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Diversity of Alkane Hydroxylase Systems in the Environment

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Résumé — Diversité des systèmes alcane hydroxylase dans l'environnement — La première étape dans la dégradation aérobie des alcanes par les bactéries, les levures et les champignons est catalysée par des oxygénases, une classe d'enzymes capables d'introduire des atomes d'oxygène issus de l'oxygène moléculaire dans le substrat alcane. Ces enzymes jouent un rôle important dans la biodégradation du pétrole et dans la biodégradation cométabolique de composés tels que le trichloroéthylène et les éthers-carburants. De plus, ce sont des biocatalyseurs très utiles qui peuvent également servir de modèles pour caractériser une réaction chimique difficile : l'activation régio- et stéréospécifique de la liaison C-H. Plusieurs autres classes d'enzymes catalysent l'oxydation des alcanes. Les souches de levures capables de dégrader les alcanes contiennent plusieurs alcanes hydroxylases appartenant à la superfamille des P450, alors que différentes bactéries contiennent des enzymes similaires au système alcane hydroxylase membranaire de *Pseudomonas putida* GPo1. Les alcanes à courte chaîne sont probablement oxydés par des alcanes hydroxylases solubles similaires aux méthanes monooxygénases. La présence dans l'environnement de ces oxygénases a été étudiée dans des échantillons de sols et aquifères uniquement pour les alcanes hydroxylases associés aux membranes.

Abstract — Diversity of Alkane Hydroxylase Systems in the Environment *— The first step in the aerobic degradation of alkanes by bacteria, yeasts, and fungi is catalyzed by oxygenases. These enzymes, which introduce oxygen atoms derived from molecular oxygen into the alkane substrate, play an important role in oil bioremediation and in the cometabolic degradation of compounds such as trichloroethylene and fuel oxygenates. In addition, they are useful biocatalysts and important models for a difficult chemical reaction: the regio- and stereospecific activation of C-H bonds. Several unrelated enzyme classes catalyze the oxidation of alkanes. Alkane-degrading yeast strains contain multiple alkane hydroxylases belonging to the P450 superfamily, while many bacteria contain enzymes related to the* Pseudomonas putida *GPo1 membrane-bound alkane hydroxylase system. Short-chain alkanes are probably oxidized by alkane hydroxylases related to the soluble and particulate methane monooxygenases. Only the membrane-bound enzymes have been studied with respect to their prevalence in environments such as soils or aquifers.*

INTRODUCTION

Saturated hydrocarbons such as linear, branched, and cyclic alkanes are highly reduced forms of carbon that are produced by geochemical processes from decaying plant and algal material. Alkanes constitute about 20-50% of crude oil, depending on the source of the oil. In addition, alkanes (predominantly long-chain compounds) are produced throughout the biosphere by living organisms (plants, algae and bacteria) as a waste product, a structural element, a defense mechanism, or as a chemoattractant. The assimilation of biogenic alkanes is a major process in geochemical terms, but has not been quantified. Recycling of alkanes from natural oil seepage, oil-spills and runoff due to countless dispersed sources has been estimated to amount to several million tons per year [1, 2].

The microbial biodegradability of alkanes by microorganisms is well established. Almost a century ago, Söhngen was able to isolate bacteria that degrade methane [3] and longer alkanes [4]. Later in the 20th century, alkanes were considered for use as a feedstock for the production of single cell protein. This led to a flood of research in the fifties and sixties, which ended abruptly in response to the oil-crisis in 1973. Alkane-degrading microbes were also investigated by the oil industry for many other purposes, such as petroleum exploration (the presence of alkane degraders was thought to indicate the presence of oil- or gas fields), the treatment of environmental pollution, deparaffinization of jet-fuel and diesel oil to reduce viscosity, microbially enhanced oil recovery, and because of corrosion of fuel lines or oil tanks due to bacteria able to grow on oil or petroleum (see for example [5-7], and references therein). More recently, alkane hydroxylase genes received increasing attention as markers to predict the potential of different environments for oil degradation (see references in Table 2). An emerging theme is the cometabolic degradation of recalcitrant chemicals such as methyl *tert*-butyl ether (MTBE) or trichloroethylene (TCE) by bacterial strains growing on short-chain alkanes [8, 9]. Methane oxidation has often been regarded as a special case of alkane utilization, and was treated separately [10]. However, it now appears that at least some of the enzymes that are involved in the degradation of short-chain alkanes are very similar to methane monooxygenases [11]. Therefore, some information on these enzymes is included in this review. For a more detailed overview on the molecular biology and biochemistry of methane monooxygenases see Murrell *et al.* [12].

Alkanes are also known as paraffins (which can be translated as "not enough affinity") as they are chemically quite inert [13]. For biodegradation, the alkane molecules have to be activated to allow further metabolic steps. In the absence of oxygen, this is done by the addition of a C_1 (CO_2) or a C_4 (fumarate) compound [14]. If oxygen is available, the initial activation step is carried out by oxygenases: enzymes that introduce oxygen atoms derived from molecular oxygen in the alkane substrate [15]. As the regio- and stereoselective oxidation of nonactivated methyl or methylene-group is a difficult but important chemical reaction, alkane hydroxylases are also studied as model systems for biomimetic catalysts [13]. From a biotechnological perspective, alkane hydroxylases are versatile biocatalysts, which carry out a wide range of useful oxidation reactions [16, 17]. This review summarizes current knowledge on the diversity of alkane hydroxylase systems, their occurence in the environment, and their applications in biocatalysis and bioremediation.

1 AEROBIC ALKANE DEGRADATION PATHWAYS

Many microbial genera include strains that are able to grow on alkanes. Table 1 lists a small selection of genera that include strains from which genes involved in alkane degradation were cloned, or genera that are often mentioned in alkane degradation studies. Alkanes are usually activated by terminal oxidation to the corresponding primary alcohol, which is further oxidized by alcohol and aldehyde dehydrogenases. The resulting fatty acids enter the β-oxidation cycle (for general reviews see [6, 10, 18]). Short-chain alkanes are metabolized via terminal as well as subterminal oxidation [19], and many methanotrophs co-oxidize short-chain alkanes at terminal as well as subterminal positions [7, 20]. Subterminal oxidation has also been detected for longer alkanes, *e.g.* in *Penicillium*, *Bacillus*, *Pseudomonas* [10], and more recently in *Rhodococcus* sp. Q15 [21]. The secondary alcohols are converted to the corresponding ketone [22], which is oxidized by a Baeyer-Villiger monooxygenase to an ester [23-25]. The ester is subsequently hydrolyzed by an esterase to an alcohol and a fatty acid [19, 26]. In some cases, both ends of the alkane substrate are oxidized, which has been exploited for the production of dicarboxylic acids by yeasts as well as bacteria [27, 28] *(Fig. 1).*

2 DIVERSITY OF ALKANE HYDROXYLASE SYSTEMS

Several unrelated enzyme classes that carry out the oxidation of alkanes have now been characterized (Table 2). A membrane-bound alkane hydroxylase was first discovered in a hexane-degrading fluorescent pseudomonad tentatively named Pseudomonas oleovorans [39, 40], but later identified as a P. putida [41]. Biochemical studies showed that the P. putida alkane hydroxylase system consists of an integral membrane monooxygenase [42-44], 1 or 2 rubredoxins [45-47], and a rubredoxin reductase [48, 49] (Fig. 2). Rubredoxin reductase transfers electrons from NADH (nicotinamide adenine dinucleotide) via its cofactor FAD (flavin adenine dinucleotide) to rubredoxin, which transfers

TABLE 1

Microorganisms that aerobically degrade aliphatic hydrocarbons

Bacteria	Yeasts	Fungi	Algae
Achromobacter	*Candida* (+)	*Aspergillus*	*Prototheca*
Acinetobacter (+)	*Cryptococcus*	*Cladosporium*	
Alcanivorax (+)	*Debaryomyces* (+)	*Corollasporium*	
Alcaligenes	*Hansenula*	*Cunninghamella*	
Bacillus	*Pichia*	*Dendryphiella*	
Brevibacterium	*Rhodotorula*	*Fusarium*	
Burkholderia (+)	*Saccharomyces*	*Gliocladium*	
Corynebacterium	*Sporobolomyces*	*Lulworthia*	
Flavobacterium	*Torulopsis*	*Penicillium*	
Mycobacterium (+)	*Trichosporon*	*Varicospora*	
Nocardia	*Yarrowia* (+)	*Verticillium*	
Pseudomonas (+)			
Rhodococcus (+)			
Sphingomonas			
Streptomyces			

Information mainly from [7, 18]. A (+) indicates that information on the genetics of alkane degradation is available for strains belonging to the genus.

the electrons to the alkane hydroxylase. Recently, enzyme systems related to the *P. putida* GPo1 alkane hydroxylase were cloned from bacterial strains belonging to several different genera [34, 50, 52-54]. While the *P. putida* GPo1 system acts on alkanes ranging from pentane to dodecane (C_5-C_{12}) [42, 55], most related membrane-bound alkane hydroxylases solely hydroxylate alkanes containing more than 10 carbon atoms [34] (see below).

Several cytochrome P450 isozymes involved in alkane assimilation were recently cloned from the yeasts *Candida maltosa*, *Yarrowia lipolytica* and *Debaryomyces hansenii* [29, 56, 57]. They belong to the CYP52 gene family of the microsomal class II P450s, and receive electrons from NADPH (nicotinamide adenine dinucleotide phosphate) via FAD- and FMN-containing reductases. The CYP52 enzymes were found to have slightly different substrate specificities: some act primarily on C_{12} or C_{16} alkanes, while other enzymes also oxidize fatty acids at the ω-position. The CYP52 gene family may play a significant role in the degradation of alkanes in oil-contaminated environments as yeast and fungi have been shown to overgrow bacteria in sandy soil contaminated with C_{10}-C_{15} *n*-alkanes [58].

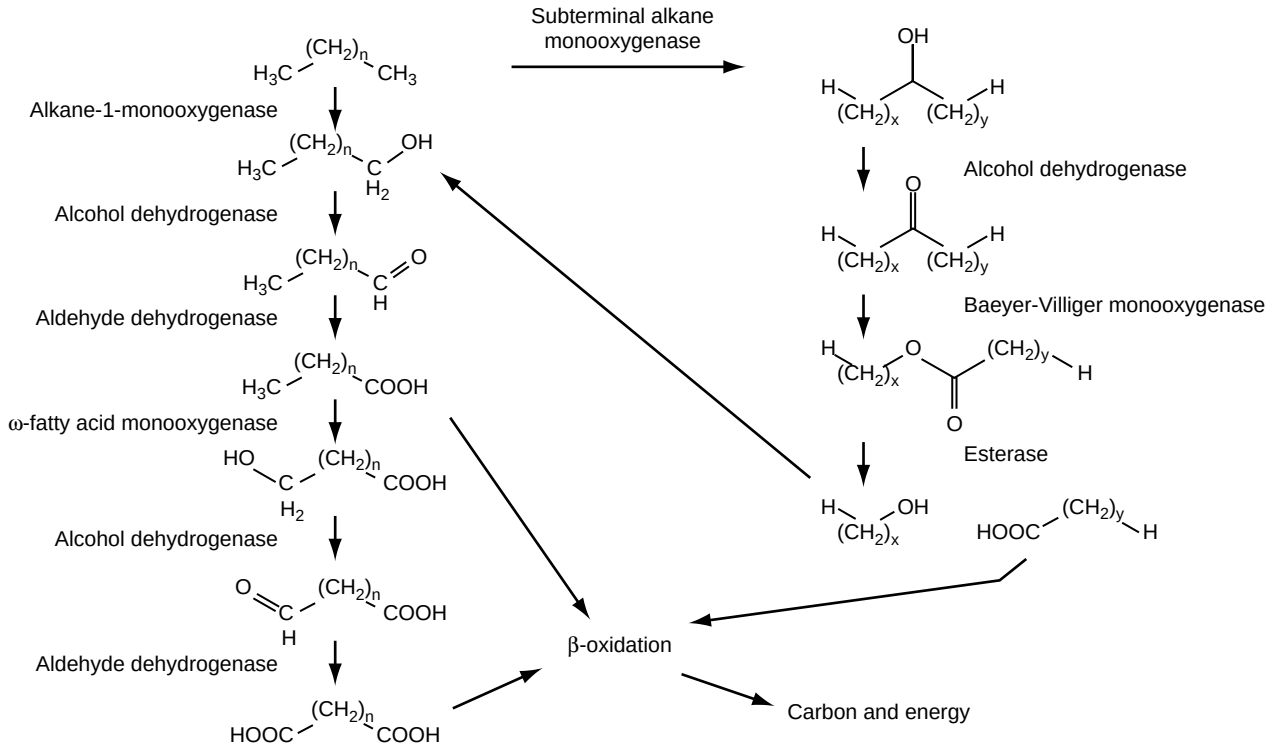

Figure 1

Pathways for the degradation of alkanes by terminal, sub- and biterminal oxidation. Terminal oxidation leads to the formation of fatty acids, which enter the β-oxidation pathway. Alternatively, ω-hydroxylation by a fatty acid monooxygenase or alkane hydroxylase may take place, leading to dicarboxylic acids. Subterminal oxidation gives rise to secondary alcohols, which are oxidized to ketones. A Baeyer-Villiger monooxygenase converts ketones to esters, which are subsequently cleaved by an esterase.

TABLE 2

Enzyme classes involved in the oxidation of alkanes

Enzyme class	Composition and cofactors	Substrate range	Presence shown in:	References
Eukaryotic P450 (CYP52, class II))	Microsomal oxygenase: P450 heme reductase: FAD, FMN, NADPH	C_{10}-C_{16} *n*-alkanes	*Candida maltosa, Candida tropicalis, Yarrowia lipolytica*	[29]
Bacterial P450 oxygenase systems (CYP153, class I)	P450 oxygenase: P450 heme ferredoxin: iron-sulfur ferredoxin reductase: FAD, NADH	C_4-C_{16} *n*-alkanes	*R. rhodochrous* 7E1C *Acinetobacter* sp. EB104	[11, 30, 31]
Butane monooxygenase (similar to pMMO)	Two polypeptides: copper, iron quinone oxidoreductase: FAD, NADH	Probably C_4-C_{10} *n*-alkanes	Short-chain alkane oxidizers, *Nocardioides* sp. CF8	[11, 12, 32]
Butane monooxygenase (similar to sMMO)	$\alpha 2\beta 2\gamma 2$ hydroxylase: bi-nuclear iron reductase: [2Fe-2S], FAD, NADH regulatory subunit	C_2-C_8 *n*-alkanes	*Pseudomonas butanovora*	[11, 12, 33]
AlkB-related alkane hydroxylases	Membrane hydroxylase: bi-nuclear iron rubredoxin: iron rubredoxin reductase: FAD, NADH	C_5-C_{16} *n*-alkanes	*Acinetobacter, Alcanivorax, Burkholderia, Mycobacterium, Pseudomonas, Rhodococcus, etc.*	[34]
Dioxygenase	Homodimer: copper, FAD	C_{10}-C_{30} *n*-alkanes	*Acinetobacter* sp. M-1	[35]
Unknown	54 kDa peptide essential for butane oxidation	C_4-C_{10} *n*-alkanes	*Pseudomonas indica* IMT37	[36, 37]
Intermolecular dioxygenase	Unknown (ratio of hydrocarbon to oxygen consumption is 2:1)	Propane, isobutane	*Rhodococcus rhodochrous*	[38]

Reports on bacterial cytochrome P450 systems involved in alkane degradation are quite scarce. Cardini and Jurtshuk described a *Corynebacterium* sp. 7E1C (in recent literature known as *Rhodococcus rhodochrous*) that contains an octane hydroxylating cytochrome P450 enzyme [59]. Another cytochrome P450 enzyme involved in alkane hydroxylation was isolated [60] and recently cloned from *Acinetobacter calcoaceticus* EB104 [30]. As this P450 showed less than 40% sequence identity to other sequences in the P450 superfamily it was assigned to a new bacterial class I P450s family named CYP153. Electron transfer to the bacterial class I enzymes is mediated by ferredoxin reductase, an FAD-containing protein which accepts electrons from NAD(P)H, and ferredoxin, an iron-sulfur protein which transfers the electrons to the P450 monooxygenase [61].

Butane-utilizing strains were shown to contain several unrelated alkane hydroxylases, based on responses to ethylene (which inactivates cytochrome P450 enzymes by alkylating the heme group), acetylene (a mechanism-based inactivator of soluble methane monooxygenases (sMMO), membrane-bound methane monooxygenases (pMMO) and ammonia monooxygenase (AMO)) and allyl thiourea (which chelates copper, and reversibly inhibits pMMO and AMO) [11]. One of these strains, *Nocardioides* sp. CF8, was shown to contain a membrane-bound alkane hydroxylase related to the *P. putida* GPo1 AlkB, and a copper-dependent light sensitive enzyme similar to pMMO [32]. A butane monooxygenase recently cloned from *Pseudomonas butanovora* is quite similar to sMMO [12, 33]; its protein A α and β subunits, and protein B show more than 60, 50, and 40% amino acid sequence identity, respectively, to the corresponding subunits of sMMOs.

A gene which allows isolates of a novel *Pseudomonas* sp. named *P. indica* IMT37 [36] to grow on butane had no detectable homology or similarity with other alkane hydroxylase systems [37]. Here, further biochemical work is necessary to elucidate the role of the encoded peptide in butane oxidation. The same holds for a new type of intermolecular dioxygenase, which appears to oxidize two molecules of propane with one molecule of molecular oxygen [38]. A copper-dependent alkane dioxygenase was reported for *Acinetobacter* sp. M-1 [35], a strain that was later shown to contain two membrane-bound alkane hydroxylases related to *P. putida* GPo1 AlkB as well [54].

3 DIVERSITY OF MEMBRANE-BOUND ALKANE HYDROXYLASE SYSTEMS

Biochemical studies and molecular genetic analysis of the *P. putida* GPo1 alkane hydroxylase system have led to a detailed understanding of C_5-C_{12} *n*-alkane metabolism by this strain [40, 41], while other alkane-degraders (except for the methanotrophs) were studied in much less detail. For example, even though hydrocarbon utilization is very common amongst strains belonging to the *Corynebacterium-Mycobacterium-Nocardia* (CMN) complex, until recently only a few CMN strains, such as the *R. rhodochrous* strains

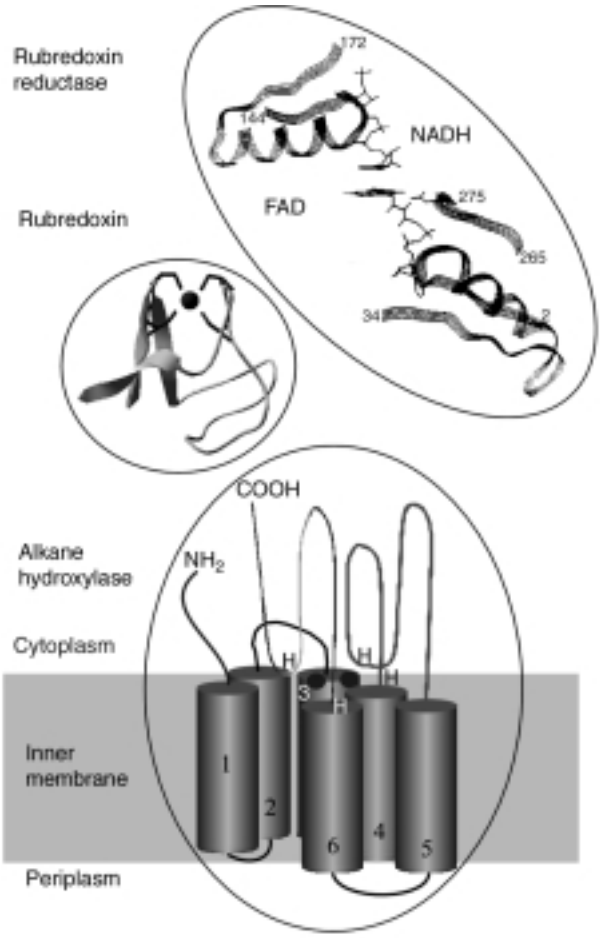

Figure 2

Structure of membrane-bound alkane hydroxylase systems. The membrane-bound alkane hydroxylases possess 6 transmembrane helices [44], and 4 conserved histidine-rich motifs (H) [50, 51]. The 2 iron atoms in the active site are marked (•). Rubredoxin contains an iron atom marked (•) that is liganded by 4 cysteines. Several strains contain more than 1 rubredoxin, or larger proteins consisting of N-terminal and C-terminal rubredoxin domains connected by a linker [47]. Rubredoxin reductase is an FAD-containing NADH-dependent reductase [49].

B29 and 7E1C, were studied in some detail [59, 62]. Several fluorescent pseudomonads that were studied in the sixties and seventies [63-66] were later shown to contain alkane hydroxylase systems that were virtually identical to the *P. putida* GPo1 enzyme [67].

In 1998, a membrane-bound alkane hydroxylase that was quite distantly related to the *P. putida* GPo1 AlkB was cloned from *Acinetobacter* sp. ADP1, and shown to act on C_{12} and longer *n*-alkanes [52]. The genome sequence of *Mycobacterium tuberculosis* H37Rv [68] was also found to encode an AlkB-homolog, which was later shown to be functional by heterologous expression [34]. Comparison of the three alkane hydroxylase sequences showed that four histidine-containing motifs were well conserved, three of which were also present in the distantly related membrane-bound desaturases [51]. Based on two of the motifs, highly degenerate primers were developed that amplified internal gene fragments of *alkB* homologs from gram-negative as well as gram-positive strains [32, 50, 53, 69] (*Table 3*). In the initial PCR study, most strains that were able to grow on alkanes yielded *alkB* gene fragments [50], and the same was found for a set of *Rhodococcus* strains isolated from soil in Bremen, Germany [86, 87]. However, in a collection of 17 hexane-degrading strains isolated from a trickling bed bioreactor that removed hexane from an airstream [88], only 5 strains possessed an *alkB*-homolog [86]. This suggests that many strains able to degrade shorter alkanes possess enzyme systems that are unrelated to the membrane-bound alkane hydroxylase of *P. putida* GPo1. On the other hand, many strains (especially *Rhodococcus* and *P. aeruginosa* strains) possess multiple—quite divergent—alkane hydroxylases [67, 86, 89] (*Fig. 3, Table 3*).

The *alkB* homologs amplified from the different strains showed high sequence divergence: the branches of a distance tree based on the amino-acid translations of the DNA sequences obtained by the PCR approach, molecular cloning, and genome sequencing had sometimes only 40% sequence identity *(Fig. 3)*. To demonstrate that the *alkB* gene homologs indeed encode functional enzymes, special expression vectors [91] and host strains for functional heterologous expression of alkane hydroxylases [34] were constructed. The host strains (*Pseudomonas fluorescens* KOB2Δ1, an *alkB* knockout derivative of *P. fluorescens* CHA0, and *P. putida* GPo12(pGEc47ΔB), a derivative of *P. putida* GPo1 lacking the *alkB* gene) supply the electron transfer components rubredoxin and rubredoxin reductase, the enzymes that carry out further metabolic steps leading to fatty acids, and, in the case of *P. fluorescens* KOB2Δ1, also an uptake system for alkanes longer than C_{12}. The host-strains made functional expression of alkane hydroxylases from strains as diverse as *Mycobacterium tuberculosis*, *Prauserella rugosa*, *Rhodococcus erythropolis*, *Burkholderia cepacia*, *Pseudomonas aeruginosa*, and *Alcanivorax borkumensis* possible [34, 53, 89] (marked by "+" in *Fig. 3*). However, they only allow functional analysis of enzymes that oxidize *n*-alkanes ranging from C_5-C_{16}; alkane hydroxylases which oxidize longer alkanes could not be investigated yet. Additional membrane-bound alkane hydroxylases were cloned from *Acinetobacter* sp. strain M-1 and *A. calcoaceticus* EB104, and shown to be functional by expression in an *alkM* knockout strain of *Acinetobacter* sp. ADP1 [50, 54]. Several genome sequences *(e. g. Burkholderia mallei, P. aeruginosa, Legionella pneumophilia, M. tuberculosis, Mycobacterium bovis*) that have recently become available also contain *alkB* homologs (marked by "*" in *Fig. 3)*. Most of these bacteria are

TABLE 3.

Bacterial strains that contain homologs of the *P. putida* GPo1 *alkB* gene

Strain	Gowth on *n*-alkanes	Number of *alkB* homologs	Homologs functional?	References
Acinetobacter sp. ADP1	C_{12}-C_{16}	1	yes	[52, 70]
A. calcoaceticus 69-V	C_{11}-C_{18}	1	n. t.	[50, 71]
A. calcoaceticus EB104	C_{6}-C_{18}	1	yes	[50, 71]
A. calcoaceticus NCIB 8250	C_{8}-C_{16}	1	n. t.	[50, 72]
Acinetobacter sp. 2796A	C_{10}-C_{16}	1	n. t.	[50]
Acinetobacter sp. M-1	C_{13}-C_{44}	2	yes	[54]
Alcanivorax borkumensis AP1	C_{10}-C_{16}	2	yes	[34, 73]
A. borkumensis SK2	C_{10}-C_{16}	2	n. t.	[73]*
Burkholderia cepacia ATCC 25416	C_{10}-C_{16}	1	n. t.	[50, 74]
B. cepacia RR10	C_{10}-C_{16}	1	yes	[53]
B. mallei	C_{10}-C_{16}	1	n. t.	[74]**
B. pseudomallei	C_{10}-C_{16}	1	n. t.	[74]**
Oleiphilus messinensis ME102	obligate	1	n. t.	[75]
Pseudomonas aureofaciens RWTH 529	C_{10}	1	n. t.	[76]
Pseudomonas sp. 7/156	n. d.	1	n. t.	[77]
Pseudomonas putida GPo1	C_{5}-C_{12}	1	yes	[43, 78]
P. putida P1	C_{8}	1	yes	[41, 50]
Pseudomonas fluorescens CHA0	C_{12}-C_{28}	1	yes	[34, 79]
Pseudomonas aeruginosa PAO1	C_{12}-C_{16}	2	yes	[34, 80]
P. aeruginosa PG201	C_{10}-C_{16}	2	n. t.	[81]
P. aeruginosa KSLA473	C_{5}- C_{16}	3	yes	[67, 82]
P. aeruginosa NCIMB 8704	C_{8}-C_{16}	3	yes	[34, 67]
P. aeruginosa NCIMB 9571	C8-C_{16}	3	yes	[34, 67]
P. aeruginosa ATCC 17423	C8-C_{16}	3	yes	[34, 67]
Mycobacterium avium	paraffins	1	n. t.	[83]**
M. avium subsp. *paratuberculosis*	paraffins	1	n. t	[83]**
M. bovis BCG	C_{12}-C_{16}	1	n. t.	[84]
M. smegmatis	C_{9}-C_{16}	1	n. t.	[84]**
M. tuberculosis H37Rv	C_{11}-C_{16}	1	yes	[34, 68, 84]
Nocardioides sp. *CF8*	C_{2}-C_{16}	1	n. t.	[32]
Prauserella rugosa NRRL B-2295	C_{8}-C_{14}	1	yes	[34, 85]
Rhodococcus sp. 1BN	C_{6}-C_{28}	1	n. t.	[69]
Rhodococcus erythropolis Q15	C_{8}-C_{32}	4	1 yes, 3 no	[21]
R. erythropolis 35-O	C_{6}-C_{16}	5	n. t.	[86]
R. erythropolis 42-O	C_{6}-C_{32}	5	n. t.	[86]
R. erythropolis 62-O	C_{6}-C_{16}	5	n. t.	[86]
R. erythropolis 23-D	C_{6}-C_{36}	5	n. t.	[86]
R. erythropolis 50-V	C_{6}-C_{32}	4	n. t.	[86]
R. erythropolis NRRL B-16531	C_{6}-C_{36}	4	1 yes, 3 no	[85]
Rhodococcus fascians 115-H	C_{6}-C_{32}	3	n. t.	[86]
R. fascians 154-S	C_{6}-C_{24}	4	n. t.	[86]

* J.B. van Beilen, unpublished data.
** data from unfinished genome sequence.

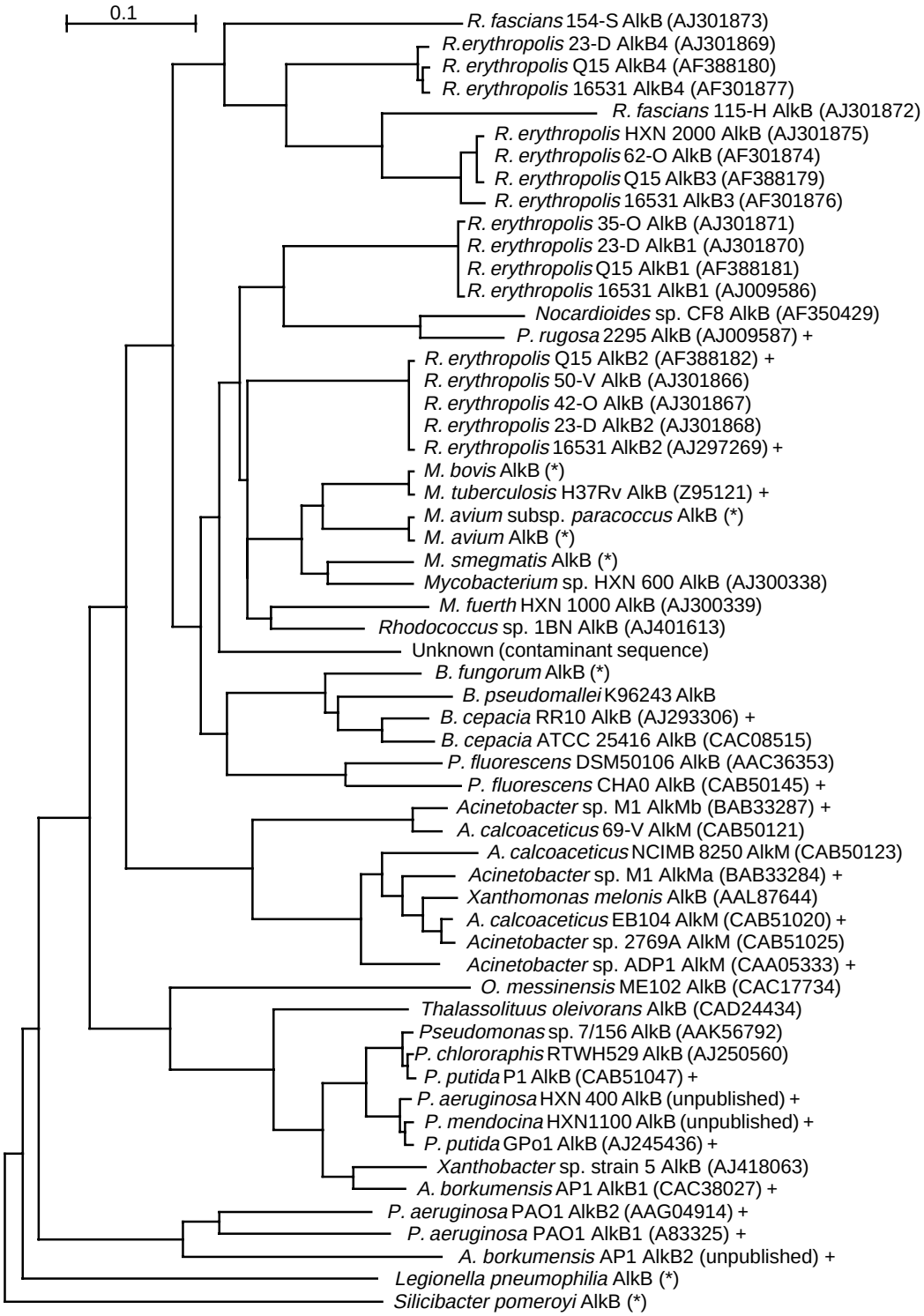

Figure 3

Unrooted phylogenetic tree based on an alignment of partial amino acid sequences of (putative) membrane-bound alkane hydroxylases. The distance tree was generated by ClustalX [90] from a manual alignment of the (putative) alkane hydroxylases. Only the segment corresponding to the 550 basepair fragments obtained in PCR experiments with highly degenerate primers based on histidine box 1 and histidine box 4 were used for the alignment. Alkane hydroxylases that were shown to be functional by heterologous expression or gene knockouts are indicated by (+), while sequences from unfinished genome sequences are indicated by (*). Scale bar, 0.1 substitution per amino-acid site.

facultative pathogens that may utilize alkanes as a carbon-source in environments such as soil or water.

A careful inspection of the distance tree shows that there is no clear linkage between the diversity of the *alkB* genes and phylogenetic lines *(Fig. 3)*. For example, AlkB homologs from fluorescent pseudomonads are almost as divergent as the entire collection, while the four AlkB-homologs in *R. erythropolis* NRRL B-16531 and Q15 are as divergent as the whole set of sequences from gram-positive strains [89]. Some strains contain multiple AlkB sequences that are on completely different branches—*A. borkumensis*, and several *P. aeruginosa* strains that are able to grow on *n*-octane as well as the longer *n*-alkanes (Smits *et al.*, submitted; van Beilen, unpublished data). On the other hand, the *Burkholderia* and *P. fluorescens* sequences are so closely related to some sequences from gram-positive strains that cross-reactions in Southern and dot-blots are possible (>70% DNA sequence identity). In part, the failing link between phylogenetic lineage and AlkB-diversity is clearly due to horizontal gene transfer. For example, the *P. putida* GPo1 *alk*-genes are located on a large broad host-range plasmid named OCT [92], while the closely related *P. putida* P1 *alk*-genes are located on a class I transposon [41].

Our present knowledge on the structure-function relationship of the class of integral membrane alkane hydroxylases is limited to the folding topology [44] and the likely involvement of conserved histidines in binding of the 2 active-site iron atoms [51]. The AlkB sequences show significant sequence divergence, but the membrane folding of these alkane hydroxylases appears to follow the same pattern: the 6 hydrophobic stretches that are likely to span the cytoplasmic membrane [51] *(Fig. 2)* are highly conserved in all sequences, while the same is true for the conserved histidines. It is not yet possible to identify residues involved

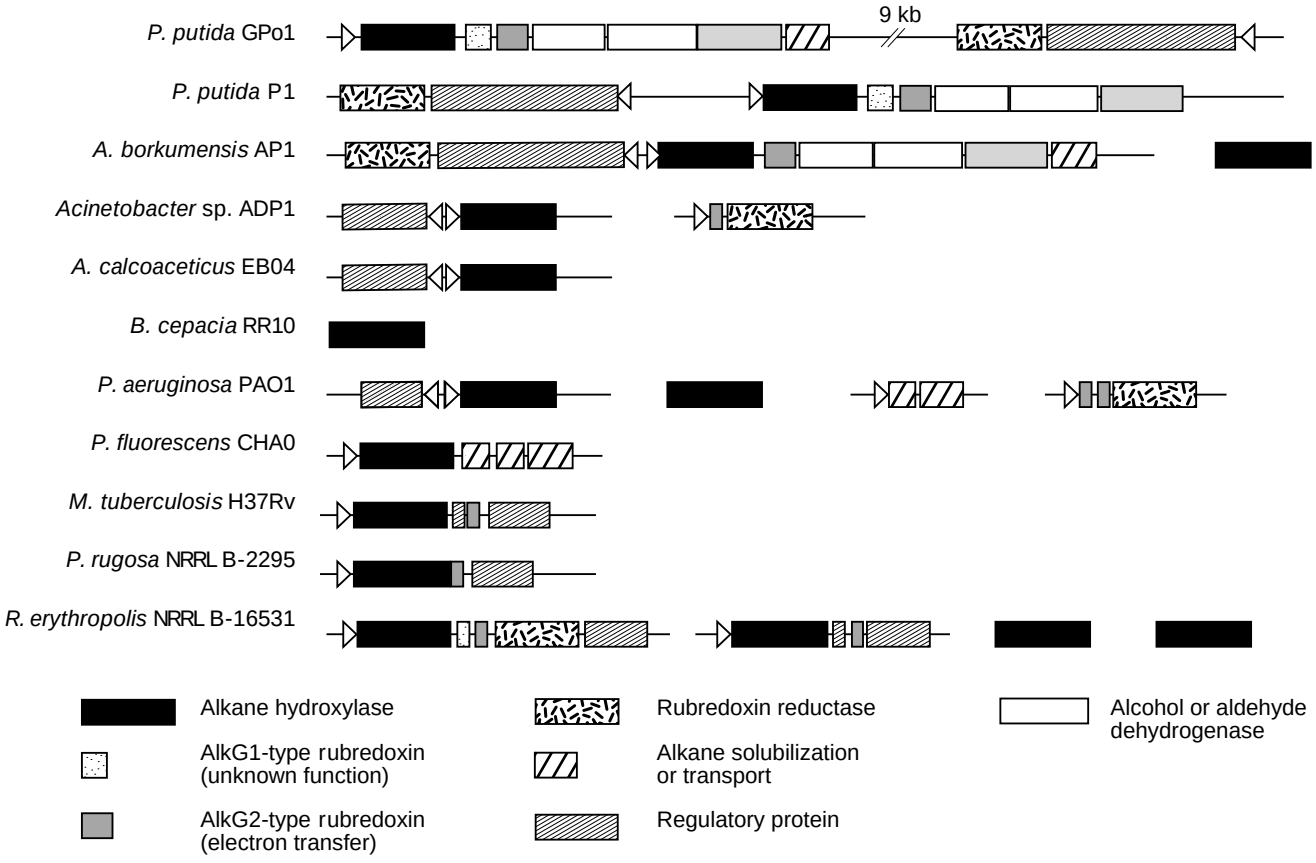

Figure 4

Organization of *alk* genes in different microorganisms. The function of the encoded proteins is indicated below. If the ORFs are linked by a line, the sequence is contiguous. If the ORFs are not linked by a line, the genes are located elsewhere on the chromosome. The triangle indicates the direction of transcription.

in other aspects of alkane hydroxylase function. Residues that are involved in binding of rubredoxin are likely to be conserved between all alkane hydroxylases, as rubredoxins can be exchanged between alkane hydroxylase systems from all strains tested [47]. However, these residues cannot be distinguished from other conserved residues. Similarly, the three medium chain-length alkanes hydroxylases from *P. putida* GPo1, *P. putida* P1, and *A. borkumensis* AP1 are too closely related to each other to distinguish residues conserved by chance from residues conserved because of functional constraints.

The organization of genes involved in alkane oxidation varies strongly among the different alkane degrading bacteria *(Fig. 4).* In most strains, genes involved in alkane degradation seem to be distributed over the genome. Except in the case of *R. erythropolis* [89], none of the rubredoxin reductase genes is located close to an alkane hydroxylase, perhaps because they are also involved in other pathways, and require a different type of regulation. In contrast, most rubredoxin genes are located immediately downstream of the alkane hydroxylase genes. Those that are located elsewhere are encoded directly upstream of rubredoxin reductase genes. Interestingly, the *alk*-gene cluster around *alkB1* of *A. borkumensis* (a recently discovered seawater bacterium that only grows on alkanes [73]) is very similar to the *alk* gene clusters of *P. putida* strains GPo1 and P1, with respect to gene organization as well as sequence. The *P. putida alk* genes have a significantly lower G+C content than the rest of the genome, and are encoded on a putative catabolic transposon [41]. These comparisons suggest that the *A. borkumensis alk* genes may have ended up in this strain by horizontal gene transfer as well. Interestingly, the alkane hydroxylase genes are accompanied by alcohol and aldehyde dehydrogenase genes only in these strains *(Fig. 4).*

4 DETECTION OF ALKANE HYDROXYLASE GENES IN THE ENVIRONMENT

Most of the enzyme systems described above have been discovered recently, and evidence that similar systems are present in other bacteria is still scarce. A gene probe based on the IMT37 sequence essential for butane oxidation detected

TABLE 4

Studies on the occurrence, abundance, and/or diversity of *alkB*-homologues in different environments or strain collections

Environment/source	Methods	Conclusions for *alkB* homologs	References
Alaskan sediments	Colony hybridization with *alkB* gene probe	39% of viable heterotrophs from uncontaminated soil contain *alkB* 67% of viable heterotrophs from contaminated soil contain *alkB*	[97]
Contaminated soil	PCR followed by Southern blot with *alkB* gene probe	Detection and quantification of *alkB* (and other genes) in soil: 1-10 gene copies per gram of soil can be detected	[98
Variety of cold ecosystems	PCR, and Southern blots with *alkB* gene probe	None of the psychrophilic alkane degraders possess genes with high homology to *P. putida alkB*	[99]
Fuel oil-contaminated site	Dot-blots	DNA extracted from soil shows no significant hybridization with an *alkB*-gene probe	[100]
Shallow aquifer	Southern blots with *alkB* gene probe	10-20% of the total bacterial community hybridizes with an *alkB* gene probe	[101]
Various sources (54 bacterial strains)	PCR with highly degenerate primers for *alkB* homologs	Most alkane-degrading strains contain distantly related *alkB* homologs (homology not sufficient for Southern or dot-blots)	[50]
Shallow aquifer (natural attenuation site)	Dot-blots with DNA extracted from aquifer samples	*alkB* genotypes start at 11% of total community, and peak at 52% after injection of synthetic jet fuel in the aquifer	[102]
Various sources (54 bacterial strains)	Southern, colony and dot-blots, PCR followed by Southern	*alkB* genes (close homologs) are widespread only in short-chain *n*-alkane degrading pseudomonads	[76]
Rhizosphere *vs.* bulk soil	Multiplex PCR	At a contaminated site *alkB* was 10 times more prevalent in the endophytic community compared to the bulk soil community	[103]
Propane and butane-utilizing bacteria	Dot-blots	8 of 15 strains (including pseudomonads and rhodococci) gave a strong signal, and 7 a weak signal with the IMT37 gene	[37]
Land treatment unit (LTU)	PCR, terminal restriction fragment length polymorphism	*alkB* increased in abundance during the first 3 weeks of LTU operation, and comprised > 80% of the total PCR products	[104]
Arctic and Antarctic soil	PCR-hybridization and colony hybridization	Rhodococcal *alkB* genes occur in contaminated and pristine soils, *P. putida alkB* occurs more frequently in contaminated soils	[105]

similar genes in other butane degraders (*pseudomonads, rhodococci* and unidentified bacteria). In a dot-blot experiment, 8 out of 15 propane or butane-degrading bacteria gave a strong signal with the gene-probe, while the remaining 7 gave a weak but detectable signal [37].

The prevalence in the environment of the CYP153 P450 alkane hydroxylase family, the first member of which was recently cloned from *Acinetobacter* sp. EB104 [30], and the yeast CYP52 P450 family, has not been investigated yet. However, hexane or octane inducible cytochrome P450s were detected in several *Rhodococcus*, *Bacillus megaterium* and *Candida apicola* strains [59, 93]. Indications that the butane monooxygenases studied by Arp and coworkers [11, 32, 33] are also widespread come from studies on the cometabolic degradation of TCE and other chlorinated compounds by bacteria growing on short-chain alkanes [9, 11]. Soluble and particular MMOs are known to oxidize the same compounds [20], and are ubiquitous in many environments (see for example [94-96]). This also implies that some of the gene diversity detected with primers that amplify pMMO and sMMO genes may well be due to short-chain alkane-degrading bacteria instead of methanotrophs.

Because the sequence of the *P. putida* GPo1 *alkB* gene was already published in 1989 [43], several research groups have used this gene as a probe in ecological studies, with conflicting results *(Table 4)*. While in some studies *alkB* homologs were detected in 10-40% of the bacterial population [97, 101, 102], other groups did not detect *alkB* homologs at all [99, 100]. AlkB homologs that are closely related to the *P. putida* GPo1 enzyme appear to be common in gram-negative strains only (probably pseudomonads) [76]. This is supported by the observation that genes almost identical to the *P. putida* GPo1 *alkB* gene were found in several *P. putida*, *P. aeruginosa*, and *P. mendocina* strains [67, 87], but not in other strains. An exception is the *alkB1* gene found in *A. borkumensis* AP1, which is sufficiently closely related to the *P. putida alkB* genes for cross-reactions in Southern blots. This supports the notion that horizontal gene transfer has played a role in spreading this genotype [34]. *Rhodococcal alkB* homologs were only cloned recently [50, 86, 89], but first reports indicate that these genotypes are widespread in Arctic and Antarctic soils, and in other environments [86, 105].

5 APPLICATIONS OF ALKANE HYDROXYLASE SYSTEMS

A strong incentive for research on alkane hydroxylases was the early observation that the *P. putida* alkane hydroxylase oxidizes alkenes to epoxides [106], while *P. aeruginosa* KSLA 473 (which contains an enzyme system that is identical to the *P. putida* GPo1 alkane hydroxylase [67]) hydroxylates a wide range of branched and cyclic alkanes, and alkylbenzenes [107]. *P. putida* GPo1 was found to oxidize many other substrates as well (including ethyl-substituted heterocyclic compounds, allyl phenyl or allyl benzyl ethers, thioethers, branched alkanes, etc.) with excellent regio- and/or stereoselectivity [16, 55, 108-111]. Screening of a collection of alkane-degrading strains for the regio- and stereoselective hydroxylation of *N*-benzyl-pyrrolidine showed that 25-30% of the strains oxidized this substrate to *N*-benzyl-3-hydroxypyrrolidine with varying enantiomeric excess [110]. The best strain in this study was a hexane-degrading *Sphingomonas* sp. strain HXN200, isolated from the trickling-bed filter mentioned earlier [88]. In subsequent work, *Sphingomonas* sp. strain HXN200 was shown to hydroxylate many 4-, 5,- and 6-ring alicyclic compounds with rates of up to 10-20 μmol min^{-1} g^{-1} cell dry weight [17, 112, 113]. The stereoselective oxidation of isopropyl moieties by alkane-grown *Rhodococcus* and *Pseudomonas* strains has also been studied in some detail [85, 114, 115].

A new application area of alkane hydroxylases is the cometabolic degradation of TCE, MTBE, and related compounds by bacteria able to grow on short-chain alkanes (typically propane to pentane) [8, 116-120]. The propane and TCE degrading strain *P. butanovora* was shown to contain butane monooxygenase genes that were closely related to sMMO [33].

CONCLUSIONS AND OUTLOOK

Alkanes are ubiquitous in the environment due to biogenic production and oil pollution, and many aerobic micro-organisms are able to use these highly reduced hydrocarbons as a sole carbon- and energy source. The oxygenases that are required for the initial activation of alkanes belong to several different enzyme classes, some of which only oxidize short-chain alkanes, while others oxidize medium and long-chain alkanes. Membrane-bound alkane hydroxylases related to AlkB of *P. putida* GPo1 have been found in many bacteria able to grow on C_5-C_{16} alkanes, and have also been detected in environments such as soils and aquifers. Other enzyme systems involved in alkane assimilation have been studied only in a limited number of strains (often just 1 or 2). Future research efforts are likely to yield additional and perhaps completely novel alkane hydroxylase systems. A major challenge will be to explain the parallel evolution of such diverse families of alkane hydroxylases with essentially the same function. For this purpose, the roles of the individual enzyme systems in their hosts and ecological niches need to be assessed in detail. Important elements in the required physiological studies will be the quantification of kinetic parameters such as the k_{cat} and K_m of various enzyme systems for a variety of alkanes and oxygen, according to standardized protocols. Potential applications in

bioremediation and biocatalysis are a strong incentive to study cometabolic oxidations by alkane hydroxylase systems in much greater detail. Studies of alkane hydroxylase gene diversity, coupled with information on substrate range, induction, enzyme kinetics and host properties, should help to optimize the biodegradative activity of indigenous hydrocarbon degrading strains, benefit biocatalytic applications, and promote fundamental research on the activation of oxygen by enzymes and biomimetic catalysts.

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