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The 2-methylpropene degradation pathway in *Mycobacteriaceae* family strains

Steffen Helbich[1] | **Israel Barrantes**[2] | **Luiz Gustavo dos Anjos Borges**[2] | **Dietmar H. Pieper**[2] | **Yevhen Vainshtein**[3] | **Kai Sohn**[3] | **Karl-Heinrich Engesser**[1]

[1]Institute for Sanitary Engineering, Water Quality and Solid Waste Management, University of Stuttgart, Stuttgart, Germany

[2]Microbial Interactions and Processes, Helmholtz Centre for Infection Research, Braunschweig, Germany

[3]Fraunhofer Institute for Interfacial Engineering and Biotechnology, Stuttgart, Germany

Correspondence
Steffen Helbich, Institute for Sanitary Engineering, Water Quality and Solid Waste Management, University of Stuttgart, Stuttgart, Germany.
Email: steffen.helbich@iswa.uni-stuttgart.de

Present addresses
Israel Barrantes, Rostock University Medical Center, Institute for Biostatistics and Informatics in Medicine and Ageing Research, Rostock, Germany; and Luiz Gustavo dos Anjos Borges, Max-Delbrück Centre for Molecular Medicine, Berlin Institute for Medical Systems Biology, Epigenetic Regulation and Chromatin Architecture Group, Berlin, Germany.

Abstract

Mycolicibacterium gadium IBE100 and *Mycobacterium paragordonae* IBE200 are aerobic, chemoorganoheterotrophic bacteria isolated from activated sludge from a wastewater treatment plant. They use 2-methylpropene (isobutene, 2-MP) as the sole source of carbon and energy. Here, we postulate a degradation pathway of 2-methylpropene derived from whole genome sequencing, differential expression analysis and peptide-mass fingerprinting. Key genes identified are coding for a 4-component soluble diiron monooxygenase with epoxidase activity, an epoxide hydrolase, and a 2-hydroxyisobutyryl-CoA mutase. In both strains, involved genes are arranged in clusters of 61.0 and 58.5 kbp, respectively, which also contain the genes coding for parts of the aerobic pathway of adenosylcobalamin synthesis. This vitamin is essential for the carbon rearrangement reaction catalysed by the mutase. These findings provide data for the identification of potential 2-methylpropene degraders.

INTRODUCTION

2-Methylpropene (isobutene, 2-MP) is a colourless gas of industrial importance. It is the precursor for the synthesis of methacrolein, butyl rubber, the gasoline oxygenates methyl *tert*-butyl ether, ethyl *tert*-butyl ether and isooctane, vitamin A and the terpenoids citral, linalool and geraniol (van Leeuwen et al., 2012). It is also used as a propellant, thus being released into the atmosphere during the use and recycling of spray cans. Its structural homology to isoprene suggests a similar impact on atmospheric chemistry. While reactions of the alkene with OH radicals and NO_x promote tropospheric ozone formation, stratospheric ozone is depleted by the generation of ozonide and Criegee intermediates (Pacifico et al., 2009).

Likewise originating from its molecular structure, various strategies for aerobic microbial degradation seem to be possible (Figure 1). A monooxygenase attack on an sp^3-carbon (Takami et al., 1999) and subsequent oxidation of the resulting alcohol and aldehyde by alcohol and aldehyde dehydrogenases, respectively, would theoretically yield methacrylic acid (Figure 1, left branch). This intermediate may be channelled into the central metabolism via the lower *L*-valine degradation pathway (Massey et al., 1976). A monooxygenase attack on the carbon = carbon double bond (Figure 1, right branch) would produce 1,2-epoxy-

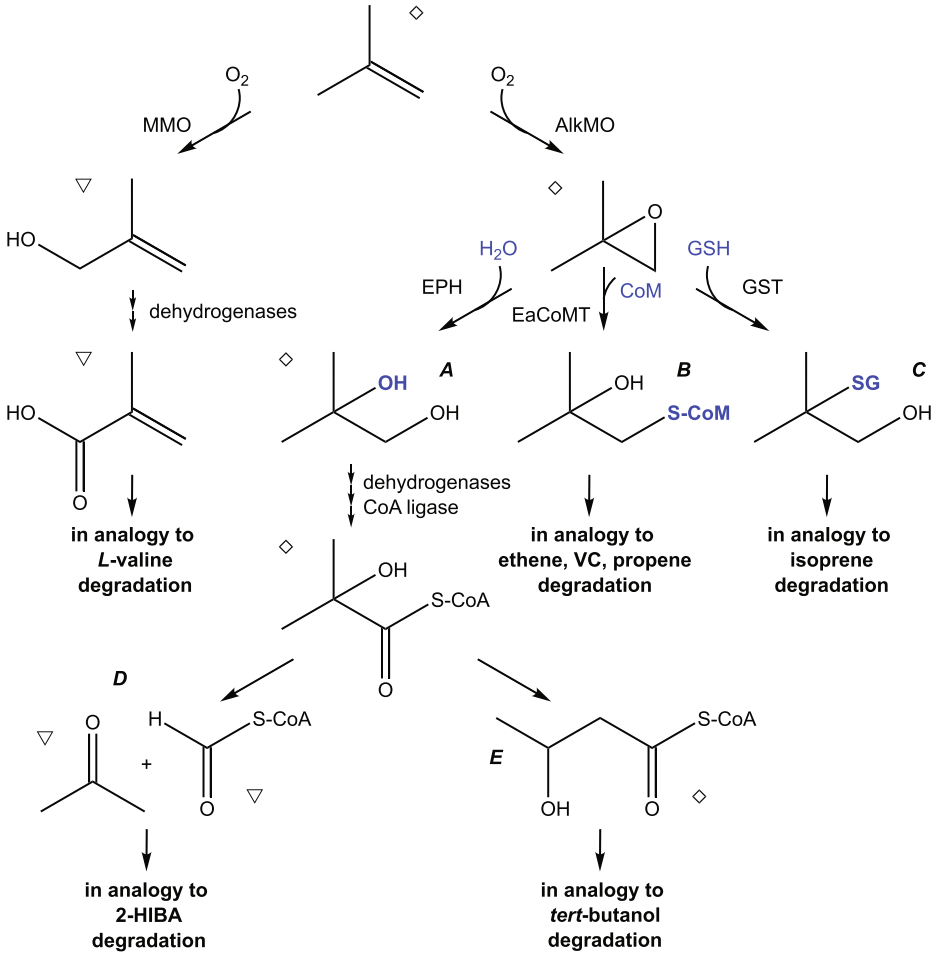

FIGURE 1 Possible initial steps in 2-MP degradation. Left branch: terminal oxidation forms methacrylic acid, which may be channelled into the *L*-valine degradation pathway. Right branch: epoxidation of the carbon = carbon double bond. The resulting epoxide may be opened by three different mechanisms: conjugation with either glutathione (B) or coenzyme M (C), or hydrolytic cleavage (A). After terminal oxidation of the latter product it could be degraded in analogy to the 2-HIBA- (D) or the *tert*-butanol (E) degradation pathway. AlkMO, alkene monooxygenase; CoA, coenzyme A; CoM, coenzyme M; EaCoMT, epoxyalkane CoM transferase; EPH, epoxide hydrolase; GSH, glutathione; GST, glutathione *S*-transferase; MMO, methane monooxygenase; VC: vinyl chloride; 2-HIBA: 2-hydroxyisobutyric acid. Substrates tested ▽ in this study and growth inducers ◇ in strain IBE100 (CoA-conjugates as free acids or Na-salts).

2-methylpropane. This highly reactive intermediate may be subject to ring-opening by coenzyme M, glutathione, or water. In cofactor-dependent reactions, as found in ethene/vinyl chloride/propene and isoprene degradation pathways, thioethers are formed (Figure 1B, C) (Krishnakumar et al., 2008; van Hylckama Vlieg et al., 2000), whereas cofactor independent hydrolysis produces vicinal diols (Figure 1A) (van den Wijngaard et al., 1989).

Up to now, a few bacterial strains are known to oxidise 2-methylpropene to its corresponding epoxyalkane. While *Xanthobacter autotrophicus* Py2 and *Nocardioides* sp. JS614 accumulate 1,2-epoxy-2-methylpropane (isobutylene oxide) as a dead-end-product (Ensign, 1996; Owens et al., 2009), *Mycobacterium* sp. ELW1, isolated from freshwater sediment, is the only bacterial strain capable to use 2-methylpropene as a sole source of carbon and energy (Kottegoda et al., 2015). The degradation pathway, according to metabolite analysis, involves a monooxygenation generating 1,2-epoxy-2-methylpropane, followed by coenzyme-independent hydrolysis to form 2-methylpropane-1,2-diol. From here on, convergence with the methyl *tert*-butyl ether (MTBE) degradation pathway in *Aquincola tertiaricarbonis* L108 (Rohwerder et al., 2006) and *Methylibium petroleiphilum* PM1 (Hristova et al., 2007) is assumed with the central 2-methylpropane-1,2-diol being oxidised via an aldehyde intermediate to 2-hydroxyisobutyric acid (2-HIBA). In *A. tertiaricarbonis* L108, this branched acid is activated by a specific CoA ligase and subsequently isomerized to 3-hydroxybutyryl-CoA (Figure 1E) by a B12-dependent mutase (Yaneva et al., 2012). The linearized CoA-thioester is supposed to enter the central metabolism via β-oxidation and subsequent cleavage into two acetyl-CoA.

Recently, another pathway for 2-HIBA degradation was discovered (Rohwerder et al., 2020). In *Actinomycetospora chiangmaiensis* DSM 45062, the CoA-thioester is cleaved by a thiamine-dependent lyase to acetone and formic acid (Figure 1D), which are further oxidised to pyruvate and CO_2, respectively.

Here, we describe the gene clusters of two novel 2-methylpropene degrading bacterial isolates belonging to the *Mycobacteriaceae* family. A combined strategy of differential expression analysis in one isolate and peptide-mass fingerprinting in the other isolate allowed the postulation of a common degradation pathway, which is distinct from other short-chain and branched alkene degradation pathways. The key enzymes identified were a multicomponent 2-MP monooxygenase of the 'isoprene monooxygenase'-type, an epoxide hydrolase, and a 2-hydroxyisobutyryl-CoA mutase. This is the first verification on the genetic level of a degradation pathway of 2-MP, which up to now has been based solely on transformation analysis with presumptive metabolites, postulated from MTBE degradative experiments.

EXPERIMENTAL PROCEDURES

Enrichment and isolation

The strains used in this study were enriched in 100 mL liquid mineral salts medium (MSM) (see below), inoculated with 5 mL activated sludge from a wastewater treatment plant in Basel (Switzerland), supplied with 2-MP, and incubated as described below. After 7 days, 1 mL of suspension was transferred to a fresh medium plus carbon source and incubated until a visible increase in turbidity was observed. This step was repeated a second time. A dilution of a 1 mL sample was plated on MSM plates and incubated in a desiccator supplied with 2-MP (see below). Single colonies were picked and streaked on fresh plates to obtain pure cultures. Two phenotypically distinct strains could be isolated and were tested again for growth in liquid culture to exclude the utilisation of agar as a carbon source.

Growth conditions

MSM contained KH_2PO_4 1.0 g, Na_2HPO_4 2.79 g, $(NH_4)_2SO_4$ 1.0 g, $Ca(NO_3)_2{\cdot}7H_2O$ 0.01 g, $C_6H_8O_7{\cdot}Fe{\cdot}NH_4$ 0.01 g, and 1 mL of trace mineral solution per litre distilled water. The trace mineral solution consisted of H_3BO_3 0.3 g, $CoCl_2{\cdot}6H_2O$ 0.2 g (left out for cobalt deficient medium), $ZnSO_4{\cdot}7H_2O$ 0.1 g, $Na_2MoO_4{\cdot}H_2O$ 0.03 g, $MnCl_2{\cdot}4H_2O$ 0.03 g, $NiCl_2{\cdot}6H_2O$ 0.02 g, and $CuCl_2{\cdot}2H_2O$ 0.01 g per litre distilled water. Solid medium contained 15 g/L agar. Cultivation in liquid culture was performed in Schott flasks with a maximum of 1/5 of flask space as liquid, sealed gastight with a butyl rubber septum. Gaseous substrates were supplied through the septum via a glass syringe to a final concentration of 8.4 mmol/L medium. Liquid substrates were supplied at a final concentration of 3 mmol/L either via a vaporizer (in case of epoxides) or directly into the liquid medium. Liquid cultures were always supplemented with Triton X-100 (5 μL/L) to prevent clumping of the cells. Incubation was carried out at 30°C in the dark on magnetic bar stirrers (250 rpm). Growth was measured via optical density at 600 nm with a spectrophotometer (Ultrospec III, Pharmacia Biotech), and liquid samples were taken with a glass syringe to prevent gas exchange. For cultivation on solid medium with gaseous substrates, plates were stored without parafilm in desiccators and gassed for 1 s. Liquid substrates were applied in five drops of 2 μL each per plate. Plates were sealed with parafilm and incubated at 30°C in the dark.

Selected substrates were tested as sole sources of carbon and energy, investigating the hypotheses on the valine pathway (*L*-valine, 3-hydroxy-2-methylpropene, methacrylate), the postulated 2-MP pathway (2-methylpropene, 1,2-epoxy-2-methylpropane, 2-methylpropane-1,2-diol, 2-hydroxyisobutyric acid, *LD*-3-hydroxybutyric acid), analogues to postulated 2-MP pathway metabolites (*LD*-1,2-epoxy-propane, *LD*-propane-1,2-diol, butane-2,3-diol (enantiomeric mixture)), the 2-HIBA pathway (acetone, formate), whether MmoXYBR is an alkane/propane monooxygenase with terminal or subterminal preference (methane, *n*-propane, *n*-butane, *n*-hexane, 1-propanol, 1-butanol, 1-hexanol, 2-propanol, 2-butanol, 2-hexanol), substrates with the 2-MP molecular structure, an unsaturated C=C bond, or short branched-chained molecules (2-methylbut-1-ene, 2-methylbut-2-ene, isoprene, *D*-limonene, α-methylstyrene, methylenecyclohexane, 1-methylcyclohexene, 1-hexene, *iso*-butanol, *tert*-butanol), the usage of cyclic alkanes and aromatics (cyclohexane, benzene, benzoate, toluene, styrene), and sugars as well as rich media (glucose, fructose, LB (tryptone 10 g/L, yeast extract 5 g/L, NaCl 10 g/L), NB (peptone 5 g/L, beef extract 3 g/L), and TB (tryptone 24 g/L, yeast extract 20 g/L, glycerol 4 mL/L, KH_2PO_4 14 mmol/L, K_2HPO_2 72 mmol/L)).

To identify the influence of cofactors on growth in MSM, coenzyme M and glutathione (each 5 mmol/L) were added and $CoCl_2{\cdot}6H_2O$ was omitted from the medium.

Phenotypic assays

Cells grown on MSM plates with 2-MP or 1-hexanol were sprayed with a 50 mmol/L indole solution (in dimethylformamide). Indigo formation indicates the

presence of a monooxygenase (Mermod et al., 1986). Bactident™ Oxidase and Aminopeptidase tests (both Merck KGaA) were performed according to the manufacturer's instructions. Catalase activity was tested by the visible formation of oxygen from 3% H_2O_2 using the slide drop method (Taylor & Achanzar, 1972). The acid-fastness of the cells was checked using Ziehl–Neelsen staining.

DNA isolation, genome sequencing, assembly, and annotation

Bacterial DNA was isolated from 2-MP grown cells using the FastDNA Spin Kit for Soil (MP Biomedicals) following the manufacturer's protocol. The quality and concentration of extracted DNA were verified on a Qubit fluorometer. Genomic paired-end libraries (2×250 bp) were prepared according to the instructions from the manufacturer (TruSeq DNA LT Sample Prep Kit, Illumina). Whole genome sequencing was then carried out on a HiSeq 2500 Sequencing System (Illumina). IBE100 reads were identified and trimmed using BBMap v.38.67 (Bushnell, 2014). For IBE200, sequencing adapters were removed from the FASTQ files with scythe, and low-quality bases were trimmed with sickle (Buffalo, 2011; Joshi & Fass, 2011). For IBE100, the qualified reads were assembled by *de novo* assembly using Unicycler (Wick et al., 2017) in PATRIC (Davis et al., 2019). Contigs shorter than 300 bp were discarded. The assembly quality was evaluated by comparison to corresponding complete reference genomes using QUAST (Gurevich et al., 2013). The resultant assembly was annotated using RASTtk (Brettin et al., 2015). For IBE200, the filtered sequences were assembled and scaffolded using multiple k-mers (automatic selection based on read lengths) using SPAdes (Bankevich et al., 2012). The obtained contigs were annotated with Prokka (Seemann, 2014). The novel genomic sequences were submitted to GenBank, under the BioProject PRJNA814055, accession number JAKZMO000000000 (IBE100), and BioProject PRJNA590116, accession number WNWV00000000 (IBE200).

Taxonomy

16S rRNA gene sequences from genome sequencing (MNO81_29630, GKP29_RS26675) were classified with the megablast algorithm against the rRNA/ITS database of 16S ribosomal RNA sequences (Bacteria and Archaea). Partial *hsp65* gene sequences (MNO81_27715, GKP29_RS22280) enclosed by primers HSPF3 and HSPR4 (Kim et al., 2005) were classified with the megablast algorithm against the standard database nucleotide collection (nr/nt). Genome-based taxonomy was performed with the Type (Strain) Genome Server, https://tygs.dsmz.de/ (Meier-Kolthoff & Göker, 2019).

Evolutionary analyses

Evolutionary analyses were conducted in MEGA X (Kumar et al., 2018). The evolutionary history was inferred using the Neighbour-Joining method (Saitou & Nei, 1987). The trees are drawn to scale, with branch lengths in the same units as those of the evolutionary distances used to infer the phylogenetic tree. In case of the 16S rRNA phylogenetic tree, the evolutionary distances were computed using the Maximum Composite Likelihood method (Tamura et al., 2004) and are in the units of the number of base substitutions per site. In case of the peptide sequence comparison, the evolutionary distances were computed using the Poisson correction method (Zuckerkandl & Pauling, 1965) and are in the units of the number of amino acid substitutions per site. All ambiguous positions were removed for each sequence pair (pairwise deletion option).

Protein isolation, SDS-PAGE and mass spectrometry analysis

Processing steps were conducted with ice-cooled cultures, buffers and containers. Protein was isolated from 100 mL of cells (IBE100) grown in liquid culture to an $OD_{600nm} \approx 0.8$, harvested and washed two times with PBS (4°C, 18,600 × *g*, 10 min). The pellet was suspended in PBS to yield a final OD of 20 which was disrupted with a French pressure cell press FA-078 (SLM Aminco, USA) in an HTU-180 3/8″ cell (G. Heinemann ULT, Germany) at 137.9 MPa in three passages. The cell extract was centrifuged (4°C, 18,600 × *g*, 30 min), 30 μL of supernatant mixed with 10 μL RotiLoad (Carl Roth GmbH), and denatured at 95°C for 5 min. Samples were loaded onto an acrylamide gel (stacking gel: 5%, separation gel: 10%), and run at 50 V (stacking) and 80 V (separation), respectively. Protein bands were made visible with Coomassie staining.

Trypsin digest was performed according to a modified version of Shevchenko and colleagues (Shevchenko et al., 1996). Bands of interest were excised from the gel, milled and washed with water (LC–MS UHPLC grade), acetonitrile, 100 mM NH_4HCO_3/10 mM DTT, acetonitrile, 100 mM NH_4HCO_3/55 mM chloroacetamide, 100 mM NH_4HCO_3, and acetonitrile at room temperature. Samples were incubated on ice for 30 min with trypsin (10 ng/μL trypsin in 40 mM NH_4HCO_3) and digested overnight at 37°C. Gel pieces were incubated with 66% acetonitrile/1.7% acetic acid and incubated for 15 min at 37°C. The supernatant was pooled with the tryptic digest and dried in a SpeedVac (Thermo Fisher Scientific, Germany). Samples were suspended in 0.1 TFA for analysis.

Nano-LC-ESI-MS/MS experiments were performed on an EASY-nLC 1200 system (Thermo Fisher Scientific, Germany) coupled to a Q Exactive HF mass spectrometer (Thermo Fisher Scientific, Germany) using a NanosprayFlex source (Thermo Fisher Scientific, Germany). Tryptic peptides were directly injected into a NanoEase analytical column (NanoEase M/Z HSS C18 T3, 1.8 μm 100 Å 75 μm × 250 mm column, Waters GmbH, Germany) operated at a constant temperature of 35°C. Gradient elution was performed at a flow rate of 250 nL/min using a 30 min gradient with the following profile: 2%–55% solvent B in 30 min, 55%–95% solvent B in 10 min, 5 min isocratic at 95% solvent B, re-equilibration for 10 min from 95% to 2% B and 10 min isocratic with 2% B. Solvents used were 0.1% formic acid (solvent A) and 0.1% formic acid in 80% acetonitrile (solvent B). The Q Exactive HF was operated under the control of XCalibur software (version 4.0.), Thermo Fisher Scientific Inc., USA). Survey spectra ($m/z = 200–2000$) were detected in the Orbitrap at a resolution of 60.000 at $m/z = 200$. Data-dependent MS/MS mass spectra were generated for the 20 most abundant peptide precursors in the Orbitrap using high energy collision dissociation (HCD) fragmentation at a resolution of 15,000 with a normalised collision energy of 27. Internal calibration was performed using lock-mass ions from ambient air as described by Olsen et al., (2005).

Mascot 2.6 (Matrix Science, UK) was used as a search engine for protein identification. Spectra were searched against the proteome of *M. gadium* IBE100 as deduced from the genome. Search parameters specified trypsin, allowing three missed cleavages, a 5 ppm mass tolerance for peptide precursors, and 0.02 Da tolerance for fragment ions. Methionine oxidation was allowed as a variable modification and carbamidomethylation of cysteine residues was set as a fixed modification. The Mascot results were transferred to Scaffold™ Software 4.10.0 (Proteome Software).

RNA isolation, library preparation, differential expression analysis

Liquid cultures of strain IBE200 (100 mL) were grown in triplicate on 2-MP or 1-hexanol, supplemented with Triton X-100 (5 μL/L). Cells were harvested by centrifugation, washed twice with PBS (4°C, 10,000 × *g*, 10 min) and resuspended in 5 mL PBS. Cell harvesting steps were conducted with ice-cooled cultures, buffers and containers. Pellets were created by pipetting droplets (~100 μL) directly into liquid nitrogen.

Cell pellets were disrupted with a Mixer Mill MM 200 (RETSCH, Germany) and RNA isolated with the QIAGEN RNeasy Plus Mini Kit via QiaCube (QIAGEN, Hilden, Germany) followed by library preparation with the ScriptSeq Bacteria Low Input Kit (Illumina, USA). Quality of the library was controlled using the HS NGS Fragment Analysis Kit on a Fragment Analyser (AATI, USA). The library was sequenced by a HighSeq2500 (Illumina, USA) in a single-end mode for 65 cycles.

The raw Illumina reads were de-multiplexed according to the sequencing barcodes introduced during library preparation, using Illumina's bcl2fastq v1.84 software with default settings for adapter trimming and allowing no mismatch per sequencing bar code. All reads for every sample were cleared from potential adapter contamination, quality controlled, and, if necessary, trimmed in single-end mode using BBDuk from the BBMap v34.41 package (https://sourceforge.net/projects/bbmap/). To pass the quality filter, read quality (Phred score) should be above 20 and every read should be at least 50 bp long after trimming of low-quality and adapter bases. The average library size among all biological replicates and samples after quality-based trimming was 4.34 million of reads per sample (Table 1).

In addition, each sample was tested before and after trimming to evaluate per base sequence quality, average base composition, GC content, sequence length distribution and adapter contaminations (http://www.bioinformatics.babraham.ac.uk/projects/fastqc/). After quality-based trimming and adapter removal, every sample was mapped to the *Mycobacterium paragordonae* IBE200 genome (Table 1).

Resulting properly mapped quality reads were quantified and reads per kilobase per million mapped read (RPKM) values were calculated using the RSeQC v2.3.1 package (Wang et al., 2012). The differential expression analysis was performed by EdgeR (Robinson et al., 2009; McCarthy et al., 2012) on three

TABLE 1 Sequencing and mapping statistics.

Samples	Library size	Unmapped reads	Mapped reads
IBE200 2-Methylpropen, br1	4,378,863	2.53%	97.47%
IBE200 2-Methylpropen, br2	4,435,001	3.33%	96.67%
IBE200 2-Methylpropen, br3	4,585,072	0.86%	99.14%
IBE200 1-Hexanol, br1	4,567,665	3.17%	96.83%
IBE200 1-Hexanol, br2	4,119,620	3.97%	96.03%
IBE200 1-Hexanol, br3	3,951,317	3.25%	96.75%

Note: Total library size—Nr. of reads after quality-based trimming; % Mapped/Unmapped—mapping to *Mycobacterium paragordonae* IBE200 reference genome.

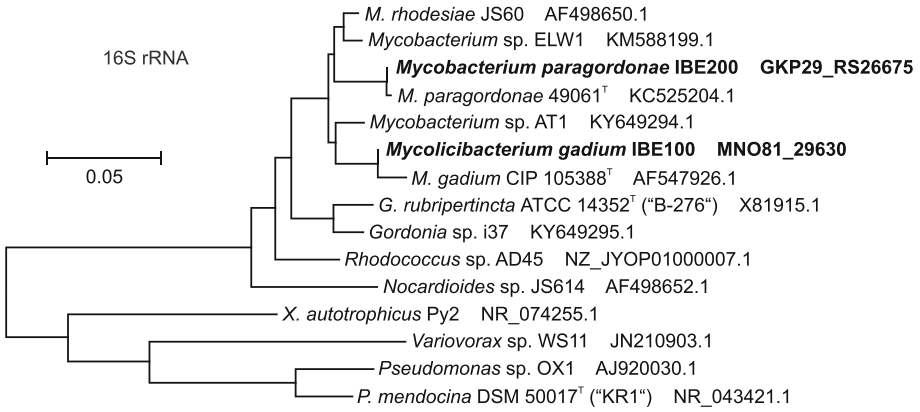

FIGURE 2 Evolutionary relationships of selected alkene degraders based on their 16S rRNA sequences. Type strains of *M. paragordonae* and *M. gadium* are not known to oxidise alkenes.

biological replicates for both growth conditions. For further analysis, only genes were chosen satisfying the following selection criteria: |logFC| (logarithm of fold change) value ≥1, adjusted *p*-value (FDR) ≤ 0.05.

RESULTS

Characterization of the microbial isolates

Strains IBE100 and IBE200 were isolated from activated sludge from a wastewater treatment plant using 2-methylpropene as the sole source of carbon and energy. IBE100 is of coccoid, and IBE200 of coccoid rod-shaped morphology. Both strains are acid-fast, non-motile bacteria, which form circular flat colonies. IBE100 colonies show pale yellow colouring with a rough and dry surface, while colonies of IBE200 are exhibiting a more intense yellow colour with a smooth surface. The colour intensifies with progressing age of the colonies. Due to the waxy nature of the cells, liquid cultures were supplemented with Triton X-100 to mitigate severe clumping. Both strains are oxidase and catalase positive as well as aminopeptidase negative. These obligately aerobic bacteria stop growth when oxygen is depleted but substrate still present, and continue to grow when oxygen is reapplied. On a MSM plate, 2-MP grown cells sprayed with an indole-solution showed indigo formation within 1 h of incubation, while 1-hexanol grown cells did not. This strongly suggests the presence of a 2-MP-inducible monooxygenase.

Taxonomy

Based on 16S rRNA gene sequence (MNO81_29630, GKP29_RS26675) and the partial *hsp65* gene sequence (MNO81_27715, GKP29_RS22280) comparison (Kim et al., 2005), as well as genome-based taxonomy using the Type (Strain) Genome Server (Meier-Kolthoff & Göker, 2019), the strains IBE100 and IBE200 were identified as *Mycolicibacterium gadium* and *Mycobacterium paragordonae*, respectively. The evolutionary relationships of selected alkene degraders based on their 16S rRNA gene sequences are depicted in Figure 2.

Characterization of the growth properties

Strains IBE100 and IBE200 differ in the variety of substrates which can be used as sources of sole carbon and energy. Besides the isolation substrate 2-methylpropene (IBE100: $\mu = 0.018\ h^{-1}$ and IBE200: $\mu = 0.025\ h^{-1}$), 1-propanol, 1-butanol, and 1-hexanol allow both strains to grow. Strain IBE100 is able to grow on presumed metabolites of 2-MP, 1,2-epoxy-2-methylpropane, 2-methylpropane-1,2-diol, 2-hydroxyisobutyric acid, 3-hydroxybutyric acid as well as on *L*-valine, *iso*-butanol, glucose, fructose, and rich media (LB, NB, and TB), while strain IBE200 is not. Strain IBE200 is able to grow on *n*-hexane, while strain IBE100 is not. No growth was observed in both strains on methane, *n*-propane, *n*-butane, 1-hexene, 2-propanol, 2-butanol, 2-hexanol, *tert*-butanol, 1,2-epoxy-propane, propane-1,2-diol, butane-2,3-diol, presumed metabolites of 2-HIBA acetone and formate (Figure 1D), presumed metabolites 3-hydroxy-2-methylpropene and methacrylate as well as 2-methylbut-1-ene, 2-methylbut-2-ene, isoprene, limonene, cyclohexane, benzene, benzoate, toluene, styrene, α-methylstyrene, methylenecyclohexane, and 1-methylcyclohexene.

The addition of coenzyme M (required for alkene degradation, Figure 1B) and glutathione (required for isoprene degradation, Figure 1C) to the growth medium did not alter growth behaviour with 2-MP as the carbon source in both strains, whereas the omission of $CoCl_2$ (pointing to a presumptive mutase reaction, Figure 1A) from the medium prevented cells from growth (data not shown).

Genome analysis

The *M. gadium* IBE100 genome is approximately 6,036,732 bp in size with a GC content of 65.5%. It contains 5831 protein-coding genes, 47 tRNA-encoding genes and 3 rRNA-encoding genes. The novel genomic sequence consisting of 87 contigs was submitted to GenBank, under the BioProject PRJNA814055, accession number JAKZMO000000000.

The *M. paragordonae* IBE200 genome is approximately 7,136,091 bp in size with a GC content of 66.8%. It contains 6279 protein-coding genes, 50 tRNA-encoding genes and 6 rRNA-encoding genes. The novel genomic sequence consisting of 262 contigs was submitted to GenBank, under the BioProject PRJNA590116, accession number WNWV00000000.

As 2-MP is a four-carbon branched alkene, the genome was searched for genes encoding monooxygenases with similarity to those reported to be involved in short-chain alkene oxidation (AAO48576.1, ACZ56346.1, ACM61846.1, AAV52084.1 were used as queries) from the NCBI nucleotide collection database via the tblastn algorithm (Altschul et al., 1997). In each strain, homologous genes (*mmoX* and *isoA*) could be identified within a cluster of genes possibly associated with the degradation of alkenes. These clusters contain genes encoding two multi-component monooxygenases *mmoXYBR* and *isoABCDEF*, two alcohol dehydrogenases *mdpB* and *fadB*, an aldehyde dehydrogenase *mdpC*, a multi-component mutase *hcmAB* and its chaperone *meaH*, a CoA ligase *hcl*, genes for cobalamin synthesis, a two-component cobalt transporter *cbtAB*, an acetyl-CoA acetyltransferase *phaA*, and several transcriptional regulators. Clusters are depicted in Figure 3B and genes with predicted functions are listed in Table 2.

Clusters are similar in size in both strains, 61.0 and 58.5 kbp, respectively, and have a similar composition and order of genes, with three distinctions. First, strain IBE100 contains several transposon elements upstream of *isoABCDEF* and *mmoXYBR*, and upstream (not shown) as well as within the cobalamin synthesis gene cluster, while those were absent from strain IBE200. The second difference is the location of the *mdpB* alcohol dehydrogenase, *mdpC* aldehyde dehydrogenase, and *eph* epoxide hydrolase encoding genes, which are located between the two monooxygenase encoding genes in strain IBE100 and upstream of the cobalamin synthesis genes in IBE200. Third, whereas the IBE100 gene cluster encodes two putative transcriptional regulators downstream of the mutase encoding gene and *mmoXYBR*, IBE200 encodes an additional putative regulator downstream of the *mdpB*/*mdpC*/*eph* genes.

Soluble diiron monooxygenases (SDMs)

Genes encoding two soluble oxo-bridged diiron monooxygenases (SDMs), each potentially involved in 2-methylpropene transformation, could be identified in the genomes of IBE100 and IBE200. The *mmoXYBR* gene cluster encodes three components, a hydroxylase consisting of an α-subunit MmoX (IBE100: 513 aa; IBE200: 514 aa) and a β-subunit MmoY (366 aa; 366 aa), a coupling protein MmoB (111 aa; 112 aa), and a reductase MmoR (342 aa; 344 aa), all with high sequence similarity to those encoding methane monooxygenases and particularly those involved in propane oxidation (Figure 4).

The α-subunits MmoX show 99.81% (IBE100) and 84.4% (IBE200) identity with monooxygenase (QEN17575.1) from *Mycobacterium* sp. ELW1. A possible product of terminal 2-MP oxidation is 2-methylprop-2-en-1-ol, which may be further oxidised to methacrylic acid and funnelled into the *L*-valine degradation pathway (Figure 1 left branch).

Downstream of *mmoXYBR*, a second SDM is encoded. According to its six-component operon structure and amino acid sequence, it is closely related to isoprene monooxygenases and termed *isoABCDEF* (Figure 4). It consists of a monooxygenase α-subunit IsoA (505 aa; 506 aa), a γ-subunit IsoB (92 aa; 92 aa), a Rieske-type ferredoxin IsoC (113 aa; 113 aa), a coupling protein IsoD (108 aa; 109 aa), a β-subunit IsoE (316 aa; 342 aa), and a flavin-containing NAD(P)H reductase IsoF (319 aa; 339 aa).

The α-subunits IsoA contain, according to a homology model (pdb: 5TDS), an oxo-bridged diiron centre within a four-helix bundle. They show 99.8% and 85.5% identity with isoprene monooxygenase oxygenase subunit alpha from *Mycobacterium* sp. ELW1 (QEN17701.1). Oxidation of the C=C bond would yield 1,2-epoxy-2-methylpropane (Figure 3C, reaction 1).

Epoxide hydrolase (EPH)

A search within the genomes for genes encoding proteins similar to epoxyalkane:coenzyme M transferase (Q56837.1) from the CoM-dependent propene oxide cleavage of *Xanthobacter* sp. Py2, glutathione *S*-transferase IsoI (KJF19166.1) from the GSH-dependent isoprene oxide cleavage of *Rhodococcus* sp. AD45, and epoxide hydrolase 1EHY (1EHY_A) from the cofactor-independent epichlorohydrin cleavage of *Agrobacterium tumefaciens* AD1 revealed epoxide hydrolases with 70% and 72% identity, respectively to the latter enzyme. Corresponding genes were located upstream of MmoXYBR in IBE100 (MNO81_04050) and upstream of the cobalamin synthesis genes in IBE200 (GKP29_RS08420).

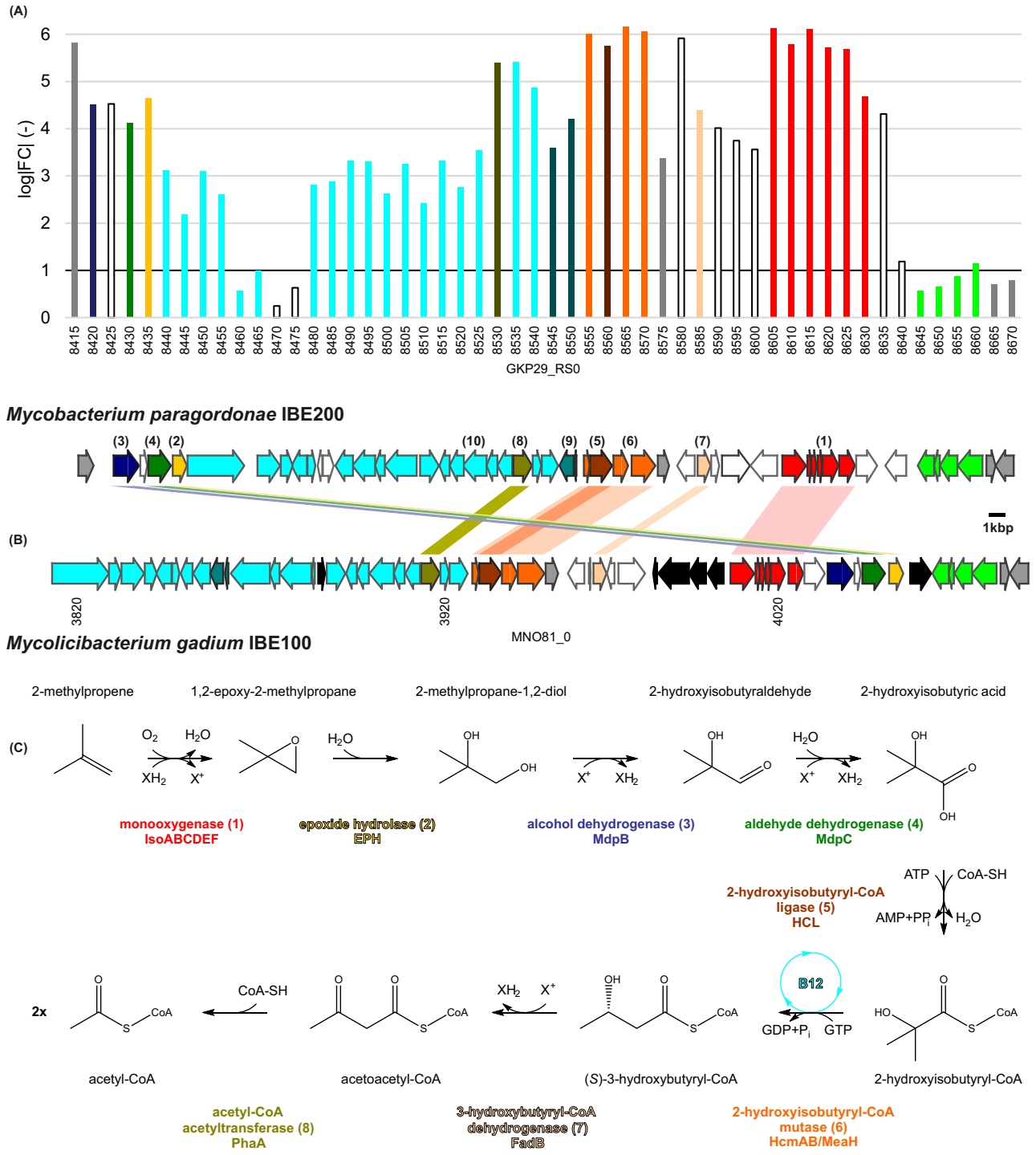

FIGURE 3 The 2-methylpropene degradation clusters and metabolism in *Mycolicibacterium gadium* IBE100 and *Mycobacterium paragordonae* IBE200 (A) Gene expression levels in *M. paragordonae* IBE200 of the proposed 2-methylpropene degradation cluster; (B) degradation gene cluster organisation in IBE200 and IBE100; and (C) proposed degradation pathway of 2-methylpropene in both strains. X: NAD(P), (1) isoprene monooxygenase, (2) epoxide hydrolase, (3) alcohol dehydrogenase, (4) 2-hydroxyisobutyraldehyde dehydrogenase, (5) 2-hydroxyisobutyryl-CoA ligase, (6) 2-hydroxyisobutyryl-CoA mutase, (7) 3-hydroxybutyryl-CoA dehydrogenase, (8) acetyl-CoA acetyltransferase, (9) cobalt transporter, (10) cobalamin synthesis lower pathway. Genes encoding enzymes with matching predicted functions are colour-coded and connected with bars between both clusters. Transcriptional regulators are indicated in grey, transposon elements in black, open reading frames not related to the degradation pathway and of unknown function in white. For details on genes, see Table 2.

The encoded EPHs (303 aa; 304 aa) showed 100% and 88.4% identity with alpha/beta hydrolase (QEN17571.1) from *Mycobacterium* sp. ELW1 (Figure 5). Hydrolytic cleavage of the proposed pathway intermediate 1,2-epoxy-2-methylpropane by EPH probably yields 2-methylpropane-1,2-diol (Figure 3C, reaction 2).

TABLE 2 List of genes associated with the degradation of 2-methylpropene in strains IBE100 and IBE200.

Description/predicted function	Gene	Locus tag IBE100 MNO81_0	Locus tag IBE200 GKP29_RS0	Similarity	Colour/ react.-no
Transcriptional regulator	*araC*	-	8415	-	
GMC family oxidoreductase	*mdpB*	4035	8420	96.5%	3
Hydroxyisobutyraldehyde dehydrogenase	*mdpC*	4045	8430	92.4%	4
Epoxide hydrolase	*eph*	4050	8435	95.7%	2
Cobalamin synthesis lower pathway	*cobX*/ *cbiX*	3820–3885 3890–3910 3920–3925	8440–8465 8480–8525 8535–8540		
Two-component cobalt transporter	*cbtAB*	3860–3865	8545–8550		
Acetyl-CoA C-acetyltransferase	*phaA*	3915	8530	92.8%	8
Hydroxyisobutyryl-CoA mutase, β-subunit	*hcmB*	3930	8555	95.7%	6
Hydroxyisobutyryl-CoA ligase	*hcl*	3935	8560	92.5%	5
G protein chaperone	*meaH*	3940	8565	90.2%	6
Hydroxyisobutyryl-CoA mutase, α-subunit	*hcmA*	3945	8570	96.0%	6
Transcriptional regulator	*gntR*	3950	8575	91.6%	
3-Hydroxybutyryl-CoA dehydrogenase	*fadB*	3965	8585	90.7%	7
Isoprene monooxygenase, α-subunit	*isoA*	4000	8605	92.3%	1
Isoprene monooxygenase, γ-subunit	*isoB*	4005	8610	87.1%	1
Isoprene monooxygenase, coupling protein	*isoC*	4010	8615	86.8%	1
Isoprene monooxygenase, 2Fe-2S	*isoD*	4015	8620	91.7%	1
Isoprene monooxygenase, β-subunit	*isoE*	4020	8625	83.7%	1
Isoprene monooxygenase; reductase	*isoF*	4025	8630	81.5%	1
Methane monooxygenase, 2Fe-2S	*mmoR*	4060	8645	75.3%	
Methane monooxygenase, coupling protein	*mmoB*	4065	8650	85.9%	
Methane monooxygenase, β-subunit	*mmoY*	4070	8655	85.4%	
Methane monooxygenase, α-subunit	*mmoX*	4075	8660	91.8%	
Two-component system regulator		4080	8665	85.7%	
Histidine kinase		4085	8670	87.9%	

Note: For comparison, the similarity of translated amino acids between enzymes of the same predicted functions are given. Colour code and reaction-numbering according to the clusters and pathway in Figure 3B, C, respectively.

Alcohol dehydrogenase (MdpB) and aldehyde dehydrogenase (MdpC)

The diols produced by hydrolysis of epoxides are assumed to be oxidised to the corresponding acids. The *tert*-butanol oxidising strains *Aquincola tertiaricarbonis* L108 (Rohwerder et al., 2006), *Mycolicibacterium austroafricanum* IFP 2012 (Ferreira et al., 2006), and *Methylibium petroleiphilum* PM1 (Hristova et al., 2007) as well as the 2-methylpropane-1,2-diol-degrading strain *Actinomycetospora chiangmaiensis* DSM 45062 (Rohwerder et al., 2020) use distinct alcohol dehydrogenases and aldehyde dehydrogenases for these steps.

Genes encoding these functions, GMC family oxidoreductase MdpB (545 aa; 545 aa) and aldehyde dehydrogenase MdpC (484 aa; 484 aa), could be identified within both clusters. MdpBs are 83.8% and 92.1% identical to GMC family oxidoreductase from *Mycobacterium* sp. ELW1 (QEN17568.1), and MdpCs have 99.8% and 87.6% identity with aldehyde dehydrogenase (QEN17570.1) from *Mycobacterium* sp. ELW1 (Figure 6 and Figure 7).

The corresponding oxidation products would be 2-hydroxyisobutyraldehyde and 2-hydroxyisobutyric acid (Figure 3C, reactions 3 and 4).

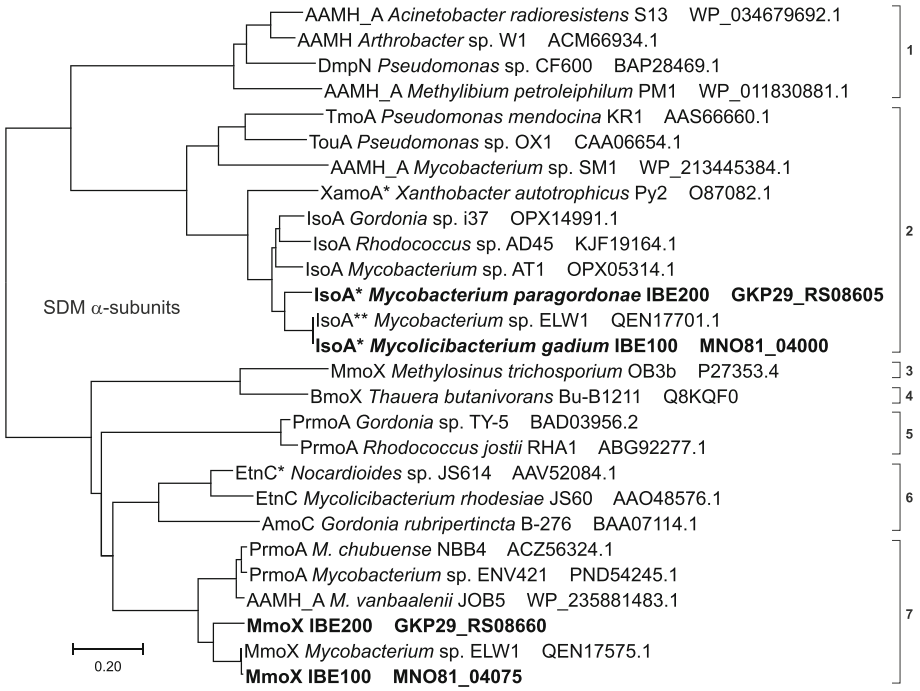

FIGURE 4 Evolutionary relationships of selected α-subunits of soluble diiron monooxygenases: phenol (1), aromatic/alkene/isoprene (2), methane (3), butane (4), 2-propane (5), ethene (6), and 1-propane (7) monooxygenases. * confirmed and ** postulated 2-MP epoxidation.

Hydroxyisobutyryl-CoA ligase (HCL), hydroxyisobutyryl-CoA mutase (HCM), and its chaperone (MeaH)

Branched-chain acids, for example, methylmalonic acid, ethylmalonic acid, isobutyric acid, and 2-hydroxyisobutyric acid (2-HIBA) usually undergo carbon skeleton rearrangement, as shown for methylmalonyl-CoA mutase MCM (Marsh et al., 1989), ethylmalonyl-CoA mutase ECM (Erb et al., 2008), isobutyryl-CoA mutase ICM (Zerbe-Burkhardt et al., 1998), and hydroxyisobutyryl-CoA mutase HCM (Rohwerder et al., 2006). This rearrangement obviously necessitates previous CoA activation by a CoA ligase HCL (Zahn et al., 2019).

Sequences coding for functions homologous to 2-hydroxyisobutyryl-CoA ligase from *Aquincola tertiaricarbonis* L108 (AFK77666.1) could be found in both strains, a CoA ligase with 56% identity (MNO81_03935) in IBE100 and 56% identity (GKP29_RS08445) in IBE200, each located in the postulated clusters. These putative 2-hydroxyisobutyryl-CoA ligases (472 aa; 473 aa) exhibit 99.6% identity with phenylacetate—CoA ligase (QEN17700.1) from *Mycobacterium* sp. ELW1, and 85.7% identity with phenylacetate—CoA ligase from *Mycobacterium* sp. 1465703.0 (OBJ09429.1), respectively (Figure 8). The reaction product of the ligase would be 2-hydroxyisobutyryl-CoA (Figure 3C, reaction 5).

The genes encoding 2-HIBA-CoA ligase HCL are surrounded by genes encoding a probable hydroxyisobutyryl-CoA mutase HcmAB and its chaperone MeaH. Hydroxyisobutyryl-CoA mutase HCM is a B12-dependent radical enzyme. It forms an $\alpha_2\beta_2$-heterotetramer, connected by the two HcmA units (Kurteva-Yaneva et al., 2015). The large subunits HcmA (575 aa; 573 aa) exhibit 99.8% and 90.7% identity with methylmalonyl-CoA mutase (QEN17551.1) from *Mycobacterium* sp. ELW1 whereas the small subunits HcmB (140 aa; 140 aa) are identical to the cobalamin B12-binding domain-containing protein from *Mycobacterium* sp. ELW1 (QEN17550.1), and share 93.5% identity to methylmalonyl-CoA mutase from *Mycobacterium goodii* (AKS33818.1).

The putative chaperone MeaH is orthologous to the methylmalonyl-CoA mutase-associated GTPase MeaB and the isobutyryl-CoA mutase-associated GTPase MeaI. Usually, the gene is expressed separately, although it can be found as a fusion protein with the CoA mutase α-subunit, for example, in *Geobacillus kaustophilus* (Cracan et al., 2010). It is described to promote the attachment of adenosylcobalamin to its binding site after hydrolysis of GTP and to serve as a chaperone to protect the mutase from oxidative inactivation during radical catalysis (Korotkova & Lidstrom, 2004; Padovani & Banerjee, 2006).

The chaperones (329 aa; 331 aa) share 84.9% and 83.9% identity with the methylmalonyl-CoA mutase-associated GTPase MeaB from *Mycobacterium* sp. PS03-16 (WP_135129297.1). The linearization of 2-HIBA-CoA would yield 3-hydroxybutyryl CoA (Figure 3C, reaction 6).

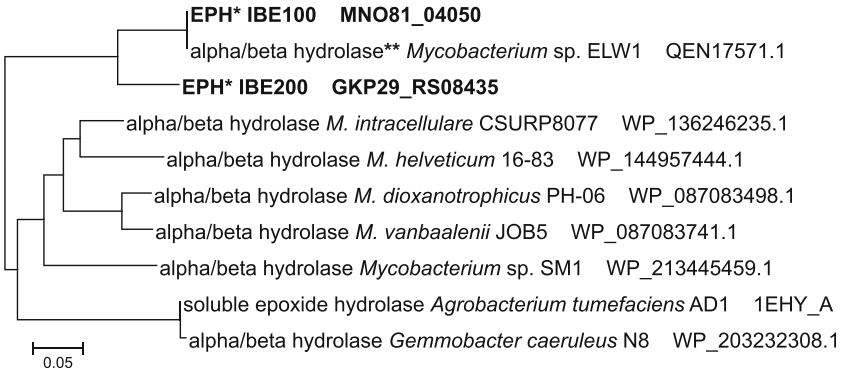

FIGURE 5 Evolutionary relationships of epoxide hydrolases. * confirmed and ** postulated 1,2-epoxy-2-methylpropane hydrolysis.

3-Hydroxybutyryl-CoA dehydrogenase (FadB) and acetyl-CoA C-acetyltransferase (PhaA)

3-Hydroxybutyryl-CoA formed after carbon skeleton rearrangement may enter the central metabolism via dehydrogenation to 3-acetoacetyl-CoA (Figure 3C, reaction 7) and thiolytic cleavage. Various genes encoding enzymes similar to 3-hydroxybutyryl-CoA dehydrogenase (QNF04532.1) from *M. tuberculosis* H37Rv could be found in both genomes with *fadB* located between the genes encoding isobutyryl-CoA mutase and *isoABCDEF* coding for proteins sharing the highest identities of 79% and 90%, respectively.

The dehydrogenases FadB (288 aa; 287 aa) have 99.3% and 82.3% identity with 3-hydroxybutyryl-CoA dehydrogenase from *Mycobacterium* sp. ELW1 (QEN17555.1).

The thiolytic cleavage into two acetyl-CoA (Figure 3C, reaction 8) may be catalysed by acetyl-CoA C-acetyltransferases MNO81_03915 in IBE100 and GKP29_RS08439 in IBE200, located within the cobalamin synthesis gene clusters. The respective acetyl-CoA C-acetyltransferases PhaA (403 aa; 393 aa) again exhibit highest sequence identity with acetyl-CoA C-acetyltransferase (QEN17548.1) from *Mycobacterium* sp. ELW1 (98.8%) and acetyl-CoA C-acetyltr ansferase (WP_048416325.1) from *Mycolicibacterium chubuense* (91.9%), respectively.

The cobalamin synthesis gene cluster

Adenosylcobalamin (AdoCbl) is required as a coenzyme for the transformation of 2-HIBA–CoA to 3-HBA–CoA as catalysed by 2-hydroxyisobutyryl-CoA mutase (Figure 3, reaction 6) (Rohwerder et al., 2006). Three main bacterial synthesis pathways are described (Fang et al., 2017), the aerobic and the anaerobic *de novo* synthesis, and the salvage pathway. While the salvage pathway demands an external cobinamide source as well as a respective ABC transporter, the porphyrin ring is synthesised from 5-aminolaevulinate in the *de novo* routes.

In strains IBE100 and IBE200, all genes for the aerobic *de novo* synthesis, *cobIJ*, *cobG*, *cobM*, *cobF*, *cobK*, *cobL*, *cobH*, *cobB*, *cobN*, *cobS*, and *cobT* and transport (cbtAB) are present in the genomes and located downstream of the mutase encoding gene *hcmB*. Genes encoding enzymes of the anaerobic route are also present, though irrelevant, due to the aerobic nature of the degradation pathway.

As an example, the cobalt chelatase subunits CobN (1191 aa; 1199 aa) share 99.2% and 81.3% identity with the cobaltochelatase subunit CobN from *Mycobacterium* sp. ELW1 (QEN17531.1).

Identification of proteins induced during growth of IBE100 on 2-MP

To identify proteins involved in 2-MP degradation in strain IBE100, cell extracts obtained after growth on this substrate and fructose were separated by SDS-PAGE (Figure 9). Selected bands were excised from lane 2-MP, digested and analysed by mass spectrometry.

Proteins of the postulated 2-MP degradative pathway (Figure 3C) could be identified from 2-MP grown cells by unique peptides covering 24%–95% of the predicted proteins (Table 3). All identified proteins were > 30 kDa and comprised the IsoA, IsoE, and IsoF subunits of isoprene monooxygenase, whereas methane monooxygenase subunits (MmoX, MmoY, and MmoR with predicted masses of 59.6, 40.9, and 37.9 kDa, respectively) were not observed. Also, subsequent enzymes of the proposed pathway (epoxide hydrolase EPH, alcohol- (MdpB) and aldehyde- (MdpC) dehydrogenase, 2-hydroxyisobutyryl-CoA ligase HCL, 2-hydroxyisobutyryl-CoA mutase α-subunit HcmA as well as its chaperone MeaH, 3-hydroxybutyryl-CoA dehydrogenase FadB and acetyl-CoA C-acetyltran sferase PhaA) were expressed. Only subunits IsoB, IsoC, and IsoD of isoprene monooxygenase and HcmB of 2–hydroxyisobutyryl-CoA mutase were not detectable by this approach due to their small size (10.2, 12.5, 12.3, and 15.3 kDa, respectively).

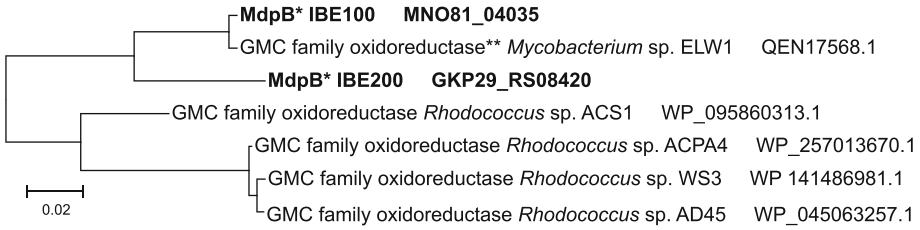

FIGURE 6 Evolutionary relationships of alcohol dehydrogenases. * confirmed and ** postulated 2-methylpropane-1,2-diol dehydrogenation.

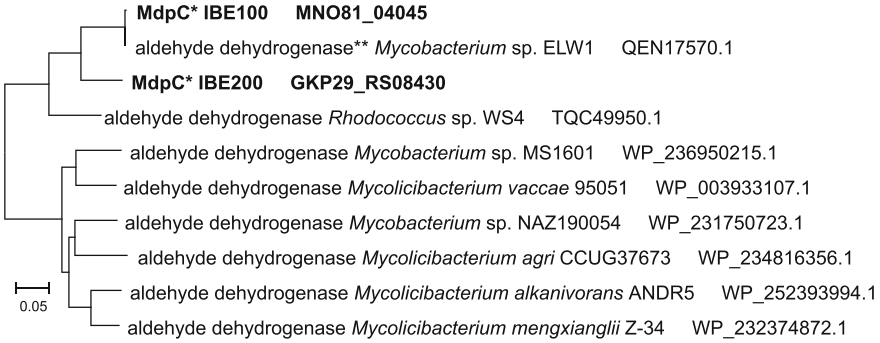

FIGURE 7 Evolutionary relationships of aldehyde dehydrogenases. * confirmed and ** postulated 2-hydroxyisobutyraldehyde dehydrogenation.

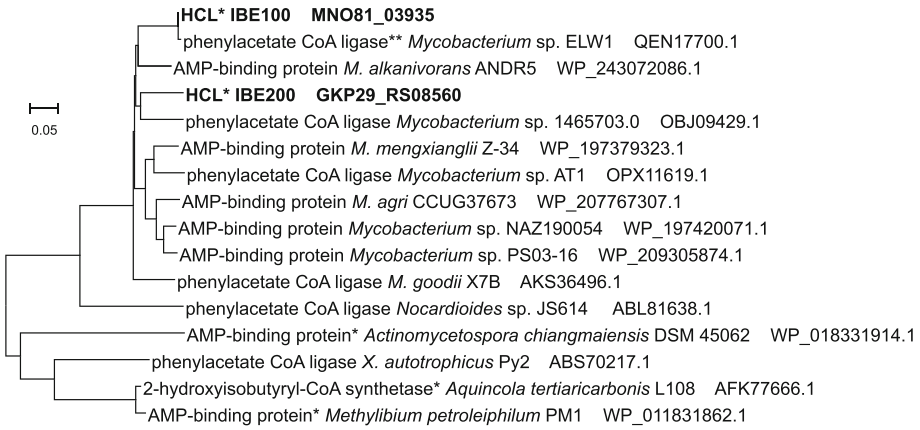

FIGURE 8 Evolutionary relationships of hydroxyisobutyryl-CoA ligases. * confirmed and ** postulated 2-hydroxyisobutyric acid CoA-activation.

Differential expression analysis in IBE200

To verify the involvement of gene products encoded by the 2-MP gene cluster in strain IBE200 (Figure 3B and Table 2), a gene expression analysis was performed with 2-MP-grown cells versus 1-hexanol-grown cells. Hexanol was chosen as a negative control because no overlap between the two degradation pathways was expected, and because of its similar cell mass yield and growth rate compared to 2-MP grown cells. Here, 936 out of 6,292 genes were differentially expressed, with 817 genes being upregulated and 119 genes being downregulated.

Upregulation of genes within the cluster

Analysis revealed that all genes from the postulated 2-MP degradation cluster were upregulated, ranging from 17.4 to 71.5-fold (Figure 3A). Among these highly upregulated genes were all those encoding the putative isoprene monooxygenase subunits (70.1 to 25.7-fold). In contrast, the genes encoding methane monooxygenase subunits showed no or only slightly increased transcript levels (up to 2.2-fold). This clearly indicated the six-component isoprene monooxygenase to be responsible for the initial oxidation of 2-MP in strain IBE200. The high induction level of all postulated subsequent

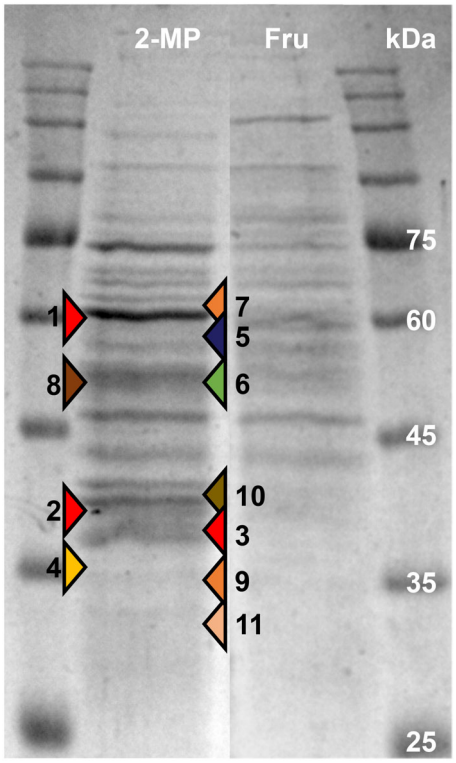

FIGURE 9 Coomassie stained SDS-PAGE (10%) of soluble extracts from *M. gadium* IBE100 cells grown on 2-methylpropene (2-MP) and fructose (Fru). Triangles mark the expected location of proteins of interest, which were excised and digested from lane 2-MP. Colour code according to Table 2, for band numbering see Table 3.

pathway genes makes this degradation pathway highly probable.

Also, genes for aerobic cobalamin synthesis, including the cobalt transporter, were upregulated at least 4.5-fold, emphasising their role in 2-MP degradation.

Upregulation of genes outside the cluster

Several other genes outside the cluster showed induction. These could be attributed to the functions of stress response (several general stress response proteins and universal stress proteins), cell wall modification common in *Mycobacteriaceae* (trehalose phosphatase and precursor synthesis by gluconeogenesis, teichoic acid synthesis), branched-chain amino acid synthesis (ketol acid reductoisomerase, acetolactate synthase, methylmalonyl semialdehyde dehydrogenase), lantibiotic production (lantipeptidase), and fatty acid as well as polyketide synthesis (branched-chain alpha-keto acid dehydrogenase complex, acyl carrier proteins, phosphopantetheine binding protein). This indicates the somewhat stressful nature of growth with 2-MP as a water-insoluble, low molecular weight compound.

DISCUSSION

Several critical reaction steps must be overcome in bacterial 2-MP degradation, in particular initial oxidation, epoxide cleavage, and branched-chain acid metabolization (Figure 1). Genomic sequencing and refined blast searches on two bacterial isolates capable of growing on 2-MP identified clusters of putative degradation genes, encoding enzymes achieving alkane/alkene oxidation, epoxide hydrolysis, branched-chain isomerization and cobalamin synthesis. Peptide-mass fingerprinting of protein extracts of *M. gadium* IBE100, together with the analysis of the whole transcriptome of 2-MP-grown cells of *M. paragordonae* IBE200, complemented these findings and confirmed those genes to be involved in the transformation of 2-MP to the central metabolite acetyl-CoA.

Characterization of SDMs in IBE100 and IBE200

IBE100 and IBE200 each encode two SDMs, designated MmoXYBR and IsoABCDEF. Phylogenetic analysis of the α-subunits grouped MmoXYBR with propane monooxygenases (Figure 4). Initially, MmoXYBR was assumed to hydroxylate 2-MP to form 2-methylprop-2-en-1-ol, a reaction demonstrated with heterologously expressed cumene dioxygenase (Takami et al., 1999). The allyl alcohol could then be further oxidised to methacrylic acid and after CoA-activation, channelled into the central metabolism via the lower *L*-valine degradation pathway (Massey et al., 1976). Kottegoda and colleagues assumed that this initial step is a minor side reaction in parallel with epoxidation, as ELW1 can grow slowly on the allyl alcohol (Kottegoda et al., 2015). Although IBE100 can grow on *L*-valine, neither IBE100 nor IBE200 can grow on 2-methylprop-2-en-1-ol or methacrylate (metabolite during valine degradation). Transcriptomic and proteomic analysis showed that MmoXYBR was not upregulated or expressed at a detectable level after growth on 2-MP, contradicting the hypothesis. The original function of this supposed propane monooxygenase remains to be determined, as the isolates were unable to utilise propane (as well as methane and butane) but were able to grow on 1-propanol, propanal, and propionic acid.

Numerous SDMs exhibit epoxidation activity towards alkenes, as shown with cells grown on the respective substrates (Furuhashi et al., 1981; Johnston et al., 2017; Kottegoda et al., 2015), resting cells (Cheung et al., 2013; Coleman & Spain, 2003; McClay et al., 2000; Ono & Okura, 1990; Owens et al., 2009; van Hylckama Vlieg et al., 1998), and cell extracts (Hou et al., 1983; Patel et al., 1982). However, 2-MP epoxidation was demonstrated only with resting cells of

TABLE 3 Mass-spectrometric identification of proteins of the proposed 2-MP degradation pathway in *M. gadium* IBE100.

Band-no	Protein	Coverage	Unique peptides	Predicted mass (kDa)	Colour/ react.-no
1	IsoA	278/505 aa (55%)	29	58.1	1
2	IsoE	205/340 aa (60%)	16	38.3	1
3	IsoF	109/340 aa (32%)	7	36.3	1
4	EPH	182/303 aa (60%)	11	34.6	2
5	MdpB	516/545 aa (95%)	41	59.8	3
6	MdpC	392/484 aa (81%)	32	52.2	4
7	HcmA	342/575 aa (59%)	29	64.0	6
8	HCL	193/472 aa (41%)	17	53.1	5
9	MeaH	106/329 aa (32%)	7	34.8	6
10	FadB	101/288 aa (35%)	8	30.6	7
11	PhaA	98/403 aa (24%)	5	41.7	8

Note: Colour code and reaction-numbering according to the clusters and pathway in Figure 3B, C, respectively.

X. autotrophicus Py2 (Ensign, 1996) and *Nocardioides* sp. JS614 (Owens et al., 2009), with heterologously expressed dimethylsulfide monooxygenase (Takami et al., 1999) as well as in cell extracts of *Methylobacterium* sp. CRL-26 (Patel et al., 1982) and *Brevibacterium* sp. CRL56 (Hou et al., 1983). Although 1,2-epoxy-2-methylpropane was not detected in growing and resting cells of strain ELW1 (Kottegoda et al., 2015), the presented data strongly suggest epoxidation of 2-MP in this strain.

IsoABCDEF is an SDM of the aromatic/alkene/isoprene type (Figure 4), whose inducibility by 2-MP could be shown via indigo formation, transcriptomic and proteomic analysis. Within this group of SDMs, IsoA of strains IBE100, IBE200, and ELW1 were assorted close to isoprene monooxygenases, but on a separate sub-branch. IBE100 and IBE200 are able to grow on 2-MP, but not on the structural homologues isoprene, 2-methyl-1-butene, or 2-methyl-2-butene, consistent with the findings for ELW1. In addition, there are no reports of isoprene-epoxidizing SDMs or strains capable of oxidising 2-MP. Further research may classify the 2-MP epoxidizing SDM to be a new subtype of the aromatic/alkene/isoprene monooxygenase group, with the term isobutene monooxygenase IbeABCDEF proposed here. However, it has to be considered that a lack of growth may also be due to uptake limitations or constraints of subsequent metabolising enzyme(s). The detailed specificity of the 2-MP oxidising SDMs remains to be defined.

Epoxide cleavage

Epoxides are common metabolites in bacterial alkene degradation, and must be metabolised rapidly due to their reactive nature. Of the three introduced mechanisms, coenzyme M conjugation in short-chain alkene degradation (Allen et al., 1999; Allen & Ensign, 1998; Krishnakumar et al., 2008; Mattes et al., 2005), glutathione conjugation in degradation of isoprene and styrene (Heine et al., 2018; Johnston et al., 2017; Larke-Mejía et al., 2019; van Hylckama Vlieg et al., 1998), and hydrolysis in epoxy alkane, alkene, and terpene degradation (de Bont et al., 1982; Kottegoda et al., 2015; Nakamura et al., 1992; van den Wijngaard et al., 1989; van der Werf et al., 1998), genetic evidence for the latter was found in strains IBE100 and IBE200. Genes encoding epoxide hydrolases EPH were located in the postulated cluster and confirmed to be involved in 2-MP degradation by transcriptomic analysis and peptide-mass fingerprinting. The use of an epoxide hydrolase in 2-MP degradation represents a major difference compared to isoprene and short-chain alkene degradation pathways. Phylogenetic analysis clustered those enzymes closest to the alpha/beta hydrolase from the 2-MP degrader *Mycobacterium* sp. ELW1 (100% and 88.4% identity) as well as to putative alpha/beta hydrolases of unknown function from other *Mycobacteriaceae* (70%–77% identity) (Figure 5).

Convergence with the tert-butanol degradation pathway

2-Methylpropane-1,2-diol, produced by epoxide hydrolysis, is further oxidised to 2-hydroxyisobutyraldehyde and 2-hydroxyisobutyric acid, followed by CoA-activation. The enzymes catalysing these reactions in strains IBE100 and IBE200 are the GMC family oxidoreductase MdpB, the aldehyde dehydrogenase MdpC, and the 2-hydroxyisobutyrate-CoA ligase HCL, as confirmed by transcriptomic analysis and peptide-mass fingerprinting. The genes encoding MdpB and MdpC were not upregulated in 1-hexanol-grown cells, indicating that they do not catalyse the oxidation of 1-hexanol.

Again, the closest relative to MdpB is a GMC oxidoreductase from *Mycobacterium* sp. ELW1, which most

likely has the same function (Figure 6). Several isoprene-degrading Rhodococci possesses similar enzymes (83.5%–86.0% identity), but no link to a metabolic function was identified. In contrast, functional homologues of 2-methylpropane-1,2-diol-degrading *A. chiangmaiensis* DSM 45062 and *tert*-butanol-degrading *M. austroafricanum* IFP 2012, with confirmed dehydrogenation of the diol, are only distantly related (40.2% and 41.5% identity). Furthermore, the assigned MdpB (ABM97329.1) from MTBE-degrading *Methylibium petroleiphilum* PM1 belongs to a different class of enzymes and is not related.

A similar picture can be drawn for the relationships of the aldehyde dehydrogenases MdpC (Figure 7) and 2-hydroxyisobutyryl-CoA ligases HCL (Figure 8). Several closely related enzymes without assigned function and obviously without involvement in 2-MP degradation are found in other strains, whereas enzymes with confirmed identical catalytic function from 2-methylpr opane-1,2-diol- and *tert*-butanol-degrading strains are not considered homologues (MdpC: *A. chiangmaiensis* DSM 45062 (66.2%, WP_018331915.1), *M. austroaf ricanum* IFP 2012 (70.6%, WP_011827962.1), *Methylibium petroleiphilum* PM1 (36%, WP_011827962.1), HCL: *A. tertiaricarbonis* L108 (55.9%, AFK77666.1), *Methylibium petroleiphilum* PM1 (55.5%, WP_01 1831862.1), *A. chiangmaiensis* DSM 45062 (53.2%, WP_018331914.1)).

2-Hydroxyisobutyryl-CoA can be cleaved either by a thiamine pyrophosphate (TPP)-dependent lyase (Rohwerder et al., 2020) to form acetone and formyl-CoA or be subject to a cobalamin-dependent mutase reaction (Kottegoda et al., 2015; Hristova et al., 2007; Rohwerder et al., 2006). A TPP-dependent cleavage as reported in *A. chiangmaiensis* DSM 45062 can be excluded for strains IBE100 and IBE200 because neither metabolite produced by this reaction served as growth substrate, nor were genes encoding enzymes necessary for this pathway (TPP-lyase, acetone monooxygenase, and formate dehydrogenase) found in the genomes.

Instead, convergence with the degradation pathway of *tert*-butanol (Hristova et al., 2007; Rohwerder et al., 2006) was demonstrated. The 2-hydroxyisobutyryl-CoA mutase HcmAB and its chaperone MeaH, the 3-hydroxybutyryl-CoA dehydrogenase FadB and the acetyl-CoA C-acetyltransferase PhaA catalyse the formation of acetyl-CoA in strains IBE100 and IBE200. Highly similar enzymes are found in strain ELW1 (Figure 10). Genes encoding FadB and PhaA in strain IBE200 were not upregulated in cells grown on 1-hexanol, indicating their specific role in 2-MP degradation.

Despite this overlap in the lower parts of the 2-MP and *tert*-butanol degradation pathways, *tert*-butanol itself could not be used as a sole source of carbon and energy by IBE100 and IBE200. Oxygenases required for the conversion to 2-methylpropane-1,2-diol were identified as *tert*-butanol-specific MdpJK-type oxygenase-reductase system (Schäfer et al., 2007) or AlkB-type oxygenase (Lopes Ferreira et al., 2007). Similar enzymes to the latter, but with only partially available sequence (ABB13506.2, 114 aa), were found in IBE100 (MNO81_RS03470, 405 aa) and IBE200 (GKP29_RS14140, 406 aa; GKP29_RS12070, 411 aa). Apparently, they are not involved in *tert*-butanol oxidation.

Cluster comparison

Blast searches for homologous enzymes to the ones in IBE100 and IBE200 showed relatives in a variety of bacteria, although most of the species belonged to the *Mycobacteriaceae* family. Consequently, the question arises of which strains could potentially be 2-MP degraders. Comparison of the IBE100 and IBE200 clusters revealed striking similarities in size, composition, and order of genes (Figure 10, Table 2). In addition, a nearly identical copy of the entire cluster of IBE100 is found on plasmid pELW1-1 from strain ELW1 (CP032156.1), the up to now only other strain reported to grow on 2-MP. The identity even goes down to the nucleotide level, with two stretches of 40,754 nucleotides (identities: 40,566, gaps: 22) and 35,773 nucleotides (identities: 35,513, gaps: 36), flanked and interrupted by transposon elements (Figure 10). This strongly indicates horizontal gene transfer.

With the apparent patterns of conservation, an *in silico* analysis was conducted on the available whole genomes of the strains represented in the phylogenetic trees (Figures 4–8).

Mycobacterium sp. NAZ190054 (Figure 10) harbours the dehydrogenases (MdpB: WP_067953590.1, MdpC: WP_231750723.1) as well as the CoA-lyase/mutase (HCL: WP_197420071.1, HcmAB/MeaH: WP_082753666.1, WP_067953579.1, WP_06795 3577.1) subclusters. With FadB (WP_067953589.1) and PhaA (KWX57522.1) also present, conversion down to acetyl-CoA is possible. Due to the absence of homologues to an epoxide hydrolase and an SDM, 2-MP degradation is unlikely in this strain. Instead, it encodes an AlkB-type oxygenase (KWX69091.1) with 96% identity to a partial sequence of AlkB (ABB13506.2) from *M. austroafricanum* IFP 2012, involved in *tert*-butanol hydroxylation (Lopes Ferreira et al., 2007). Therefore, this clinical isolate from abdominal cerebrospinal fluid pseudocyst is most likely a *tert*-butanol degrader.

Similarly, *Mycolicibacterium alkanivorans* ANDR5 (Figure 10), a propane degrading strain isolated from a natural gas seep (Farhan Ul Haque et al., 2022), possesses enzymes for the degradation of 2-methylprop ane-1,2-diol to acetyl-CoA, homologous to those of the

2-MP degrading strains (MdpB: WP_252393995.1, MdpC: WP_252393994.1, HCL: WP_243072086.1, HcmAB/MeaH: WP_243072084.1, WP_243072087.1, WP_243072085.1, FadB: WP_252394578.1, PhaA: WP_243072091.1). Here, homologues to AlkB or MdpJK are lacking, such that the function of previously mentioned enzymes in a putative *tert*-butanol degradative pathway is unlikely. Three SDMs and four EPHs of unknown function are encoded in the genome, however, whether one of these could be recruited for 2-MP degradation remains to be elucidated.

Mycobacterium sp. SM1 (Figure 10), an environmental isolate from a mud volcano, possesses an SDM of the aromatic/alkene/isoprene-type (WP_213445384.1). It clusters (Figure 4) with toluene-4-monooxygenases TouA and TmoA with epoxidation activity towards alkenes of *Pseudomonas stutzeri* OX1 and *Ps. mendocina* KR1, respectively (McClay et al., 2000; Sazinsky et al., 2004). Furthermore, homologues to EPH (WP_213445459.1), MdpB (WP_236057206.1), MdpC (WP_213445463.1), HCL (WP_213445446.1), HcmAB/MeaH (WP_213445451.1, WP_236057202.1, WP_236056917.1), FadB (MBS4728511.1), and PhaA (MBS4727285.1) are present in the genome, covering every required reaction step from 1,2-epoxy-2-methylpropane to acetyl-CoA. However, this strain encodes an enzyme (WP_213441950.1) with 91% similarity to epoxyalkane CoM transferase EaCoMT of *Gordonia rubripertincta* B-276. In this strain, 1,2-epoxy-2-methylpropane served as an inducer and simultaneously acted as an irreversible inhibitor of EaCoMT (Allen et al., 1999), which might scavenge the epoxide and prevent the expression of the needed epoxide hydrolase. To date, no information is available on the degradative potential of strain SM1, although the ability to mineralise short-chain alkenes, or even 2-MP is possible.

CONCLUSION

The aerobic degradation pathway of gaseous, branched, short-chain 2-methylpropene has so far only been found in strains of the *Mycobacteriaceae* family: *Mycobacterium* sp. ELW1 as well as in the novel isolates *Mycolicibacterium gadium* IBE100 and *Mycobacterium paragordonae* IBE200. In contrast, the initial epoxidation of 2-MP is not unique to these family members but is shared by species from other families harbouring SDMs of groups 2 and 6 (Figure 4). Mineralisation was found to require, besides the isoprene-type soluble diiron monooxygenase IsoABCDEF, a cofactor-independent epoxide hydrolase EPH, 2-methylpropane-1,2-diol dehydrogenase MdpB, 2-hydroxyisobutyraldehyde dehydrogenase MdpC, 2-hydroxyisobutyryl-CoA ligase HCL, cobalamin-dependent 2-hydroxyisobutyryl-CoA mutase HcmAB and its chaperone MeaH, 3-hydroxybutyryl-CoA dehydrogenase FadB, acetyl-CoA C-acetyltransferase PhaA, and cobalamin synthesis, catalysing the oxidation via 1,2-epoxy-2-methylpropane, 2-methylpropane-1,2-diol, 2-hydroxyisobutyraldehyde, 2-hydroxyisobutyric acid, 2-hydroxyisobutyryl-CoA, 3-hydroxybutyryl-CoA, acetoacetyl-CoA, and acetyl-CoA. Convergence with the *tert*-butanol degradation pathway became apparent. The genes encoding these enzymes are localised in clusters of approximately 60 kbp in size with a surprisingly highly conserved operon structure (Figure 10). The presence of transposon elements and the identity of the clusters down to the nucleotide level in strains IBE100 and ELW1 inferred horizontal gene transfer between these strains. These findings also allow for the *in silico* identification of other potential 2-MP degraders. Blast search in the NCBI database revealed putative *tert*-butanol and 2-MP degrading clinical and environmental isolates from the

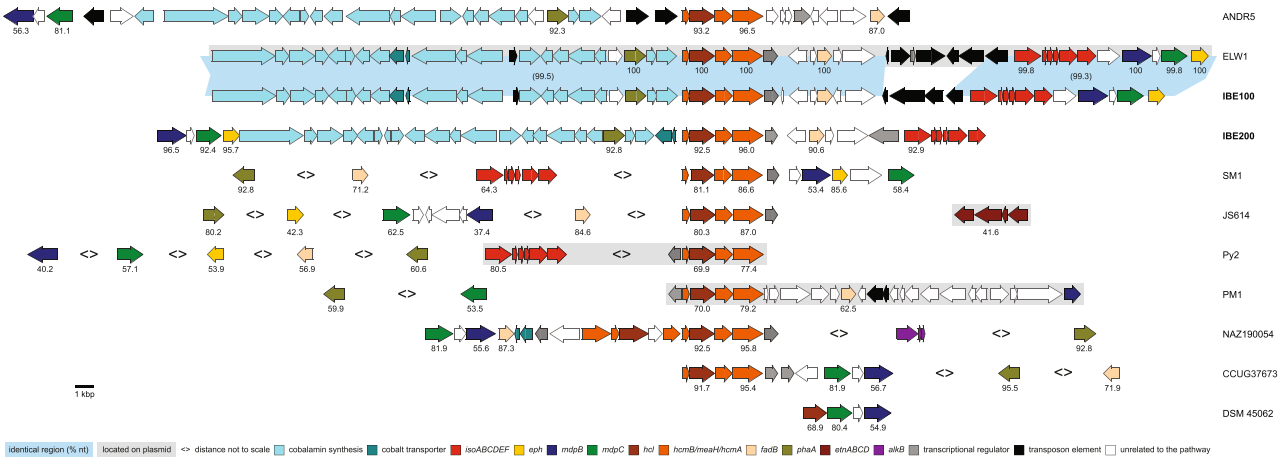

FIGURE 10 Comparison of gene clusters of strains encoding homologous enzymes related to 2-MP degradation appearing in phylogenetic trees from Figure 4 to Figure 8. Percentage similarity of peptides related to IBE100 below CDS. Confirmed (*Methylibium petroleiphilum* PM1) and postulated *tert*-butanol degraders (*Mycobacterium* sp. NAZ190054). Confirmed (IBE100, IBE200, *Mycobacterium* sp. ELW1) and potential 2-MP degraders (*Mycobacterium* sp. SM1). Strains with epoxidation activity towards 2-MP (*Nocardioides* sp. JS614, *X. autotrophicus* Py2).

Mycobacteriaceae family of previously undetermined degradation potential. Why such a specific degradation pathway for 2-MP has evolved, although no significant natural sources are known to exist on this planet, remains a puzzle to be solved.

AUTHOR CONTRIBUTIONS

Steffen Helbich: Conceptualization (lead); project administration (lead); visualization (lead); writing – original draft (lead); writing – review and editing (equal). **Israel Barrantes:** Data curation (equal); writing – original draft (supporting). **Luiz Gustavo dos Anjos Borges:** Data curation (equal); writing – original draft (supporting). **Dietmar H. Pieper:** Data curation (equal); writing – review and editing (equal). **Yevhen Vainshtein:** Data curation (equal); writing – original draft (supporting). **Kai Sohn:** Data curation (equal). **Karl-Heinrich Engesser:** Resources (lead); supervision (lead); writing – review and editing (equal).

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CONFLICT OF INTEREST STATEMENT

The authors declare no competing interests.

DATA AVAILABILITY STATEMENT

Genomic and transcriptomic data are publicly available at GenBank, under the BioProject PRJNA814055, accession number JAKZMO000000000 (IBE100), and BioProject PRJNA590116, accession number WNWV00000000 (IBE200).

ORCID

Steffen Helbich https://orcid.org/0000-0002-2740-270X
Israel Barrantes https://orcid.org/0000-0002-5252-4485
Luiz Gustavo dos Anjos Borges https://orcid.org/0000-0002-6987-0309
Dietmar H. Pieper https://orcid.org/0000-0002-3106-3421
Yevhen Vainshtein https://orcid.org/0000-0001-7944-8892
Kai Sohn https://orcid.org/0000-0002-6682-6409
Karl-Heinrich Engesser https://orcid.org/0000-0001-7273-2962

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