in the MTBE oxidation step. Actually, only two monooxygenase systems have been shown to be involved in MTBE oxidation by co-metabolism: (1) The cytochrome P-450 monooxygenase (encoded by the *ethRABCD* genes) induced after growth on ETBE in *Rhodococcus ruber* IFP 2001 (Chauvaux et al. 2001). This monooxygenase is able to oxidise ETBE and MTBE, but not TBA. The *eth* genes cluster was highly conserved in *Rhodococcus zopfii* IFP 2005 and *Mycobacterium* sp. IFP 2009, two other strains isolated from activated sludge with capacity similar to that of *R. ruber* IFP 2001 (Béguin et al. 2003), (2) The non-heme alkane hydroxylase of the well-known *Pseudomonas putida* GPo1 that oxidises MTBE but not TBA after growth on *n*-octane (Smith and Hyman 2004), but with a low affinity for MTBE (20 mM<Ks<40 mM). These authors showed that the loss of the OCT plasmid resulted in the inability to degrade MTBE.

Up to now, no data were available regarding the monooxygenase involved in TBA oxidation. TBA is a key intermediate in MTBE degradation, it can be found in MTBE-impacted aquifers as the result of a partial MTBE biodegradation, as an additive itself or as an impurity present in MTBE (Wilson 2003). The possible accumulation of TBA makes it important to get data about the monooxygenase involved in its oxidation. Several gram-positive microorganisms belonging to the *Nocardia–Corynebacterium–Mycobacterium–Rhodococcus* complex showed degradation capacity towards TBA. Among them, *Mycobacterium vaccae* JOB5, isolated for its ability to grow on 2-methylbutane (Ooyama and Foster 1965) oxidised MTBE and also TBA after growth on propane (Hyman et al. 1998; Johnson et al. 2004; Smith et al. 2003; Steffan et al. 1997), hexane or 2-methylbutane (Hyman and O'Reilly 1999). Smith et al. (2003) showed at a physiological level that a propane-induced monooxygenase was responsible for both MTBE and TBA oxidation in this strain. *M. vaccae* JOB5 was recently re-classified as *Mycobacterium austroafricanum* (CIP105723 or ATCC 29678) (M. Hyman, personal communication).

Moreover, a few strains were able to grow on MTBE and therefore on TBA; *Methylibium petroleiphilum* PM1 (Hanson et al. 1999; Nakatsu et al. 2006), *Hydrogenophaga flava* ENV735 (Hatzinger et al. 2001), *M. austroafricanum* IFP 2012 (François et al. 2002) and, more recently, *M. austroafricanum* IFP 2015 (Lopes Ferreira et al. 2006b) and *Aquincola tertiaricarbonis* L10 (Lechner et al. 2007). These strains have been studied at the physiological level, but the genes and enzymes involved in the oxidation of MTBE and in the oxidation of TBA are still unknown. *M. austroafricanum* IFP 2012 was isolated for its capacity to grow on TBA. Both MTBE and TBA were inducers of MTBE and TBA degradation activity (François et al. 2002). More recently, *M. austroafricanum* IFP 2015 was isolated for its capacity to grow on MTBE (Lopes Ferreira et al. 2006b). The product of TBA oxidation, 2-methyl 1,2-propanediol (2M1,2PD), was identified during the oxidation of TBA by resting cells of *M. austroafricanum* IFP 2012 and IFP 2015; the conversion of 2M1,2PD to hydoxisobutyric acid (HIBA) was also shown (Lopes Ferreira et al. 2006b). Finally, the genes involved in the step from 2M1,2PD to HIBA were isolated (Lopes Ferreira et al. 2006a).

But up to now, no data were available regarding the oxygenase involved in the previous step from TBA to 2M1,2PD. TBA is an obligate intermediate in the MTBE biodegradation pathway (Fayolle and Monot 2005) and it is important to identify and characterise the enzymatic system responsible for its oxidation. Therefore, the objective of the present study was to identify the monooxygenase responsible for the capacity to oxidise TBA in mycobacteria belonging to the *M. austroafricanum* species.

Materials and methods

Microorganisms and preservation

Stock cultures of *M. austroafricanum* IFP 2012, IFP 2015 and IFP 2173, *Mycobacterium* sp. IFP 2009, *M. smegmatis* mc2 155 and *M. vaccae* JOB5 (*M. austraoafricanum* CIP105723 or ATCC 29678) were kept frozen at −80°C in the mineral medium (MM) previously described (Piveteau et al. 2001) containing 20% glycerol (*v*/*v*).

Growth medium and culture conditions

All strains were grown at 30°C in Luria–Bertani (LB) medium or in MM supplemented with the required substrate. When volatile substrates (hexane or octane) were used as carbon sources, the cultures were grown in tightly closed flasks and when gaseous substrates (ethane, propane, butane) were used as a carbon source, the cultures were grown in sealed serum bottles. In both cases, the headspace volume was sufficient to prevent any O_2 limitation during growth. Growth was followed by measuring the absorbance at 600 nm (OD_{600}).

Growth and mineralisation measurements

Cell suspensions of the different mycobacteria grown on LB medium were harvested by centrifugation (23,000×*g* for 15 min), washed twice and suspended in MM. Serum bottles (250 ml) containing 40 ml-MM were seeded at an initial OD_{600} of 0.1. Substrates were added to a final concentration of 200 mg l^{-1} and the bottles were closed with a butyl rubber stopper and sealed. When the carbon source was a gas, it was injected through the rubber stoppers using a gas syringe. Flasks were incubated under agitation at 30°C. Growth was estimated by measuring OD_{600} at t=0 and after 3 or 15 days of incubation.

The mineralisation of the different alkanes was calculated by measuring the production of CO_2 under experimental conditions similar to those described above. After incubation (15 days), 2 ml of HNO_3 (68%, *v*/*v*) were added to each serum bottle through the stopper to strip the CO_2 dissolved in the aqueous phase. The headspace was then sampled with a gas-tight syringe for analysis by GC/TCD to determine the total amount of CO_2 produced in each bottle. The endogenous respiration was estimated on similar flasks without addition of any substrate and this value was subtracted for further calculations.

Degradation assay using resting cells

Cells of the different mycobacteria grown on LB or on MM containing the appropriate *n*-alkane as the carbon source

were harvested by centrifugation (23,000×*g* for 15 min), washed twice and suspended in phosphate buffer (20 mM, pH 7). Cells were suspended in 30 ml phosphate buffer containing the test substrate (TBA) in 160-ml sealed flasks to obtain an initial OD_{600} of ca. 0.3. After inoculation, the flasks were incubated at 30°C on an orbital shaker. Filtered samples were analysed by GC. Substrate concentration was measured at 0 and 24 h. The dry weight of the cells was measured by filtration through a 0.22 μm filter at the end of the experiment. The filters were dried and weighed to calculate the biomass (dry weight) concentration. The rate of TBA degradation (μmol of TBA degraded g^{-1} biomass dry weight d^{-1}) was calculated from the residual substrate concentration measured after 24 h. All experiments were performed in triplicate.

Analytical procedures

TBA was quantified by flame ionisation detection on a Varian 3300 gas chromatograph (Varian, France) equipped with a 0.32 mm×25 m Porabond-Q capillary column (J&W Scientific, Chromoptic, Auxerre, France), using a two-step temperature gradient ranging from 105°C to 210°C at 10°C min^{-1}, then maintained at 210°C for 20 min. Helium (1.6 ml min^{-1}) was used as the carrier gas. Samples, filtered through 0.22 μm filters (Prolabo, Fontenay-sous-Bois, France), were injected without further treatment.

Gaseous carbon dioxide was quantified by thermal conductivity detection (GC/TCD) on a Varian 3800 gas chromatograph fitted with a Porapak Q column (1830×2 mm). Oven and detector temperatures were 100°C and 130°C, respectively. The carrier gas was helium (30 ml min^{-1}) and the column was maintained at 50°C.

Total DNA extraction

Total DNA (tDNA) was isolated from the mycobacteria according to the method of Pospiech and Neumann (1995) with the following modifications: a 50 ml culture grown on LB medium to an OD_{600} ~0.7 was centrifuged (8,000×*g* at 4°C for 15 min) and re-suspended in 5 ml of SET buffer (75 mM NaCl–25 mM EDTA–20 mM Tris–HCl, pH 8.0). Lysozyme (final concentration of 1 mg ml^{-1}) and 500 μl of lysostaphin at 112 U μl^{-1} (Sigma) prepared in 10 mM Tris–HCl, pH 7.5, were added to the cell suspension before incubation at 37°C for 1 h. After the SDS/Proteinase K treatment, an extraction step with NaCl–CTAB replaced the addition of 1/3 volume of 5 M NaCl:1/4 volume of 5 M NaCl (1 M final) and 1/7.5 volume of 10% CTAB–0.7 M NaCl (pre-heated to 65°C) were added sequentially and the mixture was incubated 10 min at 65°C. Then, 1 volume of $CHCl_3$ was added and the subsequent steps were carried out according to the original protocol.

16S rRNA gene and *alkB* amplification and analysis

PCR amplification of the 16S rRNA gene was performed using the universal primers for bacteria: the forward primer 8F, 5′-AGAGTTTGATYMTGGCTCAG-3′, and the reverse primer 1492R, 5′-CGGTTACCTTGT TACGACTT-3′. The final 50-μl reaction mixture contained 150 ng μl^{-1} of template DNA, 0.5 μM of each primer, 1x DNA polymerase buffer, 1.5 mM $MgCl_2$, 0.25 mM (each) deoxynucleoside triphosphates, and 1.25 U of Taq polymerase (Promega). Polymerase chain reaction cycles were performed on a iCycler (BioRad) as follows: after 5 min of initial denaturation at 94°C, nucleic acids were amplified for 30 cycles (30 s of denaturation at 95°C, 30 s of annealing at 53°C and 2 min of elongation at 72°C), followed by a final extension step at 72°C for 8 min. PCR products were checked on 1% (*w*/*v*) agarose gel stained with ethidium bromide. The amplification products were purified with the Qiagen Kit (Qiagen, Mississauga, Ontario, Canada) and the nucleotide sequence was determined using primer 8F.

PCR amplification of the partial *alkB* gene was performed using the forward primer Rhose2 (5′-ACG-GSC-CAY-TTC-TAC-RTC-G-3′) and the reverse primer Rhoas1 (5′-CCG-TAR-TGY-TCG-AGR-TAG-3′), previously designed (Heiss-Blanquet et al. 2005; Solano-Serena et al. 2004). Rhose2 and Rhoas1 were located between positions Tyr160/Ile166 and Tyr270/Gly275, respectively, in the *alkB* sequence of *P. putida* GPo1. The conditions of the PCR amplification were previously described (Heiss-Blanquet et al. 2005). The amplification product (expected size of 343 bp) was purified with the Qiagen Kit and the sequence was determined using primer Rhose2.

The sequencing reactions were performed using the BigDye terminator cycle sequencing kit (Version 3.1, Applied Biosystems, Foster City, CA, USA) as described by the manufacturer with 25 ng of purified DNA and 15 μmol of the appropriate primers. The reaction programme consisted of 25 cycles of 10 s at 96°C, 5 s at 50°C and 4 min at 60°C. The sequencing products were purified with Centri-Sep columns as described by the manufacturer (Princeton Separations, Adelphia, NJ, USA) to remove excess terminators. Sequences were determined using the ABI Prism 377 automated fluorescence sequencer (Applied Biosystems, Foster City, CA, USA). The nucleotide sequences were submitted to the EMBL/GenBank database and similarities were evaluated using the BLAST alignment tool (Altschul et al. 1997).

Another primer pair was also tested for PCR amplification: forward primer TS2S (5′-AAY-AGA-GCT-CAY-GAR-YTR-GGT-CAY-AAG-3′) and reverse primer deg1RE (5′-GTG-GAA-TTC-GCR-TGR-TGR-TCI-GAR-TG-3′) (Smits et al. 1999) using the conditions described by these authors.

Southern hybridisation

Southern blot analysis was carried out on the total DNA of *M. austroafricanum* IFP 2012 digested with 7 different restriction enzymes (*Pst*I, *Stu*I, *Cla*I, *Bcl*I, *Eco*RI, *Kpn*I, *Sfo*I) and probed with a 343-bp PCR fragment (*alkB* probe) labelled with digoxigenin-dUTP (DIG DNA Labeling and Detection Kit, Roche Diagnostics, Laval, Quebec, Canada) according to Lopes Ferreira et al. (2006a).

RNA extraction

All glassware and solutions were made RNase-free according to standard procedures (Sambrook and Russell 2001). The total RNA of *M. austroafricanum* IFP 2012 was extracted from a 20-ml culture grown to an OD_{600} of ca. 0.7 in MM medium supplemented with the required substrate. After incubation on ice for 30 min, cells were harvested (8,000×*g* at 4°C for 10 min) and kept on ice. Lysozyme (600 µl of 3 mg ml^{-1}) (Roche Diagnostics, Laval, Canada) and 600 µl of 50 µg ml^{-1} lysostaphin (Sigma, St. Louis, Missouri) were added to the cell pellet and incubated at 37°C for 10 min. RNAwiz™ (10 ml) (Ambion, Austin, Texas) was added and the mixture was mixed vigorously for 15 s in a vortex mixer equipped with a vortex adapter (Ambion). The resulting mixture was divided into 2-ml tubes (1,400 µl per tube) containing 250 mg of zirconium–silica beads (0.1 mm diametre) and mixed for 10 min on the vortex mixer. After centrifugation (13,000×*g* at 4°C for 5 min), the bacterial lysates were recovered from each tube, transferred to a clean 2-ml tube and 0.2 volume of $CHCl_3$ were added. The tubes were then vortexed for 30 s, incubated at room temperature (10 min), centrifuged (13,000×*g* at 4°C for 5 min) and the aqueous supernatants were transferred to another 2-ml tube. RNA extracts were precipitated by sequentially adding 0.5 volume of H_2O treated with DEPC, 1 volume of isopropanol and 1/50 volume of RNase-free glycogen at 5 mg ml^{-1} (Ambion), mixed and incubated at room temperature for 10 min. The RNA was pelleted (13,000×*g* at 4°C for 15 min), washed twice with 70% ethanol, dried for 30 s (DNA Speed Vac, Savant) and re-suspended in sterile tubes in 25 µl H_2O–DEPC. The quality of the pooled RNA was assessed by electrophoresis on a non-denaturing 1% agarose gel in 0.5X TBE. RNA was stored at −80°C, precipitated with 1/10 volume of 3 M sodium acetate, pH 7.0 and 2.5 volume of 95% ethanol.

DNase treatment and reverse transcription PCR (RT-PCR)

Two micrograms of RNA extracted from *M. austroafricanum* IFP 2012 were treated with DNA-free (Ambion, Austin, Texas) according to the manufacturers' instructions. DNA removal was assessed by PCR with the Rhose1 and Rhoas2 primers. The final concentration of RNA was quantified at 260 nm using NanoDrop (NanoDrop Technologies, Wilmington, USA). RT-PCR was performed using the Qiagen One step RT-PCR kit (Qiagen, Mississauga, Canada) with 120 ng of RNA. Positive and negative controls, i.e. with or without 100 ng of *M. austroafricanum* IFP 2012 tDNA and minus RT-PCR controls were carried out for each RNA preparation.

The conditions of RT-PCR were those recommended by the manufacturer: reverse transcription step: 50°C, 30 min; ∞ at 50°C to allow the addition of the positive (+tDNA), negative (−RNA) and (−) RT-PCR (no reverse transcription step) controls. The PCR amplification step with primer pair Rhose2/Rhoas1 was: 95°C, 15 min; 35 cycles of 94°C, 30 s; 55°C, 1 min; 72°C, 1 min followed by a final extension at 72°C for 10 min. Samples were then stored at 4°C.

Nucleotide sequence accession numbers

The total 16S rRNA gene sequences of *M. autroafricanum* IFP 2012, IFP 2015 and IFP 2173, *Mycobacterium* sp. IFP 2009 and *M. smegmatis* mc2 155 have been deposited in the GenBank nucleotide sequence database under accession numbers AY786471, AY786470, AF190800, DQ227003 and DQ227004, respectively.

The partial *alkB* gene sequences of *M. austroafricanum* IFP 2012, IFP 2015 and IFP 2173, *Mycobacterium* sp. IFP 2009 and *M. smegmatis* mc2 155 were similarly deposited under accession numbers DQ226998, DQ226999, AY360960, DQ227000 and DQ227001, respectively.

Results

Capacity of *M. austroafricanum* IFP 2012 to grow on *n*-alkanes

M. austroafricanum IFP 2012 was pre-cultivated in LB broth and was then tested for the capacity to grow in a mineral medium (MM) in the presence of gaseous (methane, ethane, propane and butane) or liquid *n*-alkanes (hexane, octane, dodecane and hexadecane) (Table 1). *M. austroafricanum* IFP 2012 was simultaneously tested for the capacity to mineralise these *n*-alkanes under similar conditions (Table 1). This strain was neither able to grow on methane nor to mineralise it, but it had a broad spectrum of *n*-alkane utilisation from C_2 to C_{16}.

Presence of an *alkB* gene in the genome of *M. austroafricanum* IFP 2012

Using the primer pairs TS2S/deg1RE used by Smits et al. (1999), no amplification product was obtained using the

tDNA extracted from *M. austroafricanum* IFP 2012 as the template. These primers had previously been used to amplify *alkB* genes from various gram-negative and gram-positive microorganisms able to grow on *n*-alkanes, and also from *Nocardioides* sp. strain CF8, grown on C_2 to C_{16} *n*-alkanes (Hamamura et al. 2001).

The presence of an *alkB* gene was then investigated by PCR amplification with the degenerate primer pair Rhose2/Rhoas1, previously successfully used to amplify a partial *alkB* gene from *M. austroafricanum* IFP 2173 (Solano-Serena et al. 2004). The resulting amplicon with the expected 343-bp size was purified and sequenced.

The 343-bp *alkB* PCR fragment amplified from *M. austroafricanum* IFP 2012 was used as a probe in Southern hybridisation. Seven restriction enzymes were used to digest the tDNA of this strain and a unique band was detected in all cases (Fig. 1) demonstrating that the *alkB* gene is present as a single copy in the genome of *M. austroafricanum* IFP 2012.

Expression of *M. austroafricanum* IFP 2012 *alkB* gene

The expression of alkane hydroxylase in *M. austroafricanum* IFP 2012 was investigated by RT-PCR after growth on propane, hexadecane, TBA or LB (Fig. 2a) or after growth on hexane (Fig. 2b) using the primer pair Rhose2/Rhoas1. Expression of the *alkB* gene was seen after growth on propane, hexane, hexadecane and TBA, whereas no RT-PCR product was detected after growth in LB. This result shows that the alkane hydroxylase is induced during growth on *n*-alkanes including the gaseous alkane, propane. Moreover, the strong expression of the *alkB* gene after growth on TBA indicates its specific induction during growth on TBA, thus suggesting its involvement in the TBA oxidation step of the MTBE metabolic pathway.

TBA degradation capacities of the mycobacteria after growth on *n*-alkanes

The possible similar role of the alkane hydroxylase in the other mycobacteria was therefore investigated. *M. austroafricanum* IFP 2015 and IFP 2173, *Mycobacterium* sp. IFP 2009, *M. vaccae* JOB5 and *M. smegmatis* mc2 155, pre-cultivated in LB broth, were tested for their capacity to grow in MM in the presence of the gaseous *n*-alkanes, methane, ethane, propane and butane or the liquid *n*-alkanes, hexane, octane, dodecane and hexadecane (Table 1). All strains were simultaneously tested for their capacity to mineralise these *n*-alkanes under similar conditions (Table 1). None of the strains were able to grow on or mineralise methane. *M. austroafricanum* IFP 2015, *M. vaccae* JOB5 (*M. austroafricanum* ATCC 29678) and *M. austroafricanum* IFP 2012

Table 1 Growth on *n*-alkanes and mineralisation yield of *n*-alkanes by fast-growing mycobacteria

Strains		Growth[a] on *n*-alkanes and mineralisation yield[b] (%) of *n*-alkanes							
		Methane	Ethane	Propane	Butane	Hexane	Octane	Dodecane	Hexadene
M. austroafricanum IFP 2012	Growth	–	++[c]	+[c]	+[c]	++[c]	++[c]	+	++[c]
	Mineralisation yield	0	58	25	40	51	61	57	46
M. austroafricanum IFP 2015	Growth	–	++[c]	±	+[c]	++[c]	++[c]	±	++
	Mineralisation yield	0	58	48	39	54	53	43	86
M. vaccae JOB5 (re-classified *M. austroafricanum*)	Growth	–	++	+	+[c]	++	±	+	++
	Mineralisation yield	0	48	38	32	63	53	28	70
M. austroafricanum IFP 2173	Growth	–	–	–	–	++	++	–	+(clumps)
	Mineralisation yield	0	0	43	45	56	59	37	77
Mycobacterium sp. IFP 2009	Growth	–	–	–	–	+	++	++	±
	Mineralisation yield	0	0	0	0	36	77	6	66
M. smegmatis mc2 155	Growth	–	–	–	–	–	–	+(clumps)	–
	Mineralisation yield	0	0	0	0	0	0	34	43

All cultures were seeded at an OD_{600} of ~0.1 and the growth was measured after 15 days of incubation except when mentioned. Each substrate was added to a final concentration of 200 mg l^{-1} (calculated from the liquid volume of the culture).

[a] OD_{600} was measured and growth was reported as follows: −, no growth; ±, 2×(initial OD_{600}); +, 3×(initial OD_{600}); ++, ≤4×(initial OD_{600}).

[b] Percent of mineralisation=(moles of carbon in the CO_2 produced/moles of carbon in the substrate added)×100

[c] Corresponds to the measure of growth after 3 days of incubation.

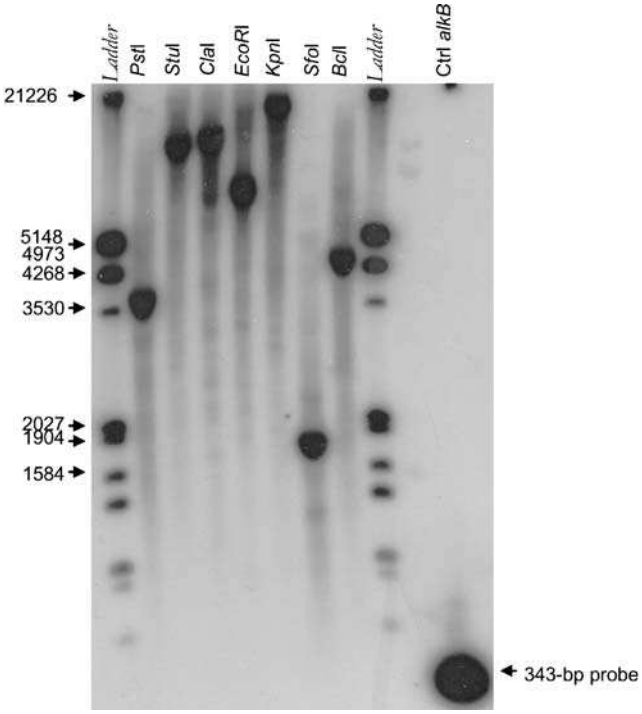

Fig. 1 Southern blot hybridisation on the restricted tDNA of *M. austroafricanum* IFP 2012. The 343-bp *alkB* sequence labelled with digoxigenin-dUTP was used as the probe. The tDNA of *M. austroafricanum* IFP 2012 was digested with *Pst*I, *Stu*I, *Cla*I, *Bcl*I, *Eco*RI, *Kpn*I or *Sfo*I

had the broadest spectrum of *n*-alkane utilisation from C_2 to C_{16}. Although *M. austroafricanum* IFP 2173 did not grow on *n*-propane, *n*-butane or dodecane, it partially mineralised these three compounds and was able to grow on hexadecane. *Mycobacterium* sp. IFP 2009 only grew on liquid *n*-alkanes (from C_6 to C_{16}). *M. smegmatis* mc2 155 only grew on dodecane, but was able to partially mineralise hexadecane.

The capacity to degrade TBA was tested using resting cells of the different mycobacteria after growth on LB or on the *n*-alkanes that they could use as carbon sources. Thus, propane, hexane and hexadecane for *M. austroafricanum* IFP 2012, IFP 2015 and *M. vaccae* JOB5, hexane or hexadecane for *M. austroafricanum* IFP 2173 and *Mycobacterium* sp. IFP 2009 and dodecane for *M. smegmatis* mc2 155 were used as carbon sources to prepare resting cells. After 24 h incubation, the amount of TBA degraded was calculated, the biomass (dry weight) was measured and the amount of TBA degraded was expressed in μmol of degraded g^{-1} biomass (dry weight) d^{-1}.

Resting cells of *M. smegmatis* mc2 155, *Mycobacterium* sp. IFP 2009 and *M. austroafricanum* IFP 2173 that had been grown on *n*-alkanes did not produce any TBA degradation activity.

M. austroafricanum IFP 2012 and IFP 2015 degraded TBA after growth on propane or hexadecane and to a greater extent after growth on hexane (Fig. 3). Propane was a good inducer of TBA degradation activity in *M. vaccae* JOB5 (Fig. 3), as previously determined by Smith et al. (2003).

Presence of an *alkB* gene in the genome of the mycobacteria

The presence of an *alkB* gene in the other mycobacteria included in our study was investigated by PCR amplification with the slightly degenerate primer pair Rhose2/Rhoas1, previously used to amplify and sequence the partial *alkB* gene from *M. austroafricanum* IFP 2012. In all the tested mycobacteria, the expected fragment size

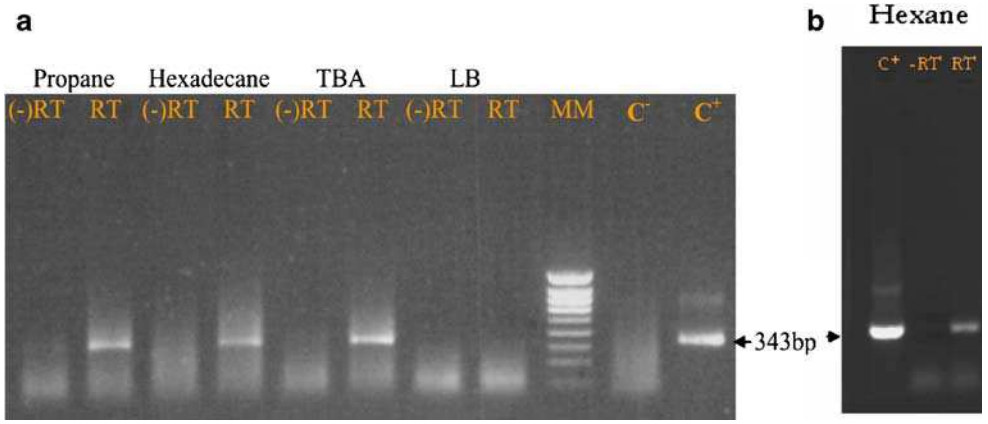

Fig. 2 Gene expression analysis in *M. austroafricanum* IFP 2012 by RT-PCR. **a** After growth on propane, hexadecane, TBA and LB. **b** After growth on hexane. Total RNA of *M. austroafricanum* IFP 2012 cells grown on MM supplemented with TBA, propane, hexane, hexadecane or LB were reverse transcribed. The reverse transcripts were subjected to PCR amplification using the *alkB* primer pair Rhose2/Rhoas1 (5′-ACGGSCCAYTTCTACRTCG-3′/5′-CCGTART GYTCGAGRTAG-3′). RNA samples were subsequently analysed by RT-PCR (*RT*). C^+ positive control (+tDNA), C^- negative control (−RNA), *−RT* control without reverse transcription step

(343 bp) was amplified. These amplicons were purified and sequenced. The deduced amino acid sequences were compared with the corresponding AlkB polypeptide sequences of *M. austroafricanum* IFP 2012, *M. tuberculosis* H37Rv and *P. putida* GPo1 (Fig. 4a). We also included in the alignment the partial sequence of a putative desaturase encoded by a gene from *M. petroleiphilum* PM1. This strain was the first one isolated for its capacity to grow on MTBE and its genome has recently been sequenced. The sequence was found by screening the genome of *M. petroleiphilum* PM1 for a homologue of the *P. putida alkB* gene.

The topology of all the mycobacterial homologues of AlkB was determined using the TopPred online prediction software (Claros and von Heijne 1994) and compared to the *P. putida* GPo1 AlkB topology. We detected the same putative AlkB protein structure (Fig. 4a) as that already described in known AlkB proteins (for the structure of AlkB, see Van Beilen et al. 2005). It included the two *trans*-membrane (TM) helices TM5 and TM6, constituting one of the three helix pairs of AlkB connected by a very short loop in the periplasm. We also detected the consensus motifs Ib [EHXXGHH] and C [NYXEHYG] (Shankling and Whittle 2003) containing two of the four highly conserved sequences of the AlkB "eight-histidine motif". The Ib region, associated with two other consensus regions (Ia and II), was previously shown to be essential for the activity of AlkB (Shankling and Whittle 2003), whereas the single conserved histidine of the motif C located between the Ib and II regions, always detected in AlkB polypeptidic sequences (Smits et al. 1999; Van Beilen et al. 2001), was identified as an additional potential ligand (Van Beilen et al. 2004). Hydropathy diagram comparison (data not shown) showed highly conserved profiles of the mycobacteria homologues of AlkB using the topology model proposed by Van Beilen et al. (1992) for the *P. putida* GPo1 AlkB.

A phylogenetic tree was built using the 16S rRNA complete gene sequences of the different mycobacteria included in our study and the 16S rRNA gene of *M. tuberculosis* H37Rv and *P. putida* GPo1. We also included the 16S rRNA gene sequence (AF176594) of *M. petroleiphilum* PM1 (Nakatsu et al. 2006) to visualise the phylogenetic differences between the studied strains (Fig. 4b). This phylogenetic tree was compared to the one built from the partial *alkB* gene sequences of the same strains (Fig. 4c). There is no divergence degree among *M. austroafricanum* species based on their 16S rRNA gene and among their partial *alkB* sequences, which strongly clustered in both trees (Fig. 4b,c) suggesting the relative ancient presence of the *alkB* gene in the genome of mycobacteria.

The partial *alkB* gene sequence of *M. austroafricanum* IFP 2012 was used to screen the genomes of the fully sequenced mycobacteria, *M. avium* ssp. *paratuberculosis* K-10, *M. bovis* AF2122/97, *M. leprae* TN, *M. tuberculosis* CDC1551 and *M. tuberculosis* H37Rv (Microbial Genome BLAST Research) and also the partially sequenced genome of *M. smegmatis* mc2 155 (http://www.tigr.org/tigr-scripts/CMR2/GenomePage3.spl?database=gms). In all cases, only one homologue of an *alkB* gene was identified in these genomes, suggesting the presence of a unique *alkB* gene in *Mycobacterium* (data not shown). A similar finding was previously reported for *M. tuberculosis* H37Rv by Van Beilen et al. (2003). Moreover, the *alkB* gene homologue amplified in

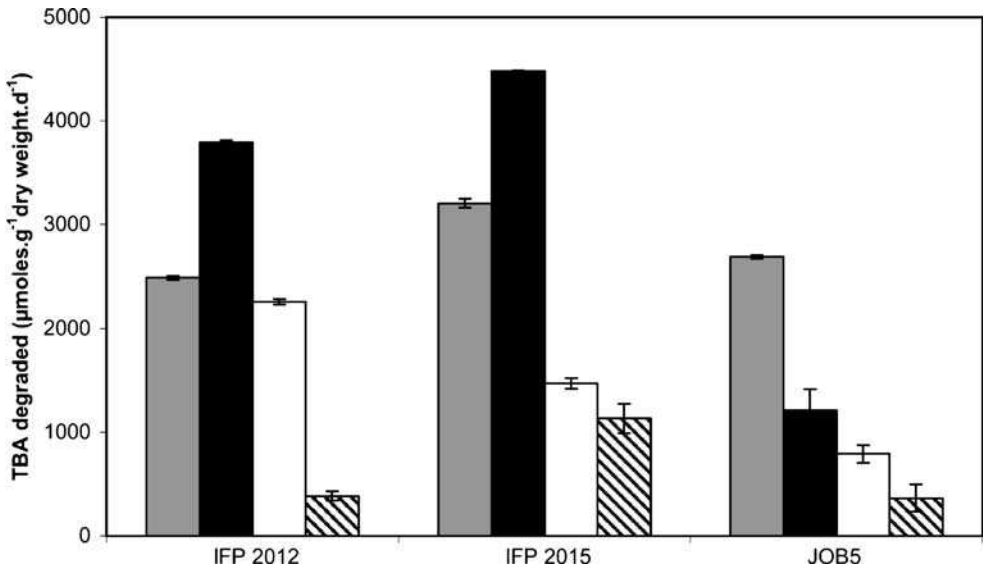

Fig. 3 TBA degradation capacities of the different fast-growing mycobacteria after growth on various *n*-alkanes. Experimental conditions were those described in the "Materials and methods" section. The residual concentrations of TBA were measured after 24 h of incubation. The results are expressed in µmol of TBA degraded g^{-1} (dry weight) d^{-1}. , growth on propane; , growth on hexane; , growth on hexadecane; , growth on LB

M. smegmatis mc2 155 was identical (100% homology) to the corresponding sequence screened in the partially sequenced genome of this strain. Near the *alkB* genes (generally downstream) of the above cited mycobacteria, except for *M. avium*, one putative *tetR* regulatory gene and, at least, one putative rubredoxin gene were detected. This situation was previously described by Van Beilen et al. (2003).

Discussion

Capacity of environmental mycobacteria to degrade *n*-alkanes

The capacity to grow on *n*-alkanes is a property shared by many microbial genera (Van Beilen et al. 2003), although this capacity is not equally documented among bacteria. Van Beilen et al. (2002) detected the *alkB* gene in two among six environmental mycobacteria able to grow on *n*-alkanes. In our study, we found that the capacity to degrade *n*-alkanes was common to all the fast-growing mycobacteria tested but the spectrum of *n*-alkanes used was highly dependent on the strains. *M. smegmatis* mc2 155 was able to grow only on dodecane. This narrow range of substrate use was previously found in *P. putida* P1, which grew on octane (Smits et al. 1999) and in *P. aureofasciens* RWTH 529, which only grew on decane (Vomberg and Klinner 2000). Johnson and Hyman (2006) recently demonstrated a new capacity of *P. putida* GPo1 to also grow on the gaseous *n*-alkanes, propane and butane, confirming the broad alkane spectrum of the alkane hydroxylase in this strain. Among the strains of *M. austroafricanum* examined in this study, three of them were able to use C_2 to C_{16}*n*-alkanes as their sole carbon and energy source: *M. austroafricanum* IFP 2012 and IFP 2015, isolated for their capacity to grow on TBA and MTBE (François et al. 2002; Lopes Ferreira et al.

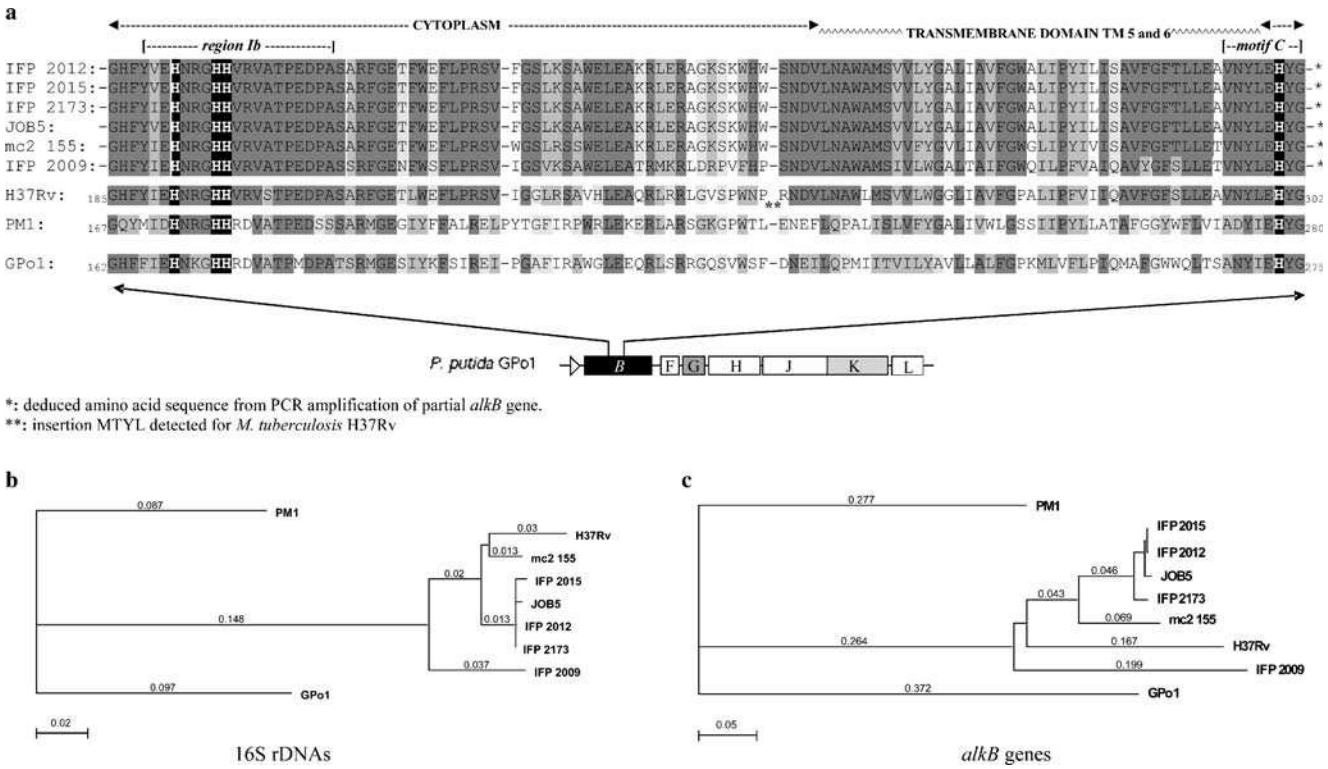

Fig. 4 **a** Alignment of the partial amino acid sequences of the PCR-amplified *alkB* genes from mycobacteria. **b** Phylogenetic tree based on the 16S rRNA gene sequences of the investigated strains. **c** Phylogenetic tree based on the partial *alkB* sequences of the investigated strains. The 16S rRNA gene was PCR-amplified and sequenced when it was not available in databases, i.e. in the cases of *M. vaccae* JOB5, *M. smegmatis* mc2 155 and *Mycobacterium* sp. IFP 2009. The 16S rRNA gene and the *alkB* amino acid sequences of *M. tuberculosis* H37Rv *alkB* (accession numbers: X58890 and Z95121, respectively) and of *P. putida* GPo1 (accession numbers: AJ249825 and CAB54050, respectively) were included for comparison. The topology of the *alkB* sequences of mycobacteria was obtained by TopPred prediction (Claros and von Heijne 1994) and compared to the topology of *P. Putida* GPo1 *alkB* gene (Van Beilen et al. 1992). The determination of the Ib and C regions was carried out by similarity according to Shankling and Whittle (2003). Phylogenetic trees of 16S rRNA gene and partial *alkB* gene sequences were built using the neighbour-joining method of Saitou and Nei (1987) and evolutionary distances were calculated according to the model of Tamura and Nei (1993). *P. putida* Gpo1 was chosen as an outgroup to root the tree. Bootstrap analysis (Felsenstein 1985) with 1,000 replicates was performed to determine the statistical significance of the branching order. Percentage of occurrence over 70% for a node in the phylogenetic tree is indicated. Branch lengths are shown to scale, indicating evolutionary distance

2006b) and *M. austroafricanum* JOB5, isolated for its capacity to grow on 2-methylbutane (Ooyama and Foster 1965). The ability to grow on such a wide range of substrates was also observed with *Nocardioides* sp. CF8 (Hamamura et al. 2001). In this strain, RT-PCR experiments performed on cells grown on C_4, C_5, C_6, C_7 or C_8 *n*-alkanes demonstrated that the *alkB* gene was only induced during growth on C_6, C_7 or C_8 *n*-alkanes (Smits et al. 1999), suggesting the presence of two different monooxygenase systems.

Alkane hydroxylases in mycobacteria

Using a specific *alkB* primer set, PCR fragments from the *alkB* gene were amplified from all the fast-growing mycobacteria used in this study. This result can be partially attributed to the efficiency of the PCR primer pair (Rhose2/Rhoas1). These primers, previously designed from the *alkB* sequence of *P. putida* GPo1 and located between positions Tyr160/Ile166 and Tyr270/Gly275 (Heiss-Blanquet et al. 2005; Solano-Serena et al. 2004), were less degenerate than the TS2S/deg1RE primer pair (Smits et al. 1999) and amplified a smaller DNA fragment (343 bp instead of 525 bp). The polypeptide sequences, deduced from the nucleotide sequences of the PCR amplified fragments, were compared to the corresponding polypeptide fragments of AlkB from *M. tuberculosis* H37Rv and *P. putida* GPoI. This alignment revealed conserved patterns comprising functional consensus sequences of the "eight-histidine motif". A highly conserved topology, characterised by the detection of two of the six *trans*-membrane domains always found in AlkB, was also observed.

From a phylogenetic point of view, the four *Mycobacterium* strains belonging to the species *austroafricanum* (IFP 2012, IFP 2015, JOB5 and IFP 2173), are indeed very closely related as confirmed by the 16S rRNA gene phylogenetic tree (Fig. 4b). The *alkB* phylogenetic tree, built on the partial *alkB* sequences, showed an overlap with the 16S rRNA gene phylogenetic tree. This suggests the relatively ancient presence of the *alkB* gene in the genome of these mycobacteria. This hypothesis is also supported by the high (G+C) content of all these *alkB* sequences (≥63.5%), which correlates well to the high (G+C) content of the mycobacterial genome.

Results from our screening of sequenced genomes demonstrated the presence of a putative *alkB* homologue in *M. petroleiphilum* PM1, although the partial sequence of this *alkB* was highly divergent from both *P. putida* GPo1 and mycobacteria *alkB* genes (Fig. 4c). The divergence between the AlkB sequences of the mycobacterial strains and *P. putida* GPo1 does not seem to affect the global organisation or function of AlkB because activity towards *n*-alkanes was shown in all cases. Further studies on the conformation of these different membrane-bound alkane hydroxylases are likely to reveal local modifications in the structures.

Role of the alkane hydroxylase in TBA degradation by *M. austroafricanum* species

In this study, we also demonstrated the possible link between the capacity to grow on *n*-alkanes and the capacity to degrade TBA in the *M. austroafricanum* strains tested. The involvement of an alkane hydroxylase with extended capacities towards *n*-alkanes in TBA oxidation was also shown by the amount of TBA degraded after growth on propane, hexane and hexadecane (Fig. 3). In *M. austroafricanum* IFP 2012, the *alkB* gene was expressed after growth on propane, hexane and hexadecane and also on TBA. Moreover, it has to be emphasised that the *alkB* gene was present as a single copy in the genome of *M. austroafricanum* IFP 2012. This is the first report providing information on the monooxygenase involved in TBA oxidation in a strain able to grow on MTBE.

Considering its capacity towards TBA, the alkane hydroxylase induced in *M. austroafricanum* strains IFP 2012, IFP 2015 and JOB5 is clearly different from the one induced on *n*-octane in *P. putida* GPo1 as the latter is not active towards TBA (Smith and Hyman 2004).

Role of the alkane hydroxylase of *M. austroafricanum* IFP 2012 in propane oxidation

Our results showed that the single genomic copy of the *alkB* gene was strongly expressed after growth on propane in *M. austroafricanum* IFP 2012 and, to our knowledge, this ability has only very recently been reported by Johnson and Hyman (2006) when studying the capacity of the alkane hydroxylase of *P. putida* Gpo1 towards gaseous *n*-alkanes. Our report confirms the involvement of a "classical" (i.e. acting on liquid *n*-alkanes) non-hemic alkane hydroxylase having a substrate range extended to propane oxidation. Studies to date show that the ability to oxidise propane appears to be mostly confined to the gram-positive *Corynebacterium–Nocardia–Mycobacterium–Rhodococcus* complex (Ashraf et al. 1994). The only monooxygenase systems that have been shown to be involved in propane and butane oxidation are: (1) a soluble NADH-dependent multi-component enzyme system belonging to the family of dinuclear-iron oxygenases in *Gordonia* sp. strain TY-5 (Kotani et al. 2003), (2) a 54-kDa polypeptide involved in butane oxidation in *Pseudomonas* sp. IMT37 and encoded by a gene displaying no sequence similarity with alkane hydroxylases oxidising liquid alkanes (Padda et al. 2001) and (3) a soluble butane monooxygenase (sBMO), which is a multimeric hydroxylase (61, 45 and 19 kDa) encoded by the *bmoXYZ* genes of *Pseudomonas butanovora* growing on butane (Sluis et al. 2002). In the case of *Nocardioides* sp. CF8, two distinct

systems were involved in the degradation of C_2 to C_{16} *n*-alkanes (Hamamura et al. 2001) including one alkane hydroxylase, but the oxygenase system was not further characterised.

In conclusion, we report that the alkane hydroxylases of *M. austroafricanum* strains IFP 2012, IFP 2015 and JOB5 isolated from different locations and at different times shared similar properties and that there is a relationship between the capacity to grow on a wide range of *n*-alkanes (C_2 to C_{16}) and the capacity to oxidise TBA. Moreover, *M. austroafricanum alkB* genes are strongly related phylogenetically, which suggests the ancient presence of this gene in this species.

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