Enzymes for Aerobic Degradation of Alkanes in Bacteria

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Contents

Abstract

Alkanes are major constituents of crude oil but they are also present at low concentrations in diverse noncontaminated habitats since many living organisms produce them as chemoattractants or as agents that help to protect against water loss. Although the metabolism of these compounds poses problems (mainly to do with their hydrophobicity), many microorganisms can use them as a carbon and energy source. This chapter examines how bacteria metabolize *n*-alkanes

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aerobically, paying particular attention to the enzymes involved in the initial oxidation of the alkane molecule – the most critical step given that *n*-alkanes are chemically rather inert.

1 Introduction

Alkanes are saturated hydrocarbons. They can be linear (*n*-alkanes), cyclic (*cyclo*-alkanes), or branched (*iso*-alkanes). These compounds are major constituents of crude oil but are also produced by many living organisms, including bacteria, green algae, plants and animals, in which they serve as chemoattractants or in protection against water loss, insects, or pathogens (Post-Beitenmiller 1996; Schirmer et al. 2010; Lea-Smith et al. 2015; Pedrini et al. 2013). Alkanes are therefore found in small amounts in most soils and water, in which ongoing biosynthesis and biodegradation keep their concentrations relatively constant. Alkanes are highly reduced molecules with a high energy and carbon content; they are therefore good carbon and energy sources for any microorganisms able to metabolize them. However, alkane metabolism is in no way straightforward. These compounds are very hydrophobic and their solubility in water therefore very reduced, hindering their uptake. The compounds generated during alkane metabolism are often hydrophobic too, leading to their accumulation in the plasma membrane and alterations in the latter's fluidity. Further, alkanes are chemically rather inert, and need to be activated (an energy-costly process) before they can be metabolized. Even so, many bacteria, filamentous fungi, and yeasts have acquired the ability to degrade alkanes and use them as a carbon source (van Beilen et al. 2003; Wentzel et al. 2007).

A typical soil, sand, or ocean sediment contains 10^4–10^6 hydrocarbon degrading microorganisms per gram (Rosenberg 1993), and considerably more in oil-polluted sites (Harayama et al. 2004). Many alkane-degrading bacteria have a very versatile metabolism, and alkanes are but some of many substrates that can serve as carbon sources (Harayama et al. 2004; Margesin et al. 2003). In fact, alkanes are not usually the most preferred substrates; cells tend to use other compounds before turning to them. How this is achieved is analyzed in detail in the following chapter in this book by Moreno and Rojo. However, hydrocarbonoclastic bacteria are highly specialized in the degradation of hydrocarbons, and play a key role in their removal from polluted environments (Harayama et al. 2004; Head et al. 2006; Yakimov et al. 2007). A particularly well-studied example is that of *Alcanivorax borkumensis*, a marine bacterium that can assimilate linear and branched alkanes, but which is unable to metabolize the aromatic hydrocarbons, sugars, amino acids, or fatty acids, etc., commonly used as carbon sources (Schneiker et al. 2006; Yakimov et al. 1998). *Alcanivorax* spp. are present in low numbers in nonpolluted sea water, probably living on the alkanes continuously produced by algae and other marine organisms. After a spill of crude oil, however, *Alcanivorax* strains become predominant and are believed to play an important role in the natural bioremediation

process (Hara et al. 2003; Harayama et al. 2004; Kasai et al. 2002; McKew et al. 2007a, b; Yakimov et al. 2007). Hydrocarbonoclastic alkane-degrading bacteria of the genera *Thalassolituus* (Yakimov et al. 2004), *Oleiphilus* (Golyshin et al. 2002), and *Oleispira* (Yakimov et al. 2003) also play an important role in this respect (Coulon et al. 2007; McKew et al. 2007a, b).

Alkanes can be metabolized aerobically or anaerobically. The present chapter deals only with aerobic degradation; anaerobic degradative pathways are covered in a separate chapter in this book series. Several reviews are available that focus on different features of the pathways and enzymes involved in alkane degradation (Coon 2005; van Beilen and Funhoff 2007; van Hamme et al. 2003; Wentzel et al. 2007; Rojo 2009; Wang and Shao 2013). The present chapter emphasizes recent developments in *n*-alkane metabolism in Eubacteria; although some Archaea can use alkanes and other hydrocarbons as carbon sources under aerobic conditions (Tapilatu et al. 2010; Fathepure 2014), the enzymes involved have not been studied in detail and are not covered here. The regulation of the expression of the genes involved in alkane degradation is treated in a separate chapter in this volume. The degradation of methane, which is oxidized via a specialized enzyme, is also covered in a separate chapter.

2 Uptake of *n*-Alkanes

2.1 The Low Water Solubility of Hydrocarbons Presents a Problem for their Uptake by Microbes

The water solubility of *n*-alkanes decreases exponentially as their molecular weight increases (Eastcott et al. 1988; see Table 1); the solubility of *n*-alkanes with more than nine carbon atoms is so low that their uptake by microorganisms is clearly compromised. This poses a problem for their biodegradation. The precise manner in which *n*-alkanes enter the cell is poorly understood, although the mechanisms involved probably differ depending on species, the molecular weight of the alkane, and the physicochemical characteristics of the environment (Wentzel et al. 2007).

Table 1 Water solubility of representative *n*-alkanes (at 25 °C)

n-Alkane	Carbon atoms	Molecular weight	Solubility (mol L^{-1})
Propane	3	44.1	5×10^{-3}
Hexane	6	86.2	1.4×10^{-4}
Nonane	9	128.3	10^{-6}
Dodecane	12	170.3	2×10^{-8}
Hexadecane	16	226.4	2×10^{-10}
Eicosane	20	282.6	10^{-12}
Hexacosane	26	366.7	4×10^{-16}

Data obtained from Eastcott et al. (1988)

Only low molecular weight *n*-alkanes are sufficiently soluble to be taken up by diffusion from the water phase. Microorganisms gain access to medium- and long-chain *n*-alkanes in the form of small, pseudosolubilized hydrocarbon droplets, or by adhering to droplets much larger than themselves (a process used by cells able to develop a hydrophobic surface) (Beal and Betts 2000; Hua and Wang 2013, 2014).

Most bacteria able to degrade *n*-alkanes secrete surfactants of different chemical nature that facilitate the emulsification of hydrocarbons (Hommel 1990; Ron and Rosenberg 2002). Biosurfactants are believed to increase the surface area that hydrophobic compounds can expose to the water phase, thereby improving the access of microorganisms to the oil phase (Ron and Rosenberg 2002). In liquid cultures, surfactants have been reported to increase the uptake and assimilation of *n*-alkanes such as hexadecane (Beal and Betts 2000; Noordman and Janssen 2002). However, in soils and elsewhere, the usefulness of surfactants in the uptake of *n*-alkanes is less evident (Holden et al. 2002). *Pseudomonas aeruginosa* produces rhamnolipid surfactants that stimulate the uptake of hexadecane via a process that requires energy (Beal and Betts 2000; Noordman and Janssen 2002). Rhamnolipids can disperse hydrocarbons in liquid cultures to generate hexadecane droplets in the sub-micromolar range, which clearly increases the availability of the hydrocarbon to the bacterium (Cameotra and Singh 2009).

Efficient emulsification requires the production of relatively large amounts of the surfactant, which in turn requires high population densities of surfactant-producing microorganisms. This suggests that the role of surfactants at low cell densities might be different to emulsification (Ron and Rosenberg 2001). Indeed, surfactants facilitate adhesion to and detachment from surfaces or from biofilms (Boles et al. 2005; Neu 1996), as well as cell motility on solid surfaces (Caiazza et al. 2005; Kohler et al. 2000). In the case of *n*-alkane-degrading bacteria that also behave as opportunistic pathogens, such as *P. aeruginosa*, these properties of biosurfactants might facilitate infection; they might therefore also be considered virulence factors (Zulianello et al. 2006). The uptake of hydrocarbons is thus just one of the processes in which the properties of surfactants may be useful.

2.2 Transport of Hydrocarbons Through the Cell Envelope

After contact is established between cells and hydrocarbon molecules, the latter need to gain access to the plasma membrane where they will be processed by enzymes that initiate their oxidation (see Sects. 3 and 4). Three mechanisms might be involved: (a) passive diffusion of the hydrocarbons through the cell envelope, (b) passive uptake facilitated by protein transporters, or (c) energy-dependent active transport (reviewed by Hua and Wang 2014). In Gram-negative bacteria, the membrane is a strong barrier to both hydrophilic and hydrophobic molecules, and contains many proteins that facilitate the import and export of different molecules across it. These can be general porins, substrate-specific transporters, or active energy-dependent transporters (reviewed in Nikaido 2003 and van den Berg 2005). General porins do

not bind their substrate, but rather allow the passive passage of small solutes by diffusion along their concentration gradients. Substrate-specific transporters have low affinity binding sites for particular compounds, and allow the entry of molecules when their concentration gradient across the membrane is shallow. Finally, active energy-dependent transporters bind substrates with high affinity and selectivity, and transport them against a concentration gradient using energy provided by the inner membrane protein TonB.

Transporters for hydrophilic molecules have been extensively studied, but much less information is available on those dealing with hydrophobic molecules such as hydrocarbons. The best known example is that of the FadL protein from *Escherichia coli*, an outer membrane protein that allows the import of long-chain fatty acids via diffusion facilitated by spontaneous conformational changes that require no exogenous energy input (van den Berg 2005). FadL also mediates the uptake of *n*-alkanes with eight or ten carbon atoms (C_8 and C_{10}, respectively, where the subindex indicates the number of carbon atoms of the alkane molecule), which is severely reduced if the *fadL* gene is inactivated (Call et al. 2016). FadL homologs are widespread in Gram-negative bacteria. One such homolog is the AlkL protein involved in the alkane degradation pathway and encoded within the OCT plasmid of *Pseudomonas putida* GPo1. AlkL has been shown to greatly improve the import of fatty acid methyl esters and C_7–C_{16} *n*-alkanes in both this bacterium and *E. coli* (Julsing et al. 2012; Grant et al. 2014). In the latter species, AlkL was found necessary for the uptake of C_{12}–C_{16} *n*-alkanes, but not for *n*-octane, probably because it can be transported by FadL (Grant et al. 2014).

In the hydrocarbonoclastic bacterium *Alcanivorax dieselolei* B5, three FadL-like outer membrane proteins named OmpT-1, OmpT-2, and OmpT-3 are required for the uptake of *n*-alkanes (Wang and Shao 2014). Compared to cultivation with acetate, the transcription of *ompT-1* was strongly induced in cells growing on C_{24}–C_{34} *n*-alkanes or pristane, while the use of C_8–C_{16} *n*-alkanes as the carbon source led to significantly reduced induction. Nevertheless, the expression of *ompT-2* and *ompT*-3 was efficiently induced by all these hydrocarbons. Inactivation of the *ompT-1* gene impaired growth on C_{28}, C_{32}, C_{36} *n*-alkanes or pristane, although C_8–C_{24} *n*-alkanes could still be efficiently used. In contrast, the inactivation of *ompT-3* impaired growth on C_8–C_{12} *n*-alkanes, but did not affect the assimilation of C_{16}, C_{24} *n*-alkanes and pristane. A mutant strain lacking OmpT-2 did not grow on C_{16}–C_{24} *n*-alkanes, and showed slow growth on *n*-alkanes with under 16 and more than 24 carbon atoms. Transport studies confirmed these proteins to be involved in the selective uptake of these hydrocarbons. The above authors concluded that OmpT-1 is used for the uptake of *n*-alkanes of over 28 carbon atoms and pristane, that OmpT-2 is preferentially used for C_{16}–C_{24} *n*-alkanes, and that OmpT-3 takes care of the uptake of C_8–C_{12} *n*-alkanes.

Finally, transport studies on the *n*-octadecane-degrading strain *Pseudomonas* sp. DG17, using ^{14}C *n*-octadecane as a substrate, showed that, when the alkane concentration was higher than 4.5 μmol/L, its uptake was driven by a facilitated passive mechanism that required no supply of external energy. It was also insensitive to chemicals such as carbonyl cyanide m-chlorophenyl hydrazone (CCCP), which

uncouples the proton gradient of the membrane and ultimately inhibits ATP synthesis. However, when the concentration of octadecane was about ten times lower, transport was strongly inhibited by CCCP (Hua et al. 2013). This suggests that the facilitated diffusion of *n*-alkanes by FadL-like proteins is probably enough to allow growth at the expense of these compounds when they are present at micromolar concentrations. At nanomolar concentrations, however, energy-driven active transport systems become necessary, but these have not yet been identified.

2.3 Chemotaxis

Many motile bacteria have systems for detecting and responding to the presence of specific chemicals in their environment, swimming towards or away from them in a process called chemotaxis (reviewed in Wadhams and Armitage 2004; Hazelbauer et al. 2008). Depending on whether the flagellum rotates in an anticlockwise or clockwise direction, cells swim in a straight line or stop and tumble. Cells sense chemicals via dedicated transmembrane receptors known as methyl-accepting chemotaxis proteins (MCPs). Studies in *E. coli* have shown that, in response to reduced binding of the attractant to the MCPs, CheA histidine kinase autophosphorylates and transfers the phosphoryl group to the CheY and CheB response regulators. Phosphorylated CheY interacts with components of the flagellar motor, promoting a change in the rotational direction of the flagellum from anticlockwise to clockwise, causing cell tumbling. Phosphorylated CheB is a methylesterase that demethylates the MCP receptors, reducing their ability to induce CheA autophosphorylation, thereby resetting the system. Binding of the specific ligands to the MCPs inhibits the autophosphorylation of CheY and, therefore, reduces the frequency of motor switching, allowing swimming towards the attractant. This basic plan seems to be conserved across bacteria, although some chemotaxis pathways are more complex.

Many chemicals eliciting an attractive response can be used as a carbon and energy source. Chemotaxis can therefore help bacteria find food supplies and, for low water-solubility chemicals, improve the rate of substrate acquisition. Several hydrocarbons elicit chemotaxic responses, although most of the literature refers to aromatic hydrocarbons such as naphthalene or toluene (Parales and Harwood 2002; Shingler 2003). Some reports indicate that *n*-alkanes also induce chemotaxis (Lanfranconi et al. 2003; Wang and Shao 2014). A recent study in *Alcanivorax dieselolei* B5 showed that the sensing of *n*-alkanes is connected to their uptake and metabolism by a signal transduction network (Wang and Shao 2014). In brief, mutagenesis studies identified an MCP protein and two additional components of the chemotaxis machinery, the inactivation of which abolished chemotaxis towards *n*-alkanes and significantly reduced growth on C_8–C_{32} *n*-alkanes and pristane. When purified, the identified MCP protein can bind all these hydrocarbons, but not acetate, which is also a carbon source for this bacterium. Several genes belonging to the chemotaxis machinery, including the mentioned MCP receptor, were expressed more strongly when cells were grown on *n*-alkanes than when grown on

acetate. An outer membrane protein named OmpS was also identified and shown to be essential for the assimilation of C_8–C_{32} *n*-alkanes and pristane. The above authors proposed that a receptor for these hydrocarbons triggers the transport/assimilation process via a mechanism still not understood. Inactivation of the gene coding for OmpT-1 porin, which transports C_{28}, C_{32}, C_{36} *n*-alkanes and pristane, abolished chemotaxis towards these hydrocarbons. Similarly, the absence of OmpT-2 or OmpT-3 abolished chemotaxis towards the *n*-alkanes they transport. It therefore seems that the sensing, chemotaxis, uptake, and assimilation of *n*-alkanes are interdependent processes in *A. dieselolei*, and probably in other *Alcanivorax* strains as well. In other bacterial species, chemotaxis can be dependent or independent of the assimilation of the attractant, depending on the compound and the strain in question (Pandey and Jain 2002; Sarand et al. 2008; Luu et al. 2015).

3 *n*-Alkane Degradation Pathways

In Eubacteria, the aerobic degradation of *n*-alkanes usually starts by the oxidation of a terminal methyl group to render a primary alcohol, which is further oxidized to the corresponding aldehyde, and finally converted into a fatty acid (see Fig. 1). Fatty acids are conjugated to CoA and further processed by β-oxidation to generate acetyl-CoA (van Hamme et al. 2003; Watkinson and Morgan 1990; Wentzel et al.

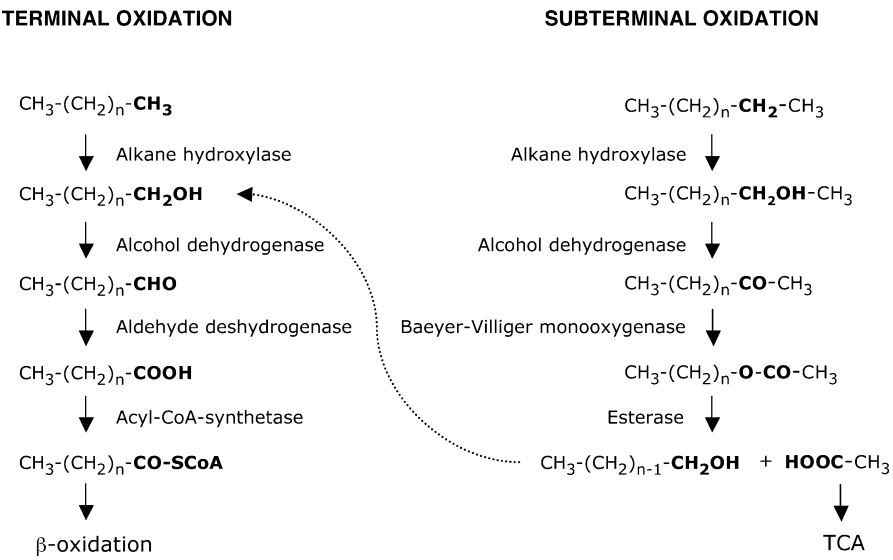

Fig. 1 The most common pathways for the degradation of *n*-alkanes by terminal and subterminal oxidation

2007; Rojo 2009). Subterminal oxidation has been reported as well in some microorganisms (Fig. 1; Britton 1984; Kotani et al. 2003, 2006; Whyte et al. 1998). While the oxidation of fatty alcohols and fatty acids is widespread among microorganisms, the activation of the alkane molecule requires enzyme systems that are much less common.

4 Hydroxylation of *n*-Alkanes

In bacteria, the initial terminal hydroxylation of *n*-alkanes can be performed by enzymes belonging to different families (Table 2; van Beilen and Funhoff 2007; van Beilen et al. 2003). Microorganisms that degrade short-chain *n*-alkanes (C_2–C_4) have enzymes related to methane monooxygenases, while those that degrade medium- (C_5–C_{11}) or long-chain (>C_{12}) *n*-alkanes commonly contain membrane, non-heme iron monooxygenases related to the well-characterized *Pseudomonas putida* GPo1 AlkB alkane hydroxylase. However, some strains contain alkane hydroxylating enzymes that belong to a family of soluble P-450 cytochromes and that are active against C_5–C_{11} *n*-alkanes. Finally, several strains that assimilate *n*-alkanes of more than 18 carbon atoms contain alkane hydroxylases that seem to be unrelated to any of those mentioned above. Several yeasts assimilate *n*-alkanes as

Table 2 Enzyme classes oxidizing *n*-alkanes

Enzyme class	Characteristics	Substrate length	Host
PRM, propane monooxygenase	Non-heme iron monooxygenase similar to sMMO	C_3	Bacteria
sBMO, butane monooxygenase	Non-heme iron monooxygenase similar to sMMO	C_2–C_9	Bacteria
pBMO, butane monooxygenase	Copper-containing monooxygenase similar to pMMO	C_2–C_9	Bacteria
CYP153	Soluble cytochrome P450 (class I)	C_5–C_{12}	Bacteria
CYP116B5	Self-sufficient cytochrome P450 (class VII)	C_{14}–C_{16}	Bacteria
CYP52	Membrane-bound cytochrome P450	C_{10}–C_{16}	Yeasts
AlkB-related	Non-heme iron monooxygenase	C_3–C_{13} or C_{10}–C_{20}	Bacteria
AlmA	Flavin-binding monooxygenase	C_{20}–C_{36}	Bacteria
LadA	Thermophilic flavin-dependent monooxygenase	C_{10}–C_{30}	Bacteria
Dioxygenase	Copper flavin-dependent dioxygenase	C_{10}–C_{30}	Bacteria
PT7_2466 monooxygenase	Rieske-type monooxygenase	C_5–C_{24}	Bacteria

The substrate range is approximate; upper and lower limits may vary in different strains. See text for details

well. In those studied, the enzymes involved in the initial oxidation of the alkane molecule belong to the microsomal cytochrome P450 family (Iida et al. 2000; Ohkuma et al. 1998; Zimmer et al. 1996). The role of yeasts in the biodegradation of *n*-alkanes in oil-contaminated sites may be more significant than previously thought, at least in some environments (Schmitz et al. 2000).

4.1 Alkane Hydroxylases Related to Methane Monooxygenase

Several bacterial strains can grow on C_2–C_4 gaseous *n*-alkanes, but not on methane (Ashraf et al. 1994). The enzymes that initially oxidize these *n*-alkanes are related to methane monooxygenases (Hamamura et al. 1999). There are two different forms of methane monooxygenases: all methanotrophs produce a membrane-bound particulate form of methane monooxygenase (pMMO) which oxidizes *n*-alkanes in the C_1–C_4 range, while some methanotrophs additionally produce a soluble form (sMMO) that is active against a wider range of substrates, oxidizing C_1–C_7 *n*-alkanes to the corresponding alcohols (Green and Dalton 1989). *Thauera butanivorans,* previously known as *Pseudomonas butanovora* (Anzai et al. 2000; Dubbels et al. 2009), can grow on C_2–C_9 *n*-alkanes using a pathway that sequentially oxidizes the terminal methyl group of the hydrocarbon (Arp 1999). The first enzyme in the pathway, butane monooxygenase (BMO), is a non-heme iron monooxygenase (similar to sMMO) that hydroxylates C_2–C_9 *n*-alkanes (Sluis et al. 2002). This enzyme has three components: a dinuclear iron-containing monooxygenase (BMOH) that in turn contains three different polypeptides, an NADH-oxidoreductase (BMOR), and a small regulatory protein (BMOB) that probably acts as an effector and that may be partly dispensable (Dubbels et al. 2007). The proper assembly of BMO may require the assistance of a chaperonin-like protein, BmoG (Kurth et al. 2008).

Gordonia sp. TY-5, which can grow on propane, contains an enzyme with sequence similarity to sMMO, but which has a very narrow substrate range: it can only oxidize propane and does so at the subterminal position, generating 2-propanol (Kotani et al. 2003). This secondary alcohol is then oxidized to acetone, which is further transformed into methylacetate and, finally, into acetic acid and methanol (Kotani et al. 2007). The genes coding for this propane monooxygenase have also been found in two propane-utilizing species, *Mycobacterium* sp. TY-6 and *Pseudonocardia* sp. TY-7 (Kotani et al. 2006). In the former, propane is oxidized at the terminal position, while in the latter both terminal and subterminal oxidation occurs. The BMOs of two other strains, *Mycobacterium vaccae* JOB5 and *Nocardioides* sp. CF8, have also been analyzed. That of *M. vaccae* JOB5 shows properties similar to sMMO (Hamamura et al. 1999), while that of *Nocardioides* sp. CF8 is a copper-containing enzyme formed by three subunits that share distant but significant similarity to other members of the pMMO family (Hamamura and Arp 2000; Hamamura et al. 1999; Sayavedra-Soto et al. 2011).

4.2 The AlkB Family of Alkane Hydroxylases

The most extensively characterized alkane degradation pathway is that encoded within the OCT plasmid of *P. putida* GPo1 (formerly *Pseudomonas oleovorans* GPo1). It was originally characterized by Coon and colleagues (Baptist et al. 1963) and has become a model system (van Beilen et al. 1994, 2001). The first enzyme of this pathway is an integral-membrane non-heme di-iron monooxygenase, named AlkB, that hydroxylates *n*-alkanes at the terminal position. This requires the assistance of two soluble electron transfer proteins named rubredoxin (AlkG) and rubredoxin reductase (AlkT). Rubredoxin reductase, via its cofactor FAD, transfers electrons from NADH to rubredoxin, which in turn transfers the electrons to AlkB (see Fig. 2).

The biochemical properties of AlkB have been analyzed in detail. Genetic studies have shown it to have six transmembrane segments and a catalytic site that faces the cytoplasm. The active site includes four histidine-containing sequence motifs that are conserved in other hydrocarbon monooxygenases and that chelate two iron atoms (Fig. 3; Shanklin et al. 1994; van Beilen et al. 1992b). The di-iron cluster allows the oxygen-dependent activation of the alkane molecule through a substrate radical intermediate (Austin et al. 2000; Bertrand et al. 2005; Shanklin et al. 1997). One of the oxygen atoms of O_2 is transferred to the terminal methyl group of the alkane, rendering an alcohol, while the other oxygen is reduced to H_2O by electrons provided by rubredoxin. Oxidation is regio- and stereospecific (van Beilen et al. 1996).

The *P. putida* GPo1 AlkB alkane hydroxylase oxidizes propane, *n*-butane (Johnson and Hyman 2006), and C_5–C_{13} *n*-alkanes (van Beilen et al. 2005b). All these *n*-alkanes support growth. Methane, ethane, and *n*-alkanes longer than C_{13} are not oxidized. Mutagenesis studies allowed the identification of a residue, Trp55, which appears to limit the size of the alkane molecules that AlkB can oxidize, since, when replaced by Ser or Cys, the substrate range increases to include C_{14} and C_{16}

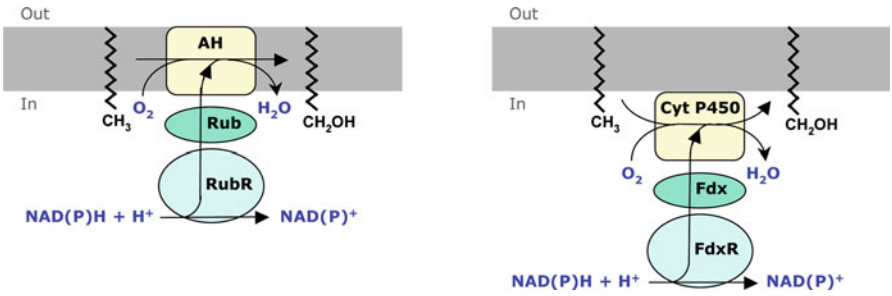

Fig. 2 Oxidation of *n*-alkanes by alkane hydroxylases of the AlkB family (*left*) and the bacterial cytochrome P450 family (*right*). AH, membrane-bound alkane hydroxylase; *Rub* rubredoxin, *RubR* rubredoxin reductase, *Cyt P450* soluble cytochrome P450, *Fdx* ferredoxin, *FdxR* ferredoxin reductase. The *gray* bar represents the plasma membrane; the phospholipid layer facing the cytoplasm is marked as "In"

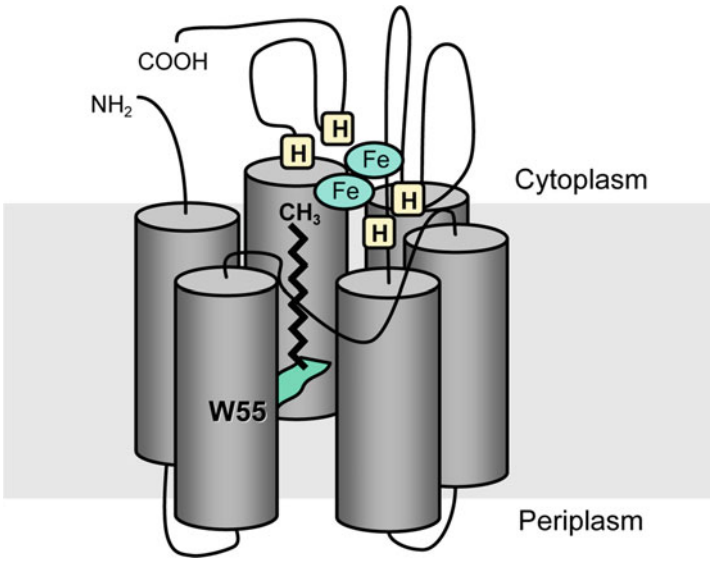

Fig. 3 Proposed structure of the *P. putida* GPo1 membrane-bound AlkB alkane hydroxylase. The *gray* bar represents the plasma membrane. The four histidine clusters (H) believed to bind the two iron atoms at the catalytic site are indicated, as is the proposed position of residue Trp55 (W55), which extends its bulky side group towards the hydrophobic pocket in which the alkane molecule is believed to fit (Adapted from van Beilen et al. (2005b) and Rojo (2005))

n-alkanes (van Beilen et al. 2005b). It has been proposed that the AlkB active site might be a deep hydrophobic pocket formed by the alignment of the six transmembrane helices, and that the alkane molecule slides into it until the terminal methyl group is correctly positioned relative to the His residues that chelate the iron atoms (Fig. 3). The estimated distance between the residue Trp55 and the His residues is similar to the length of a linear C_{13} molecule. This suggests that the bulky side chain of Trp55 protrudes into the hydrophobic pocket, impeding *n*-alkanes longer than C_{13} from entering any deeper into the pocket, and thus impairing the proper alignment of the terminal methyl group with the catalytic site. The presence at position 55 of amino acids with a less bulky side chain would allow larger *n*-alkanes to fit into the hydrophobic pocket. Recent genetic and modeling studies support this idea (Alonso et al. 2014).

More than 400 AlkB homologs are known (Marín et al. 2001, 2003; Smits et al. 1999, 2002, 2003; van Beilen et al. 2002b, 2004; Nie et al. 2014a). They have been found in both Gram-positive and Gram-negative microorganisms and show wide sequence diversity (van Beilen et al. 2003; Lo Piccolo et al. 2001; Nie et al. 2014a). Interestingly, only a few of these AlkB enzymes oxidize C_5–C_{13} *n*-alkanes, as does *P. putida* GPo1 AlkB; most members of this family prefer *n*-alkanes longer than C_{10}.

The rubredoxin that transfers electrons to the AlkB active site is a small redox-active iron-sulfur protein. The AlkG rubredoxin of *P. putida* GPo1 is unusual in that it contains two rubredoxin domains, AlkG1 and AlkG2, connected by a linker;

rubredoxins from other microorganisms have only one of these domains. Several rubredoxins present in Gram-positive and Gram-negative alkane-degrading bacteria were cloned and analyzed in complementation assays for their ability to substitute *P. putida* GPo1 AlkG. Interestingly, they clustered into two groups. AlkG1-type rubredoxins could not transfer electrons to the alkane hydroxylase, while AlkG2-type enzymes were able to do so and could therefore act as substitutes (van Beilen et al. 2002a). In some cases, the AlkB-type hydroxylase and the rubredoxin fuse into a single polypeptide, the rubredoxin domain being needed for the activity of the hydroxylase domain (Hamamura et al. 2001; Bihari et al. 2011; Nie et al. 2011; Nie et al. 2014a). In addition, fusion proteins containing an N-terminal ferredoxin domain, a central ferredoxin reductase domain, and a C-terminal alkane-hydroxylase domain have been identified during DNA sequence searches of genomic and metagenomic databanks (Nie et al. 2014a). AlkG1-type rubredoxins have other roles as well; in fact, rubredoxin-rubredoxin reductase systems are present in many other organisms that are unable to degrade *n*-alkanes. For example, they play an important role in oxidative stress responses in anaerobic microorganisms by transferring reducing equivalents from NADH to superoxide reductases, or to rubredoxin:oxygen oxidoreductases, thereby reducing oxygen or reactive oxygen species (Frazao et al. 2000). The structure of the rubredoxin-rubredoxin reductase complex, which has been resolved in *P. aeruginosa*, seems to be optimized for the rapid transport of reducing equivalents to the final receptor (Hagelueken et al. 2007).

4.3 Cytochrome P450 Alkane Hydroxylases

The cytochromes P450 are heme-thiolate proteins that catalyze the oxygenation of many compounds. Found across all the kingdoms of life, they can be grouped into more than 100 families on the basis of sequence similarity. Almost all eukaryotic P450s are membrane-bound enzymes, while most prokaryotic P450s are soluble. Several bacterial strains that degrade C_5–C_{10} *n*-alkanes contain alkane hydroxylases that belong to a distinct family of bacterial soluble cytochrome P450 monooxygenases. The first to be characterized from a biochemical and genetic perspective was CYP153A1 from *Acinetobacter* sp. EB104 (Maier et al. 2001), but similar enzymes have been found in strains of *Mycobacterium*, *Rhodococcus,* and *Dietzia*, and in several Gram-negative bacteria including hydrocarbonoclastic bacteria such as *Alcanivorax* spp. (Sekine et al. 2006; van Beilen et al. 2005a, 2006; Schneiker et al. 2006; Wang et al. 2010; Scheps et al. 2011; Nie et al. 2014b). These P450 cytochromes require the presence of ferredoxin and of ferredoxin reductase to transfer electrons from NAD(P)H to the cytochrome (Fig. 2). Complementation assays have shown many of these P450 proteins can functionally substitute for *P. putida* GPo1 AlkB, revealing them to be true alkane hydroxylases (van Beilen et al. 2006). Cytochrome P450 from *Mycobacterium* sp. HXN-1500 has been purified and shown to hydroxylate C_6–C_{11} *n*-alkanes to 1-alkanols with high affinity and regioselectivity (Funhoff et al. 2006). Fusion proteins containing an N-terminal cytochrome P450 domain, a ferredoxin reductase domain, and C-terminal ferredoxin

domain have been detected in genomic and metagenomic databanks (Nie et al. 2014a). In addition, a class VII cytochrome P450 was identified in *Acinetobacter redioresistens* S13 with an N-terminal heme domain and a C-terminal reductase domain comprising NADPH-, FMN-, and [2Fe-2S]-binding sites (Minerdi et al. 2015). This cytochrome is therefore catalytically self-sufficient and does not require ferredoxin reductase. Its heterologous expression in *E. coli* showed it to hydroxylate C_{14} and C_{16} *n*-alkanes.

As mentioned above, several yeasts can assimilate *n*-alkanes. The enzymes involved in the initial oxidation of the alkane molecule are membrane-bound cytochrome P450s of the CYP52 family (Iida et al. 2000; Ohkuma et al. 1998; Zimmer et al. 1996). These receive electrons from NADPH via FAD- and FMN-containing reductases. A detailed description of the yeast enzymes involved in the oxidation of *n*-alkanes is provided by R. Fukuda in a separate chapter in this book.

4.4 Alkane Hydroxylases for Long-Chain *n*-Alkanes

Several bacterial strains have been reported to assimilate *n*-alkanes larger than C_{20} (for a compilation see Wentzel et al. 2007). In some cases, the enzymes responsible for the oxidation of such *n*-alkanes, which are solid at room temperature, have been characterized.

In *Acinetobacter* sp. M1, which can grow on C_{13}–C_{44} *n*-alkanes, several alkane-oxidizing enzymes have been detected. Two of these, AlkMa and AlkMb, are related to *P. putida* GPo1 AlkB and are membrane-bound (Tani et al. 2001). A third enzyme has been reported that is soluble, requires Cu^{2+}, and does not receive electrons from NADH. It is therefore clearly unrelated to the AlkB family of hydroxylases (Maeng et al. 1996) and has been proposed to be a dioxygenase that oxidizes C_{10}–C_{30} *n*-alkanes generating *n*-alkyl hydroperoxides that render the corresponding aldehyde. *Acinetobacter* sp. DSM 17874 also contains at least three *n*-alkane-oxidizing enzymes. Two are AlkB paralogs similar to the AlkMa and AlkMb enzymes described above, and oxidize C_{10}–C_{20} *n*-alkanes (Throne-Holst et al. 2006). The third, a flavin-binding monooxygenase named AlmA, oxidizes C_{20} to $>C_{32}$ *n*-alkanes (Throne-Holst et al. 2007). Genes homologous to *almA* have been identified in several other long-chain *n*-alkane degrading strains, including *Acinetobacter* sp. M1 and several *Alcanivorax* species (Liu et al. 2011; Wang and Shao 2012a, b).

A different long-chain alkane hydroxylase has been characterized in the hemophilic bacterium *Geobacillus thermodenitrificans* NG80–2 (Feng et al. 2007). Termed LadA, it oxidizes C_{15}–C_{36} *n*-alkanes, generating the corresponding primary alcohols. Its crystal structure has been resolved, showing it to belong to the bacterial luciferase family of proteins, which are two-component flavin-dependent oxygenases (Li et al. 2008). LadA is believed to oxidize *n*-alkanes via a mechanism similar to that of other flavoprotein monooxygenases; its ability to recognize and hydroxylate long-chain *n*-alkanes probably lies in the way it captures these molecules.

Finally, an alkane monooxygenase different to any mentioned above was recently described in *Pusillimonas* sp. T7–7, a Gram-negative, cold-tolerant bacterium that can assimilate C_5–C_{30} *n*-alkanes. The protein was purified and shown to belong to the Rieske non-heme iron monooxygenase family (Li et al. 2013). It contains two different subunits, and works in association with a ferredoxin and an NADH-dependent reductase. The purified enzyme was shown to hydroxylate C_5–C_{24} *n*-alkanes.

Several bacterial strains can degrade $>C_{20}$ *n*-alkanes using enzyme systems that are yet to be characterized. It is likely that new enzyme classes responsible for the oxidation of these high molecular weight *n*-alkanes will be found in the near future.

4.5 Several Alkane Hydroxylases Frequently Coexist in a Single Bacterial Strain

Some bacterial strains contain only one alkane hydroxylase, as is the case for the well-characterized alkane degrader *P. putida* GPo1. However, it is rather common to find strains that contain more than one alkane oxidation system. In many cases these alkane oxidation enzymes have different substrate ranges or different induction patterns. The *Alcanivorax* species characterized to date typically contain two AlkB-related alkane hydroxylases, one to three cytochrome P450s involved in alkane degradation, and an alkane hydroxylase similar to AlmA (Hara et al. 2004; Sabirova et al. 2006; Schneiker et al. 2006; van Beilen et al. 2004; Liu et al. 2011; Wang and Shao 2012a, b). The presence of multiple alkane oxidation determinants in a single strain occurs both in hydrocarbonoclastic bacteria and in bacterial species that have a versatile metabolism. For example, *P. aeruginosa* PAO1 and RR1 contain two AlkB-related alkane hydroxylases that are differentially regulated (Marín et al. 2001; Stover et al. 2000), while genomic and proteomic analyses of *P. aeruginosa* SJTD-1 have identified two AlkB-like monooxygenases, two cytochrome P450s of the CYP153 family, and one AlmA-like monooxygenase (Liu et al. 2014, 2015). *Acinetobacter* sp. DSM17874, and probably other *Acinetobacter* strains, have at least three alkane oxidation enzymes, two of them involved in the degradation of C_{10}–C_{20} *n*-alkanes, and a third that oxidizes C_{32}–C_{36} *n*-alkanes (Throne-Holst et al. 2007). Besides carrying two AlkB-related hydroxylases, *Acinetobacter* sp. M1 also contains a dioxygenase that oxidizes long-chain *n*-alkanes (Maeng et al. 1996; Tani et al. 2001), and has a gene coding for a protein similar to AlmA (Throne-Holst et al. 2007). *Mycobacterium* sp. TY-6 and *Nocardioides* sp. CF8 also contain two different alkane oxidation systems for *n*-alkanes of different size ranges (Hamamura et al. 2001; Kotani et al. 2006). *Dietzia* sp. DQ12–45-1b has an AlkB-like alkane hydroxylase and a cytochrome P450 of the CYP153 family; the former is responsible for the hydroxylation of *n*-alkanes longer than C_{14}, while the latter deals with those shorter than C_{10} (Nie et al. 2014b). *Rhodococcus* sp. Q15 and NRRL B-16531 contain at least four AlkB-related alkane hydroxylases (Whyte et al. 2002); in the latter strain, two additional cytochrome P450s of the CYP153 family have also been detected

(van Beilen et al. 2006). It is clear, therefore, that the coexistence of several alkane degradation systems is not uncommon.

The presence of different and frequently very divergent alkane degradation genes in a single bacterial strain suggests that horizontal transfer has greatly facilitated their spread. A phylogenetic analysis of 58 AlkB-related proteins identified in different Gram-positive and Gram-negative bacteria showed that AlkB homologs from fluorescent pseudomonads were almost as divergent as the entire set of genes analyzed (van Beilen et al. 2003). The four AlkB-related proteins present in *Rhodococcus* sp. Q15 and NRRL B-16531 are as divergent as all hydroxylases analyzed from Gram-positive strains (Whyte et al. 2002). A similar conclusion was reached in a more recent analysis comparing a set of 458 AlkB-type alkane hydroxylases from 369 genomes belonging to 51 bacterial genera: the topology of a phylogenetic tree based on the AlkB sequences did not match that of the 16S rRNA genes (Nie et al. 2014a). Some alkane degradation genes have been found on transposons (van Beilen et al. 2001) and on plasmids (Sekine et al. 2006; van Beilen et al. 1994), which clearly facilitates their horizontal transfer. It is worth noting that the two AlkB genes present in *A. borkumensis* SK2 are located in two separate genome islands that were probably acquired from an ancestor of the *Yersinia* lineage, and lately transferred from *Alkanivorax* to *Pseudomonas* (Reva et al. 2008).

5 Metabolism of the Alcohols and Aldehydes Derived from the Oxidation of *n*-Alkanes

The primary fatty alcohols generated by the terminal oxidation of *n*-alkanes are further oxidized to aldehydes by alcohol dehydrogenase (ADH). There are several kinds of ADH. Some use $NAD(P)^+$ as an electron acceptor, while others use cytochromes or ubiquinone. Most $NAD(P)^+$-independent ADHs contain pyrroloquinoline quinone (PQQ) as a prosthetic group, and are commonly named quinoprotein ADHs.

Many bacteria contain several ADHs that can be used for the assimilation of distinct alcohols. For example, *T. butanivorans* can express at least four different ADHs with different specificities towards primary and secondary alcohols (Vangnai and Arp 2001; Vangnai et al. 2002). Assimilation of the alcohols derived from butane relies on two NAD^+-independent primary ADHs, named BDH and BOH. BDH contains PQQ and heme *c* as prosthetic groups, while BOH contains only PQQ. Both enzymes recognize a broad range of substrates. BDH oxidizes C_2–C_8 primary alcohols, C_5–C_9 secondary alcohols and several aldehydes (Vangnai and Arp 2001), while BOH is active against C_2–C_8 primary alcohols and C_3–C_8 secondary alcohols (Vangnai et al. 2002). Growing cells in butane leads to the induction of the genes coding for these two enzymes. Insertional inactivation of the gene coding for BDH, or of that coding for BOH, impairs but does not eliminate the assimilation of butane, although the simultaneous inactivation of both genes renders cells unable to grow on this substrate (Vangnai et al. 2002). When *T. butanivorans* was grown on 2-butanol and lactate, two additional NAD^+-dependent secondary ADHs were

detected, although their role has not been analyzed in detail (Vangnai and Arp 2001). The aldehydes generated by BOH and BDH are further oxidized to fatty acids. Genes coding for enzymes showing similarity to aldehyde dehydrogenases have been observed next to those coding for BOH and BDH, but their precise roles have not been reported (Vangnai et al. 2002). It is worth noting that BOH and BDH are active against aldehydes.

Acinetobacter calcoaceticus HO1-N contains at least two ADHs. One of them requires NAD^+ and shows a preference for decanol. The other requires $NADP^+$ and shows greater activity against tetradecanol. An aldehyde dehydrogenase active against long-chain aldehydes has also been described in this strain (Fox et al. 1992; Singer and Finnerty 1985a, b), as well as in *Acinetobacter* sp. M1 (Ishige et al. 2000).

Genes coding for alcohol and aldehyde dehydrogenases are also present in the *P. putida* GPo1 OCT plasmid. The alcohol dehydrogenase AlkJ is necessary for growth on *n*-alkanes only if the chromosomal AlcA alcohol dehydrogenase is inactivated by mutation (van Beilen et al. 1992a). This again indicates a redundancy in these enzymes. Similarly, the plasmid-encoded AlkH aldehyde dehydrogenase is not essential for growth on *n*-alkanes, which agrees with the presence of several aldehyde dehydrogenases in the *P. putida* GPo1 chromosome (van Beilen et al. 1994).

The secondary alcohols generated by subterminal oxidation of *n*-alkanes are turned into ketones by alcohol dehydrogenases (Fig. 1). *Gordonia* sp. TY-5, a bacterium that can grow on propane and C_{13}–C_{22} *n*-alkanes, metabolizes propane via 2-propanol and contains three NAD^+-dependent secondary ADHs (Kotani et al. 2003). Although 2-propanol can be oxidized by any of these three secondary ADHs, which are all expressed in propane-grown cells, ADH1 seemed to play the major role under the conditions in the latter report. NAD^+-dependent secondary ADHs have been identified in other bacteria such as *R. rhodochrous* PNKb1 (Ashraf and Murrell 1990), *M. vaccae* JOB5 (Coleman and Perry 1985), and *P. fluorescens* NRRL B-1244 (Hou et al. 1983).

The fatty acids generated by aldehyde oxidation are further metabolized by β-oxidation, generating Acyl-CoA, which enters the tricarboxylic acid cycle. However, when the carbon source is in excess relative to nitrogen, many bacteria use part of the carbon to generate storage materials such as triacylglycerols, wax esters, poly (hydroxybutyrate), or poly(3-hydroxyalkanoates), which accumulate as lipid bodies or granules (Alvarez and Steinbuchel 2002; Grage et al. 2009; Waltermann et al. 2005). These compounds can then serve as endogenous carbon and energy sources during starvation periods. The formation of storage lipids is common among hydrocarbon-utilizing marine bacteria. *Alcanivorax* strains, for example, accumulate triacylglycerols and wax esters when growing on pyruvate or *n*-alkanes (Kalscheuer et al. 2007). In addition, *P. putida* GPo1, a soil bacterium, forms intracellular inclusions of poly-β-hydroxyoctanoate when grown on *n*-octane (de Smet et al. 1983), and *Acinetobacter* sp. M-1 forms wax esters when growing on hexadecane (Ishige et al. 2000, 2002).

6 Degradation of Branched-Chain Alkanes

Branched-chain alkanes are more difficult to degrade than linear *n*-alkanes. It was observed long ago that *n*-alkanes are preferentially assimilated over branched alkanes (Pirnik et al. 1974). However, several bacterial strains can degrade simple branched-chain alkanes such as isooctane (Solano-Serena et al. 2004), and even much more complex compounds such as pristane (reviewed in Britton 1984; Watkinson and Morgan 1990). *Alcanivorax* spp. can also degrade branched alkanes such as pristane and phytane, a property that seems to provide a competitive advantage in oil-contaminated sea water (Hara et al. 2003). The metabolic pathways responsible for the assimilation of branched alkanes are less well characterized than those for *n*-alkanes, and may involve terminal and a di-terminal oxidation of the hydrocarbon molecule, rendering mono- and dicarboxylic acids (Watkinson and Morgan 1990; Nhi-Cong et al. 2010). However, a subterminal oxidation pathway for pristane has also been proposed (Nhi-Cong et al. 2009; Yang et al. 2014).

7 Applications of Alkane Oxidation Enzymes in Biotransformations of Industrial Interest

In addition to their role in alkane degradation, alkane hydroxylases can be useful in biotransformation processes. Alcohols derived from *n*-alkanes are valuable products in the pharmaceutical, cosmetics, and food industries. Alkane hydroxylases frequently oxidize not only their natural substrates but other compounds as well, albeit with reduced efficiency, further increasing their potential usefulness in industry (van Beilen and Funhoff 2005). *P. putida* GPo1 AlkB can, for example, generate epoxides from alkenes and other chemicals with a terminal double bond, oxidize alcohols to aldehydes, and catalyze demethylation and sulfoxidation reactions (van Beilen et al. 1996; Witholt et al. 1990). It can also oxidize methyl *tert*-butyl ether (Smith and Hyman 2004). Oxidation is regio- and stereospecific which, in the case of some substrates, opens doors for applications in fine chemistry. For example, when acting on a compound with a terminal double bond it produces an (*R*)-epoxide in high enantiomeric excess. Optically active epoxides can be used to generate a number of chemicals that are useful precursors from which to derive several products of added value. The set-up of a cost-effective high-scale process based on alkane hydroxylases is complicated, however, due to practical issues such as substrate uptake, the toxicity of the substrate and/or the product generated, uncoupling, oxygen mass transfer, low turnover with some compounds, regeneration of redox cofactors, and problems related to product recovery (reviewed in Soussan et al. 2016). Considerable efforts have been made in the optimization of these processes. The use of alkane hydroxylases from thermophilic microorganisms may help solve some of these problems since high temperatures can increase the solubility of *n*-alkanes and reduce their viscosity.

8 Research Needs

Despite extensive research on alkane degradation by bacteria, many features remain poorly understood, including how *n*-alkanes are incorporated or transported into the cell (which may differ between *n*-alkanes and microorganisms). The enzymes for the degradation of short- and medium-chain length *n*-alkanes are rather well characterized, although there is a paucity of structural data. However, some findings indicate that, in several microorganisms, C_{20}–C_{50} *n*-alkanes are probably oxidized by enzymes yet to be identified. The question of why bacterial strains frequently contain several different or related alkane hydroxylases that have very similar substrate specificities is also intriguing. It may be that these hydroxylases differ in aspects that are still unknown but that are important in cell biology. Finally, the use of alkane hydroxylases for biotransformations of industrial interest – an area of great potential – still has to resolve several technical issues that limit efficiency.

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