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1 **Proteomic investigation of enzymes involved in 2-Ethylhexyl nitrate biodegradation in**  
2 ***Mycobacterium austroafricanum* IFP 2173**

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25

1 **Abstract**

2 2-Ethylhexyl nitrate (2-EHN) is a synthetic chemical used as a diesel fuel additive, which is  
3 recalcitrant to biodegradation. In this study, the enzymes involved in 2-EHN degradation have  
4 been investigated in *Mycobacterium austroafricanum* IFP 2173. Using two-dimensional gel  
5 electrophoresis and a shotgun proteomic approach, a total of 398 proteins appeared to be more  
6 abundant in cells exposed to 2-EHN than in acetate-grown cells. This set of proteins includes  
7 multiple isoenzymes of the  $\beta$ -oxidation pathway, two alcohol and one aldehyde dehydrogenases,  
8 as well as four cytochromes P450, including one CYP153 which functions as an alkane  
9 hydroxylase. Strain IFP 2173 was also found to contain two *alkB*-like genes encoding putative  
10 membrane-bound alkane hydroxylases. RT-PCR experiments showed that the gene encoding the  
11 CYP153 protein, as well as the *alkB* genes, were expressed on 2-EHN. These findings are  
12 discussed in the light of a recently proposed 2-EHN degradation pathway, involving an initial  
13 attack by an alkane hydroxylase and one turn of  $\beta$ -oxidation, leading to the accumulation of a  $\gamma$ -  
14 lactone as a dead-end product.

15  
16 **Keywords :** alkane hydroxylase; cytochrome P450; CYP153; 2-ethylhexyl nitrate;  
17 *Mycobacterium austroafricanum*;  $\beta$ -oxidation.

18  
19 **Abbreviations :** ADH : alcohol dehydrogenase ; ALDH : aldehyde dehydrogenase ; 2-EHN : 2-  
20 ethylhexyl nitrate ; SDR : short-chain dehydrogenase/reductase

21  
22

## 1        1. Introduction

2        2-Ethylhexyl nitrate (2-EHN) is a xenobiotic compound used as a gasoline additive. Due to  
3 its explosive properties, 2-EHN is considered as the best cetane improver for diesel oil, including  
4 bio-diesels that might be used in the near future [1; 25]. In case of accidental release, 2-EHN is a  
5 serious health hazard, as humans exposed to this chemical were found to suffer from various  
6 symptoms, including headache, dizziness, chest discomfort, palpitations or nausea [7]. Although  
7 2-EHN was considered not readily biodegradable by US EPA [26]), it was recently reported that  
8 it could be degraded by *Mycobacterium austroafricanum* [24].

9        Soil *Mycobacteria* have been described for their ability to degrade a wide range of aliphatic  
10 and aromatic hydrocarbons, including polycyclic aromatic hydrocarbons [3; 10; 33], and fuel  
11 additives such as methyl *tertio*-butyl-ether [5; 13]. These bacteria are well equipped to degrade  
12 hydrocarbons, which they used as carbon sources. Their bacterial wall, rich in mycolic acids,  
13 confers resistance to toxic hydrophobic pollutants and, on the other hand, may facilitate access to  
14 hydrocarbons [12; 22]. Moreover, soil *Mycobacteria* contain oxygenases of different types,  
15 which play a crucial role in the degradation of both aliphatic and aromatic hydrocarbons. For  
16 instance, monooxygenases catalyze the first step in the degradation of alkanes. C<sub>5</sub>-C<sub>16</sub> alkanes are  
17 substrates of two kinds of enzymes, either integral-membrane non-heme diiron monooxygenases  
18 (AlkB) [29], or cytochromes P450 [14]. Growth on alkanes requires metabolic adaptation, as  
19 shown through a proteomic analysis of the marine bacterium *Alcanivorax borkumensis* SK2 [18].  
20 This study revealed that alkane utilisation may proceed via different pathways, involving two  
21 AlkB hydroxylases, one putative flavin monooxygenase, and three P450 cytochromes. Moreover,  
22 bacterial adaptation to alkane utilisation resulted in a strongly modified metabolism, with  
23 consequences for carbon flow and membrane lipid composition [18]. In recent years, high-  
24 throughput proteomics was implemented to identify whole sets of enzymes involved in complex  
25 bacterial metabolic pathways, such as the biodegradation of aromatic hydrocarbons [9].

1 Combined with functional genomics, proteomics helps understand cell response to environmental  
2 stimuli and may prove useful to develop efficient bioremediation strategies [34].

3 In previous studies, *M. austroafricanum* IFP 2173 was isolated on iso-octane [23], then  
4 selected for its ability to degrade 2-EHN [24]. Degradation of 2-EHN was found to be  
5 incomplete, yielding a 6-carbon  $\gamma$ -lactone, which accumulated as a dead-end product. A  
6 degradation pathway was proposed involving hydroxylation of the methyl group in distal  
7 position, then oxidation to the carboxylic acid, and further metabolism through one cycle of  $\beta$ -  
8 oxidation [17]. In order to identify the enzymes involved in this pathway, we have undertaken a  
9 proteomic analysis of cells exposed to 2-EHN. Because the genome sequence of strain IFP 2173  
10 is unknown, we tentatively identified relevant proteins by comparing their peptide sequences to  
11 those of orthologs found in the data bases. Currently, 21 genome sequences of *Mycobacterium*  
12 strains are available, six of which are from fast-growing strains isolated from soil, and genome  
13 annotation of *M. smegmatis* and related species has been assessed by proteomic analysis [6].  
14 Besides focusing on enzymes involved in 2-EHN degradation, this study gives an insight into  
15 proteins possibly involved in the response of bacteria to exposure to a toxic and hydrophobic  
16 xenobiotic compound.

17

## 18 **2. Material and Methods**

### 19 *2.1 Bacterial strain and growth conditions*

20 *M. austroafricanum* strain IFP 2173 was grown on a mineral salts medium at 30°C as described  
21 previously [17]. The carbon source was sodium acetate (4 g/l) or 2-EHN (500 mg/l). Growth was  
22 monitored by measurements of the optical density (OD) at 600 nm. To prepare 2-EHN-induced  
23 cells, acetate-grown cells were washed and resuspended to an OD<sub>600</sub> of 1.5 in culture medium,  
24 then incubated for five days with 2-EHN in conical flasks sealed with Teflon-coated screw caps.

## 1 2.2 *In vivo* <sup>35</sup>S labelling of proteins

2 For labelling experiments, bacteria were incubated with 2-EHN or acetate (control) in the  
3 presence of a mixture of <sup>35</sup>S-labelled methionine and cysteine (0.1 mCi, Easytag Express protein-  
4 labelling mix; NEN Life Science Products). Bacteria were incubated for 6 h on acetate or 30 h  
5 on 2-EHN at 30°C. In a control experiment, bacteria were incubated without exogenous C-source  
6 for 30h. Protein extracts were prepared as described below and analyzed by 2D electrophoresis  
7 and SDS-PAGE.

## 8 2.3 *Preparation of protein extracts*

9 Cell-free extracts were prepared by ultrasonication as described previously [10].  
10 Ultracentrifugation at 240,000 × g for 1 h was performed to separate soluble proteins from the  
11 membrane fraction, using an Optima TLX Ultracentrifuge (Beckman Instruments). Supernatant  
12 fractions were treated with benzonase (2,000 U; Merck), and subsequently dialysed for 4-5 h at  
13 4°C against 5 mM phosphate buffer, pH 7.5, containing 1 mM MgCl<sub>2</sub>, then overnight against  
14 ultrapure water. Samples were immediately processed as described below or stored at -20°C.

## 15 2.4 *Two dimensional gel electrophoresis*

16 Two-dimensional (2D) gel electrophoresis was carried out as described previously [10], with  
17 minor modifications. Briefly, 400 µg protein samples (or labelled extracts equivalent to 4.2 10<sup>4</sup>  
18 cpm) were applied to 18-cm IPG strips (ReadyStrip; Biorad) and fractionated in the pH range 4  
19 to 7 by isoelectric focusing for a total of approximately 70 kVh. Second dimension  
20 electrophoresis was carried out on 12.5 % polyacrylamide gels in a Protean II xi cell (Biorad) at  
21 20 mA per gel for 15-16 h, using a Tris-glycine buffer system [11]. The proteins were visualised  
22 by colloidal blue G-250 staining as described by Neuhoff [16], except that ethanol replaced  
23 methanol. <sup>35</sup>S labelled gels were stained, dried, and exposed to X-ray films for 3 weeks (Kodak  
24 BioMax MR). All gels were performed in triplicate, except for gels containing labelled proteins.  
25 Comparison of 2D gel patterns and spot intensities was carried out by visual inspection of gels.

1 Only spots that were absent in control extracts or that repeatedly showed an estimated intensity at  
2 least twice as high on 2-EHN extracts compared to control extracts were taken into consideration.  
3 Protein spots of interest were excised from the gel and processed for in-gel protein digestion and  
4 LC-MS/MS analysis as described below. Proteins up-regulated in acetate-grown cells are not  
5 discussed in this study.

#### 6 *2.5 SDS-PAGE of membrane fractions*

7 Protein pellets from high speed ultracentrifugation were homogenized in a volume equivalent to  
8 1/10 the initial volume of extract with 25 mM HEPES pH 7.5, containing 10 % of ethylene  
9 glycol. Protein samples were adjusted to 1 % SDS, 2.5 %  $\beta$ -mercaptoethanol, 10% glycerol,  
10 0.001 % bromophenol blue and 150 mM Tris-HCl, pH 7.0, prior to separation by SDS-PAGE on  
11 a 12.5 % polyacrylamide gel in a Tris-Tricine buffer system [20]. Proteins were stained with  
12 colloidal blue G-250 as described above.

#### 13 *2.6 Protein digestion*

14 Protein bands were manually excised from the gels and processed in 96-well microtitration plates  
15 using an automatic platform (EVO150, Tecan). For shotgun analysis, the protein track resulting  
16 from SDS PAGE was cut into 13 slices. Gel slices were washed in 25 mM  $\text{NH}_4\text{HCO}_3$  for 15 min  
17 and then in 50 % (v/v) acetonitrile containing 25mM  $\text{NH}_4\text{HCO}_3$  for 15 min. This washing  
18 procedure was repeated three times. Gel pieces were then dehydrated with 100 % acetonitrile and  
19 then incubated with 7 %  $\text{H}_2\text{O}_2$  for 15 min before being washed again as described above. 0.15  $\mu\text{g}$   
20 of modified trypsin (Promega, sequencing grade) in 30 $\mu\text{l}$  of 25 mM  $\text{NH}_4\text{HCO}_3$  was added to each  
21 gel slice for an overnight incubation at 37°C. Peptides were then extracted from gel pieces in  
22 three 15 min sequential extraction steps in 30  $\mu\text{L}$  of 50% acetonitrile, 30  $\mu\text{L}$  of 5% formic acid  
23 and finally 30  $\mu\text{L}$  of 100% acetonitrile. The pooled supernatants were then dried under vacuum.

1 2.7 Nano-LC-MS/MS analysis.

2 For nano-LC-MS/MS analysis, the dried extracted peptides were resuspended in water containing  
3 2.5 % acetonitrile and 2.5 % trifluoroacetic acid. A nano-LC-MS/MS analysis was then  
4 performed (Ultimate 3000, Dionex and LTQ-Orbitrap, Thermo Fischer Scientific). The system  
5 included a 300  $\mu\text{m}$  x 5 mm PepMap C18 precolumn and a 75  $\mu\text{m}$  x 150 mm C18 Gemini column.  
6 The column was developed at a flow rate of 300 nL/min with a 60-minute gradient from solvent  
7 A (5% acetonitrile and 0.1% formic acid in water) to solvent B (80% acetonitrile and 0.08%  
8 formic acid in water). MS and MS/MS data were acquired using Xcalibur (Thermo Fischer  
9 Scientific) and processed automatically using Mascot Daemon software (Matrix Science).

10 Consecutive searches against the SwissProt/TrEMBL database were performed for each sample  
11 using an intranet version of Mascot 2.0. Peptide modifications allowed during the search were N-  
12 acetylations, mono- and dioxidations (methionine), conversions to cysteic acid and methionine  
13 sulphone. Proteins showing at least two peptides with a score higher than the query threshold (p-  
14 value <0.05) were automatically validated. If one set of peptides yielded two or more proteins,  
15 and proteins were from the same organism, only the protein ranked first in the alphabetic order  
16 was validated (rejection of redundant proteins). When the proteins were from different  
17 *Mycobacterium* strains, that from *M. vanbalenii* PYR-1 was arbitrarily chosen. Proteins identified  
18 by only one peptide were checked manually using the classical fragmentation rules. The rate of  
19 false-positive protein identifications was estimated to be about 1.2% by performing a search with  
20 a SwissProt/TrEMBL decoy database according to a published procedure [4].

21 For each identified protein, the spectral count values were determined, and abundance rates were  
22 then calculated as percentages of the whole set of identified proteins according to the formula :  
23 Abundance of protein X = (spectral count protein X) / ( $\Sigma$  spectral counts for all proteins) x 100.



## 1 2.8 Cloning of the *CYP153* and *alkB* genes

2 DNA fragments containing *alkB1* alone , *alkB1-rubA1-rubA2*, *alkB1-rubA1-rubA2-tetR*, and  
3 *alkB2* were separately PCR-amplified using appropriate primers and genomic DNA from  
4 IFP2173 prepared as previously described [8]. *CYP153* was amplified using 5'-  
5 GCATATGACCGAAATGACGGTG and 5'-CGGATCCTCAGGCGTTGATGCGCAC as  
6 forward and reverse primers, respectively. The amplicons were purified, cloned into pDRIVE  
7 (Qiagen) and sequenced on both strands. Gene sequences were validated when sequencing of  
8 replicate amplicons gave identical results. Details on the amplification and cloning procedures  
9 are available upon request. Sequence analysis was performed using BLAST.

## 10 2.9 RNA extraction and RT-PCR analysis

11 Total RNA was extracted from 50-mL cultures of strain IFP 2173 using standard procedures  
12 [19]. Bacteria were grown on acetate to an OD<sub>600</sub> of 0.7 (control cells) or washed and  
13 resuspended to an OD<sub>600</sub> of 0.6, and further incubated with 2-EHN for four days. Bacteria were  
14 then centrifuged at 10,000 × g, and resuspended in 200 μL of 20 mM Tris-HCl, 5 mM EDTA,  
15 pH 8, containing lysozyme and lysostaphin, 1.5 and 0.025 mg/mL, respectively (Sigma Life  
16 Science). After 10 min at 37°C, RNA was extracted using the RiboPure™-Bacteria kit (Ambion,  
17 Austin, Texas). Crude RNA samples (2 μg) were treated with Turbo DNase (Ambion) and the  
18 resulting RNA preparations were quantified using a Nanodrop apparatus (NanoDrop  
19 Technologies). RT-PCR was performed with 10 ng of RNA preparation using the One step RT-  
20 PCR kit (Promega, France). PCR amplification of internal gene sequences was carried out with  
21 the following primer pairs: for *alkB1*, *alkB1-F* (5'-CGTGATCATGGGTGCCTAC-3') and  
22 *alkB1-R* (5'-CCAGAACGTCTCACCGAAG-3'); for *alkB2*, *alkB2-F* (5'-  
23 CCTGATGTTCCCTCGTGATCC-3') and *alkB2-R* (5'-CTTGTCGACGTCGCTCATC-3'); for  
24 *CYP153*, P450fw1 and P450rw3 [30]; for the aldehyde dehydrogenase encoding gene (*alkH*),  
25 *ALDH1-F* (5'-GCACCGTGCTGATCATCGGTGC-3') and *ALDH1-R* (5'-

1 CCAGGCGATGCGCTTGGCG-3'), for the 16S RNA gene, P16S-F (5'-  
2 GGTCTAATACCGAATACACCCTTCT-3') and P16S-R (5'-CCAGGAATTCCAGTCTCCC-  
3 3'). RT-PCR reactions were carried out as follows: 45 min at 45°C, 3 min at 95°C, then 32 cycles  
4 of 30 s at 95°C, 30 s at 62°C and 30 s at 72°C, 5 min final elongation at 72°C. Products were  
5 analyzed by electrophoresis on 2% agarose gels.

### 6 2.10 Nucleotide sequences

7 The nucleotide sequences of *alkB1rubA1rubA2tetR*, *alkB2*, *CYP153*, *alkH* (partial) were  
8 deposited under accession number FJ009005, FJ009004, FJ009003, FJ207472, respectively.

## 9 3. Results

### 10 3.1 Identification of cytoplasmic proteins up-regulated on 2-EHN

11 In order to identify proteins up-regulated on 2-EHN, protein profiles of cells incubated with this  
12 compound were compared to those of cells grown on acetate. Cytoplasmic proteins were  
13 prepared and analysed by 2D gel electrophoresis while membrane fractions from the high-speed  
14 centrifugation pellet of cell extracts were separated by SDS-PAGE. As discussed below,  
15 membrane fractions possibly included proteins loosely associated to membranes as well as  
16 cytoplasmic proteins trapped into membrane vesicles.

17 Comparison of 2D gel protein profiles revealed that 30 protein spots were either absent in  
18 acetate-grown cells or at least two-fold more abundant in 2-EHN-grown cells (Fig. 1). To  
19 confirm these results, we performed <sup>35</sup>S-labeling experiments where cells were exposed to 2-  
20 EHN for 30 h or to acetate for 6 h. This difference in incubation time was intended to reflect the  
21 much slower growth of strain IFP 2173 on 2-EHN compared to acetate. Autoradiographies of the  
22 2D gel showed markedly different patterns (Fig. S1 in supplementary data). Most labelled  
23 proteins uniquely detected in 2-EHN-exposed cells corresponded to spots previously identified  
24 based on comparison of stained gels, but two additional 2-EHN-specific polypeptides were found  
25 (E10 and E28). The position of these extra polypeptides has been reported on the 2D image in

1 Fig. 1A. The 32 protein spots of interest were subjected to trypsin digestion followed by LC-  
2 MS/MS analysis and search for peptide matches in the data bases using Mascot (see Materials  
3 and Methods for details). Thanks to the high accuracy and wide dynamic range of the mass  
4 spectrometer, several spots were found to contain 2- to 4 imperfectly separated proteins. Spots  
5 E2, E3, E4, E7, E12, E18, E22 and E24 yielded two protein identifications, spots E1, E9 and E10  
6 yielded three and spot E23 gave four. On the other hand, a few pairs of closely-located spots  
7 gave single protein identifications. As a result, the analysis of 32 spots ended up with a total of  
8 42 proteins, which matched orthologs found in *M. vanbalenii* PYR-1 and related *Mycobacterium*  
9 strains from soil (Table 1). One set of induced proteins was clearly associated with the  $\beta$ -  
10 oxidation of fatty acids. Some of the enzymes involved in this pathway were found in multiple  
11 isoforms, including acetyl-CoA acyltransferase (2 copies), acyl-CoA dehydrogenase (7 copies),  
12 enoyl-CoA hydratase/isomerase (4 copies). Consistent with this finding, the reference  
13 *Mycobacterium* strains mentioned above were found to contain multiple gene copies coding for  
14 enzymes of the  $\beta$ -oxidation in their genome (Table S1). Other proteins up-regulated on 2-EHN  
15 included dehydrogenases, diverse metabolic enzymes and proteins involved in cell response to  
16 stress (Table 1).

17

### 18 3.2 2-EHN-induced proteins associated to membrane fractions

19 Since membrane proteins are generally difficult to analyze by regular 2D gel electrophoresis, we  
20 chose to separate the proteins of the insoluble high-speed fractions of cell extracts by one  
21 dimension SDS-PAGE. When stained protein profiles of 2-EHN versus acetate-grown cells were  
22 compared no obvious differences were observed. However, <sup>35</sup>S radioactive labelling revealed that  
23 some protein bands became clearly labelled upon exposure to 2-EHN, including a prominent 45-  
24 kDa protein (Fig. S2). In order to identify proteins of interest, protein sets from 2-EHN and  
25 acetate grown cells were separated by SDS-PAGE, and subjected to trypsin-digestion and peptide

1 analysis by LC-MS/MS. Data processing using Mascot identified over 1300 proteins, most of  
2 which had counterparts in the proteome of *M. vanbalenii* PYR-1. Search for membrane-bound  
3 proteins using the HMMTOP software [27] revealed that about 30% of this set of proteins  
4 potentially showed at least one transmembrane segment. In addition, an unknown proportion of  
5 the detected proteins were probably membrane-associated through hydrophobic interactions or as  
6 part as membrane-bound complexes. However, many proteins recovered in the membrane fraction  
7 were cytoplasmic, indicating that they might have been trapped in membrane vesicles that  
8 formed upon cell lysis.

9 An inventory of proteins found to be common or specific to cells incubated with 2-EHN or  
10 acetate is presented in tables S2 and S3 in supplementary material. From the set of common  
11 proteins, a subset was selected based on abundance rates more than twice as high for the 2-EHN  
12 treated cells as compared to control cells. The proteins of this subset (65 proteins) as well as  
13 those found to be specific to the 2-EHN treatment (300 proteins) were tentatively classified in  
14 terms of enzyme category or metabolic function, with special emphasis on enzymes related to  
15 alkane degradation (Table 2). A comparison of this set of proteins with that found by the 2D gel  
16 analysis revealed that only 9 proteins were common to both sets (Table 1). A total of 17 proteins  
17 were found to be enzymes of the  $\beta$ -oxidation of fatty acids, including many redundant isoforms,  
18 five of which were also detected on 2D gels (A1TCG6, A1TDA6, A1T5U2, A1TE56 and  
19 A1TDW4). Consistent with the 2D gel data, numerous proteins up-regulated on 2-EHN were  
20 dehydrogenases including 12 short-chain dehydrogenase/reductases (SDR). Several proteins  
21 were likely involved in the response to stress, other presumably act as transcriptional regulators.  
22 The analysis highlighted two alcohol dehydrogenases (ADH) and one aldehyde dehydrogenase  
23 (ALDH) possibly implicated in the early steps of the 2-EHN degradation pathway (see below).  
24 Besides, enzymes of the central metabolism previously shown to be essential for alkane  
25 assimilation [18] have been detected, including two phosphoenolpyruvate synthases involved in

1 gluconeogenesis. Enzymes related to the metabolism of lipids were also identified, suggesting  
2 that membrane modifications might occur as part of the bacterial adaptation to growth on a  
3 hydrophobic substrate.

4 Four cytochromes P450 were identified, two of which belong to the CYP153 subclass of P450,  
5 potentially capable of alkane hydroxylation. The most abundant of the two enzymes, identified  
6 by 11 peptides (36% coverage), was found to be closely related to the CYP153 enzyme from  
7 *Mycobacterium* sp. XHN-1500 [31].

8 Membrane proteins relevant to the metabolism of alkanes were not detected in either protein  
9 extract. This is the case for the trans-membrane AlkB hydroxylase that is known to catalyze the  
10 initial oxidation reaction of alkanes in many bacteria [29].

11

### 12 3.3 Occurrence of several putative alkane monooxygenases in strain IFP 2173

13 Our proteomic analysis revealed that one cytochrome P450 with close similarity with a well  
14 characterized alkane hydroxylase (CYP153) was 2-EHN-specific. In order to learn more about  
15 this protein, its structural gene was PCR-amplified using genomic DNA from strain IFP 2173 and  
16 specific oligonucleotides designed based on the gene sequence of *CYP153A* from strain XHN-  
17 1500 [31]. A 1261 bp gene was obtained, which displayed high sequence similarity with its  
18 counterpart from strain XHN-1500 (99 % identities), resulting in a predicted protein having only  
19 two amino acid changes compared to CYP153A.

20 In a previous study, a gene potentially involved in isoalkane degradation was found in strain IFP  
21 2173 and identified as an *alkB* gene based on partial sequence determination [23]. A BLAST  
22 search showed that this gene was closely related to *alkB* from *M. vanbaalenii* PYR-1. This strain  
23 has two alkane monooxygenase genes, one of which is associated with two genes encoding  
24 rubredoxins. Primers were designed after the *alkB* gene sequences of *M. vanbaalenii* PYR-1, and  
25 used to amplify corresponding genes from strain IFP 2173 genomic DNA. Two *alkB*-like genes

1 were found in two separate loci, which displayed exactly the same gene arrangement as that  
2 found in *M. vanbaalenii* PYR-1. In particular, the *alkB1* gene was followed by two rubredoxin  
3 genes, named *rubA1* and *rubA2*, almost identical to counterpart genes of *M. vanbaalenii* PYR-1  
4 (99% identity). The second *alkB* gene (*alkB2*) was 98% identical to its counterpart in strain PYR-  
5 1.

#### 6 *3.4 RT-PCR evidence for the expression of three alkane hydroxylases in 2-EHN fed cells*

7 Since none of the AlkB-like hydroxylases was detected in extracts of strain IFP 2173 upon  
8 proteomic analysis, we carried out RT-PCR experiments to determine whether the corresponding  
9 genes were expressed under the growth conditions used in this study. Transcripts of the *alkB1*  
10 and *alkB2* genes were equally detectable in acetate and 2-EHN-fed cells (Fig. 2). Further analysis  
11 showed that a transcript specific for the gene encoding the CYP153 hydroxylase described above  
12 was also detected in both 2-EHN and acetate-grown cells (Fig. 2). This finding is consistent with  
13 the fact that the enzyme was clearly identified by proteomic analysis in 2-EHN-fed cells but  
14 contrasted with the finding that it was not found in acetate-grown cells. Perhaps cells growing on  
15 acetate produce the CYP153 protein at a low level or in a transient manner during a particular  
16 phase of growth, so that it passed undetected.

17 A single ALDH appeared to be up-regulated in 2-EHN-fed cells to a level at least 2-fold as high  
18 as in acetate-grown cells. Using primers designed after the gene encoding an orthologous ALDH  
19 from strain PYR-1 (A1P1A6), a DNA fragment that perfectly matched the target gene sequence,  
20 was PCR-amplified from IFP 2173 genomic DNA. RT-PCR indicated that this gene was  
21 transcribed in both acetate and 2-EHN fed cells (Fig. 2). The deduced sequence of the closely  
22 related PYR-1 enzyme displayed 39% sequence identity with the product of the *alkH* gene from  
23 *P. putida* GPo1.

## 1 4. Discussion

2 The present study deals with the metabolic adaptation of a bacterium which was forced to grow  
3 on a xenobiotic compound being a poor carbon source and a toxic substance. Our proteomic  
4 analysis identified over 1300 proteins based on sequence information available in the data bases  
5 even though the genome of strain IFP 2173 was unknown. Most proteins were identified as  
6 orthologs from strain *M. vanbaalenii* PYR-1 or related *Mycobacterium* species, thus reflecting  
7 the high degree of conservation of protein sequences in the proteomes from fast-growing  
8 *Mycobacterium* species isolated from various places around the world. Besides catabolic  
9 enzymes enabling the bacterium to utilize 2-EHN as carbon source, many up-regulated proteins  
10 were found to be involved in lipid metabolism, regulation and response to stress, and might help  
11 bacteria to adapt to the toxic and/or hydrophobic character of 2-EHN. The following discussion  
12 focuses on enzymes that might be implicated in 2-EHN degradation.

13 In a previous study, we showed that strain IFP 2173 partially degraded 2-EHN to a compound  
14 identified as 4-ethyltetrahydrofuran-2(3H)-one, and we proposed a degradation pathway outlined  
15 in figure 3 [17]. Every step in the pathway can be assigned at least one enzyme found among the  
16 proteins up-regulated on 2-EHN, except for the last step of the  $\beta$ -oxidation which is catalyzed by  
17 a thiolase. Since three thiolase genes are present in the genomes of three related *Mycobacterium*  
18 species (Table S1), at least one thiolase is expected to be produced by strain IFP 2173 grown on  
19 2-EHN. Perhaps, the enzyme was synthesized in small amounts and passed undetected in our  
20 proteomic analysis.

21 We identified four alkane hydroxylases that might catalyze the first step in 2-EHN degradation  
22 *i.e.* the hydroxylation of the distal methyl group. Two enzymes are similar to the classical AlkB  
23 membrane-bound enzymes that were found to play a pivotal role in alkane degradation by *P.*  
24 *putida* GPo1 [28] and *A. borkumensis* SK2 [21]. The AlkB proteins were not detected in the  
25 membrane fraction of IFP 2173 in either growth conditions, even when searching the proteomic

1 data for the expected peptides derived from their deduced protein sequence. Nevertheless,  
2 specific transcripts for the corresponding genes were found in cells grown on acetate or exposed  
3 to 2-EHN suggesting that the proteins were synthesized. Perhaps, the AlkB proteins were poorly  
4 solubilized in SDS or yielded few tryptic peptides detectable by LC-MS/MS. The two other  
5 plausible enzymes that can initiate 2-EHN degradation are soluble cytochromes P450 of the  
6 CYP153 subfamily. Although many genes encoding cytochromes P450 are present in the  
7 genomes of related *Mycobacterium* species (Table 2), sequence alignments indicated that none of  
8 the gene products was related to the CYP153 (data not shown). In addition, the CYP153 proteins  
9 identified in strain IFP 2173 were found to be mainly produced on 2-EHN, suggesting that at  
10 least one of these enzymes takes part in the degradation. CYP153 cytochromes hydroxylate linear  
11 or cyclic alkanes with medium chain length [31]. For instance, the CYP153A6 from  
12 *Mycobacterium* sp. XHN1500, which is the closest ortholog of the major CYP153 from IFP  
13 2173, preferentially utilizes octane [31], but can also hydroxylate substrates with a bulky  
14 structure like limonene [31]. CYP153-like genes have been found in other Actinomycetes as well  
15 as in  $\alpha$  and  $\beta$ -proteobacteria, and three groups were distinguished based on phylogenetic  
16 considerations [29]. Like other bacterial cytochromes, the CYP153 enzymes function with two  
17 electron carriers, a NAD(P)H-oxidoreductase and a ferredoxin. In this respect, a FAD-binding  
18 oxidoreductase that might be functionally associated with one of the CYP153 from IFP 2173 has  
19 been identified by the 2D gel approach (Table 1), and four such reductases were detected by the  
20 shotgun approach (Tables S2 & S3).

21 An esterase is also required to hydrolyze the nitro-ester bond of 2-EHN. A chloride peroxidase  
22 was found among the 2-EHN-induced proteins in the 2D gel analysis (A1T5E7), which showed  
23 the classical consensus sequence (GX<sub>2</sub>SXG) typical for the active site of esterases. This type of  
24 enzyme is active on carboxylic esters [2], but it is unknown whether it could remove the nitro  
25 group of 2-EHN. Four other putative esterases up-regulated on 2-EHN can potentially catalyze



1 this reaction (Tables S2 & S3). Since strain IFP 2173 can use 2-ethylhexanol and transform it to  
2 2-ethylhexanoic acid and 4-ethyltetrahydrofuran-2(3H)-one [17], it may be inferred that  
3 hydrolysis of the ester bond precedes the hydroxylation of the distal methyl group in the  
4 biodegradation pathway.

5 The subsequent step in the degradation pathway is the conversion of the 2-ethylpentane-1,5-diol  
6 to an aldehyde by an ADH. Three 38-kDa ADH were apparently associated to 2-EHN  
7 metabolism, which are predicted to have a zinc-binding domain and a GroES-like structure. A  
8 different and larger ADH (AlkJ; 61 kDa) is involved in alkane oxidation in *P. putida* GPo1.  
9 However, a deletion of *alkJ* did not affect alkane degradation, indicating that this reaction does  
10 not require a specific ADH [28].

11 A single 2-EHN-specific ALDH was detected by SDS-PAGE and peptide analysis. Examination  
12 of the sequence of the orthologous enzyme of strain PYR-1 (A1T1A6) showed that it might be  
13 composed of a catalytic domain and a LuxC-like domain [15]. The detected ALDH showed 39 %  
14 sequence identity with AlkH encoded by the *alk* operon, which is involved in alkane  
15 biodegradation in *P. putida* GPo1 [32].

16 The biosynthesis of multiple isoenzymes of the  $\beta$ -oxidation pathway in response to cell exposure  
17 to 2-EHN is intriguing. Many *Mycobacterium* species are known to thrive on alkanes, but the  
18 redundancy of  $\beta$ -oxidation enzymes had not been previously reported, although it could be  
19 predicted from the abundance of genes coding for such enzymes in available genome sequences  
20 of *Mycobacteria* (Table S1). In contrast, the hydrocarbonoclastic bacterium *A. borkumensis* SK2  
21 produced relatively few specific enzymes when growing on alkanes [18]. Perhaps, the greater  
22 number of isoenzymes found in soil *Mycobacteria* reflects a more versatile metabolism, adapted  
23 to a diet made of diverse hydrocarbons present in their environment.

24 In our study, we identified most of the enzymes possibly involved in 2-EHN degradation by  
25 strain IFP 2173. Since these enzymes have counterparts in other soil *Mycobacteria*, the question

1 arises whether these bacteria can degrade 2-EHN. We recently found that this ability is in fact  
2 restricted to a few *M. austroafricanum* strains [21]. Hence, 2-EHN degradation might depend on  
3 the catalytic activity of some specific enzyme such as a CYP153 hydroxylase, which is present in  
4 strain IFP 2173 but absent in related *Mycobacteria* including strain PYR-1. Accordingly, we  
5 observed that strain PYR-1 cannot utilize 2-EHN as carbon source (unpublished results).

6

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13

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24  
25  
26

1 **Legends to figures**

2 **Figure 1:** 2D gel map of soluble proteins from *M. austroafricanum* IFP 2173 induced by 2-EHN.

3 Isoelectric focusing was performed in the pH range 4 to 7. **A:** cells grown on 2-EHN, **B:** cells  
4 grown on acetate. 2-EHN-specific protein spots are numbered in panel A.

5

6 **Figure 2 :** Expression of genes relevant to 2-EHN degradation as analyzed by RT-PCR.

7 Reactions were performed as described under Materials and Methods using primers specific to an  
8 internal region of the indicated genes. *alkB1* and *alkB2* designate genes encoding two alkane  
9 hydroxylases, while *alkH* refers to a gene coding for an aldehyde dehydrogenase (see text). RNA  
10 used as template was extracted either from 2-EHN exposed cells (lanes 3 & 4) or from acetate-  
11 grown cells (lanes 5 & 6). The content of each RNA preparation was checked by carrying out a  
12 RT-PCR of a portion of 16S RNA. Lanes 3 and 5 are control reactions in which reverse  
13 transcriptase was omitted. Lane 1, DNA ladder ; lane 2, PCR amplicon generated from gDNA.

14

15 **Figure 3 :** Proposed pathway and enzymes involved in 2-EHN degradation

16 Products: **a:** 2-ethylhexyl nitrate; **b:** 2-ethylpentane-1,5-diol; **c:** 5-(hydroxymethyl)heptanoic  
17 acid; **d:** 5-(hydroxymethyl)heptanoyl CoA; **e:** 5-(hydroxymethyl)heptan-2-enoyl CoA; **f:** 3-  
18 hydroxy-5-(hydroxymethyl)heptanoyl CoA; **g:** 5-(hydroxymethyl)-3-ketoheptanoyl CoA; **h:** 3-  
19 (hydroxymethyl)-pentanoyl CoA; **i:** 3-(hydroxymethyl)-pentanoic acid; **j:** 4-ethyl-dihydrofuran-  
20 2-(3H)one

21 Enzymes: **1:** alkane hydroxylase (*AlkB1*, *AlkB2* or *CYP153*) and esterase, **2:** alcohol

22 dehydrogenase and aldehyde dehydrogenases, **3** and **8:** acyl CoA acyltransferase, **4:** Acyl CoA  
23 dehydrogenase, **5:** Enoyl CoA hydratase, **6:** Hydroxyacyl CoA dehydrogenase, **7:** Thiolase, **9:**

24 spontaneous cyclisation. ✦ Indicates that  $\beta$ -oxidation is blocked by the ethyl chain in  $\beta$   
25 position.





**Table 1:** Cytoplasmic proteins up-regulated upon incubation of *M. austroafricanum* IFP 2173 on 2-EHN

Enzyme or protein function	Spot #	2-EHN induction*	<sup>35</sup> S-labelling on 2-EHN*	pI <sup>b</sup>	Mol Mass <sup>b</sup>	Score	Coverage (%)	Peptides	SwissProt/TrEMBL	Micro-organism
<b>Fatty acid metabolism</b>										
Acyl-CoA dehydrogenase domain protein	E1	+++	+++	5,3	41116	186	10	3	A4TFJ1	<i>M. gilvum</i> PYR-GC
	E2	+++	+++	5,4		180	9	3		
	E1	+++	+++	5,3	40821	133	11	3	A3Q1G5	<i>Mycobacterium</i> sp. JLS
	E2	+++	+++	5,4	40989	702	32	10	A1TCG6 <sup>c</sup>	<i>M. vanbaalenii</i> PYR-1
	E3	+++	+++	5,15	42234	112	10	2	A4T8F2	<i>M. gilvum</i> PYR-GC
	E4	+++	+++	4,8	39744	265	18	4	A1TDA6 <sup>c</sup>	<i>M. vanbaalenii</i> PYR-1
	E7	++	++	5,87	42108	118	6	2	A4TFI8	<i>M. gilvum</i> PYR-GC
	E8	+++	+	5,2	41520	585	36	9	A1T5U2 <sup>c</sup>	<i>M. vanbaalenii</i> PYR-1
Enoyl-CoA hydratase/isomerase	E9	+++	+++	4,9	29971	133	9	2	A1TDW3	<i>M. vanbaalenii</i> PYR-1
	E10	-	+++	5,1	27387	121	11	2	A4TFL1	<i>M. gilvum</i> PYR-GC
	E9	+++	+++	4,85	27478	280	22	5	A1TE56 <sup>c</sup>	<i>M. vanbaalenii</i> PYR-1
	E9	+++	+++	4,85	26666	35	5	1	A4TDN8	<i>M. gilvum</i> PYR-GC
3-hydroxyacyl-CoA dehydrogenase, NAD-binding	E11	++	++	5,4	76177	586	19	11	A1TF87	<i>M. vanbaalenii</i> PYR-1
	E12	++	++	5,4		381	11	7		
Acetyl-CoA C-acyltransferase (EC 2.3.1.16)	E1	+++	+++	5,4	39836	591	32	9	A1TDW4 <sup>c</sup>	<i>M. vanbaalenii</i> PYR-1
	E13	++	+++	4,9	42130	189	11	3	A1TF88	<i>M. vanbaalenii</i> PYR-1
Acyl-ACP thioesterase	E10	-	+++	5,3	31192	117	8	2	A1T388	<i>M. vanbaalenii</i> PYR-1

Enzyme or protein function	Spot #	2-EHN induction*	<sup>35</sup> S-labelling on 2-EHN*	pI <sup>b</sup>	Mol Mass <sup>b</sup>	Score	Coverage (%)	Peptides	SwissProt/TrEMBL	Micro-organism
FAD-dependent pyridine nucleotide-disulphide oxidoreductase	E15	+++	+++	5,55	42755	319	18	5	A4TFL9	<i>M. gilvum</i> PYR-GC
	E7	+++	+	5,8		152	9	3		
Short-chain dehydrogenase/reductase	E17	+++	++	4,9	29920	164	15	3	A1T1A7 <sup>c</sup>	<i>M. vanbaalenii</i> PYR-1
	E18	+++	+++	4,9	30143	102	10	2	Q1BFX1	<i>Mycobacterium</i> sp. MCS
(S)-2-hydroxy-acid oxidase	E30	+++	-	7	42022	169	10	3	A1T4N1	<i>M. vanbaalenii</i> PYR-1
Dihydrolipoamide dehydrogenase	E22	++	++	5,6	49719	370	18	6	A1T382	<i>M. vanbaalenii</i> PYR-1
<b>Lysine biosynthesis</b>										
Dihydrodipicolinate synthase	E24	++	-	5,4	31436.7	163	12	3	A1T7Q1	<i>M. vanbaalenii</i> PYR-1
Dihydrodipicolinate reductase (EC 1.3.1.26)	E23	++	-	4,8	25816	59	8	1	A1T7N8	<i>M. vanbaalenii</i> PYR-1
<b>Protein synthesis</b>										
Serine-tRNA ligase (EC 6.1.1.11)	E25	+++	+++	4,8	60542.4	128	6	2	A1TGX4	<i>M. vanbaalenii</i> PYR-1
Ketol-acid reductoisomerase (EC 1.1.1.86)	E3	+++	+++	5,2	36513	158	5	2	Q1BAR7	<i>Mycobacterium</i> sp. MCS
<b>Nitrogen assimilation</b>										
Alanine dehydrogenase (EC 1.4.1.1)	E28	-	+++	5,2	38907	203	11	3	A1T7L9	<i>M. vanbaalenii</i> PYR-1
<b>Oxidative phosphorylation</b>										
ATP synthase epsilon chain (EC 3.6.3.14) (ATP synthase F1 sector epsilon subunit)	E29	+++	+++	4,8	13330	85	9	1	P45822	<i>M. leprae</i> TN
<b>CO<sub>2</sub> hydratation</b>										
Carbonic anhydrase	E31	++	++	4,8	18225	272	33	6	A1TDF0	<i>M. vanbaalenii</i> PYR-1
<b>Glycolysis / glyconeogenesis</b>										
Phosphoglycerate kinase (EC 2.7.2.3)	E32	+++	+++	4,7	42102	435	24	6	A1T8L1	<i>M. vanbaalenii</i> PYR-1
	E6	+++	+++	4,75		572	28	8		

Enzyme or protein function	Spot #	2-EHN induction*	<sup>35</sup> S-labelling on 2-EHN*	pI <sup>b</sup>	Mol Mass <sup>b</sup>	Score	Coverage (%)	Peptides	SwissProt/TrEMBL	Micro-organism
<b>Stress response</b>										
Heat shock protein Hsp20	E26	+++	+++	4,8	15648.4	177	27	3	A1T4V8 <sup>c</sup>	<i>M. vanbaalenii</i> PYR-1
UspA	E24	++	-	5,4	31354	85	5	1	A1T4W2	<i>M. vanbaalenii</i> PYR-1
<b>Miscellaneous</b>										
Putative esterase precursor	E18	+++	+++	5	35010	79	6	1	A1T6C2	<i>M. vanbaalenii</i> PYR-1
Chloride peroxidase (EC 1.11.1.10)	E19	++	-	5,7	30410	173	15	3	A1T5E7	<i>M. vanbaalenii</i> PYR-1
Antibiotic biosynthesis monooxygenase	E16	+++	+++	4,8	11741	56	16	1	Q1B2M9	<i>Mycobacterium</i> sp. MCS
Allophanate hydrolase subunit 1	E10	+++	+++	5,3	25092	142	10	2	A1T1V3	<i>M. vanbaalenii</i> PYR-1
Fumarate lyase	E20	+++	++	5,1	49944	579	24	10	A1TE24 <sup>c</sup>	<i>M. vanbaalenii</i> PYR-1
	E21	+++	++	5,1	49944	538	27	8		
HpcH/HpaI aldolase	E23	++	-	4,8	29032	202	13	4	A1TCG4	<i>M. vanbaalenii</i> PYR-1
Ribonuclease PH (EC 2.7.7.56)	E23	++	-	4,8	27449	75	5	2	A1T7Q1	<i>M. vanbaalenii</i> PYR-1
Glycyl-tRNA synthetase, alpha2 dimer	E22	++	++	5,6	59543	405	18	7	A1TBP9	<i>M. vanbaalenii</i> PYR-1
3-hydroxyisobutyrate dehydrogenase precursor	E23	++	-	4,8	29262	195	16	3	A1T4U4	<i>M. vanbaalenii</i> PYR-1
Cyclic nucleotide-binding:regulatory protein, Crp	E27	+++	-	9,6	24776	409	40	7	A1T6A5	<i>M. vanbaalenii</i> PYR-1
Phosphoribosyltransferase: Erythromycin esterase	E12	++	++	5,4	74587	49	2	1	A1T4X7 <sup>c</sup>	<i>M. vanbaalenii</i> PYR-1

\*Spot intensity was estimated from visual inspection of stained gels or autoradiographies : +, ++, +++ stand for small, medium size and large spots, respectively. (-) means undetected spot.

<sup>b</sup> Theoretical values calculated on the basis of deduced polypeptide sequences.

<sup>c</sup> These protein entries also appear in the list of 2-EHN-induced proteins identified by shotgun analysis (Table S3)

**Table 2:** Functional classification of proteins more abundant or exclusively detected on 2-EHN

	Number of proteins	
	exclusively found on 2-EHN	More abundant
Beta oxidation	17	1
P450 hydroxylases	4	0
Esterases	3	1
Dehydrogenases	2 ADH	1 ALI
SDR	10	2
Stress response	12	9
Regulation	21	3
Lipid metabolism/cell wall	13/1	1/3
Ribosomal proteins	12	2
N-metabolism	3	1
General metabolism	74	22
ABC transporter proteins	22	3
Other	64	6
Putative uncharacterized	42	10

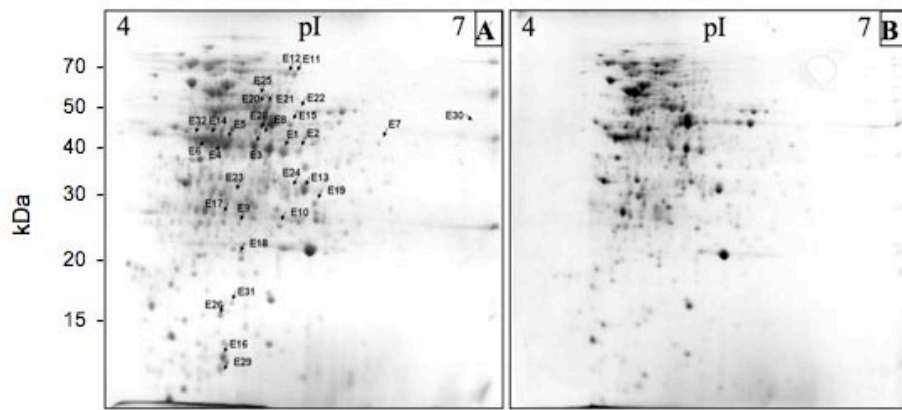


Fig. 1, Nicolau et al.

Fig. 2, Nicolau et al.

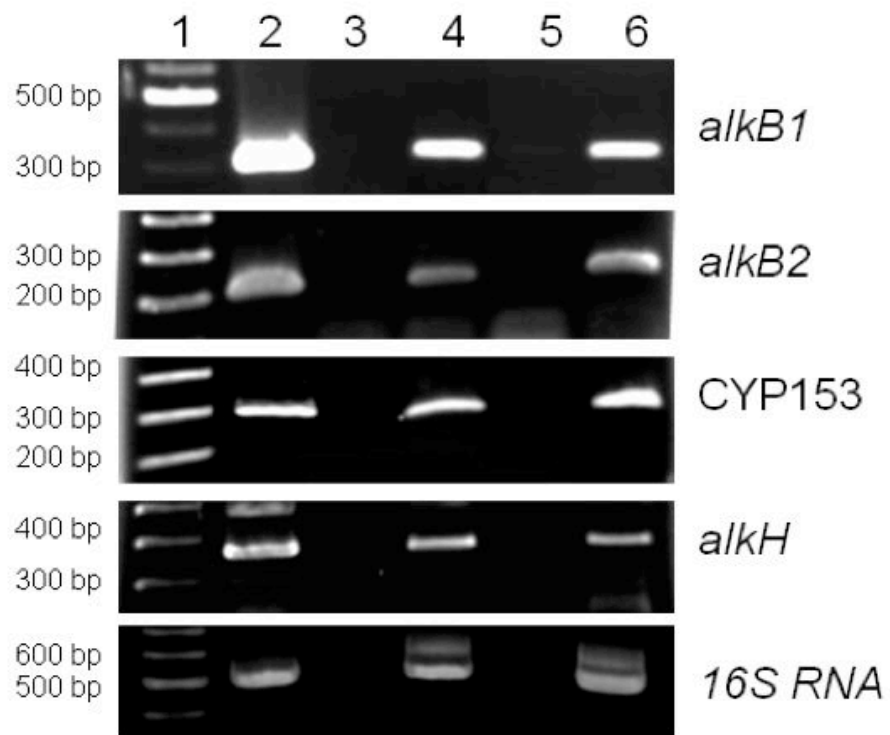


Fig. 3, Nicolau et al.

