3 Enzymes for Aerobic Degradation of Alkanes

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Abstract: Alkanes are major constituents of crude oil and are also present at low concentrations in diverse non-contaminated habitats because many living organisms produce them as chemo-attractants or as protecting agents against water loss. Although the metabolism of these compounds poses problems derived mainly from their hydrophobicity, many microorganisms can use them as carbon and energy source. This chapter is focused on how bacteria metabolize *n*-alkanes aerobically, with a particular emphasis on the enzymes involved in the initial oxidation of the alkane molecule, which is the most critical step given that alkanes are chemically rather inert.

1 Introduction

Alkanes are saturated hydrocarbons. They can be linear (*n*-alkanes), cyclic (*cyclo*-alkanes) or branched (*iso*-alkanes). Alkanes are major constituents of crude oil but are also produced by many living organisms such as plants, green algae, bacteria and animals, where they serve as chemo-attractants or participate in defense mechanisms protecting against water loss, insects or pathogens. As a consequence, alkanes are present in low quantities in most soils and waters, probably maintaining low but constant concentrations by means of ongoing biosynthesis and biodegradation processes. Alkanes are highly reduced molecules with a high energy and carbon content, and therefore can be good carbon and energy sources for those microorganisms able to metabolize them. However, alkane metabolism is not straightforward. On the one hand, these compounds are very hydrophobic, and their water solubility is extremely low. This poses a problem for their uptake. The hydrophobicity of alkanes and of several of the compounds generated during their metabolism facilitates their accumulation in the cytoplasmic membrane, which can be detrimental because it alters membrane fluidity. On the other hand, alkanes are chemically rather inert and must be activated before they can be metabolized; activation is an energy-costly process. In spite of these problems, many microorganisms (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). These values can increase considerably in oil-polluted sites (Harayama et al., 2004). Many of the alkane degraders are bacteria having a very versatile metabolism, so that alkanes are one amongst many other substrate classes that can serve as carbon sources (Harayama et al., 2004; Margesin et al., 2003). In these cases, alkanes are not preferred substrates, and cells tend to use other compounds before turning to alkanes. How this is achieved will be analyzed in detail in Chapter 23, Vol. 2, Part 5. However, some bacterial species have been characterized in the last few years that are highly specialized in degrading hydrocarbons. They are called hydrocarbonoclastic bacteria and play a key role in the removal of hydrocarbons from polluted environments (Harayama et al., 2004; Head et al., 2006; Yakimov et al., 2007). Of particular importance is *Alcanivorax borkumensis*, a marine bacterium that can assimilate linear and branched alkanes, but which is unable to use aromatic hydrocarbons, sugars, amino acids, fatty acids and most other common substrates as the carbon source (Schneiker et al., 2006; Yakimov et al., 1998). *Alcanivorax sp.* are present in non-polluted sea waters in low numbers, probably living at the expense of the alkanes that are continuously produced by algae and other sea organisms and that are present at low but constant concentrations. *Alcanivorax* strains become predominant after a spill of crude oil and are believed to play an important role in natural bioremediation of oil spills worldwide

(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 the biodegradation of oil spills in several environments (Coulon et al., 2007; McKew et al., 2007a, b).

Alkanes can be metabolized aerobically or anaerobically. This chapter deals only with aerobic degradation, since anaerobic degradative pathways are covered in a separate chapter in this book. The pathways and enzymology for the degradation of alkanes have been reviewed extensively before (Ashraf et al., 1994; Coon, 2005; Rehm and Reiff, 1981; van Beilen and Funhoff, 2007; van Hamme et al., 2001; Watkinson and Morgan, 1990; Wentzel et al., 2007). This chapter will emphasize the most recent developments on alkane metabolism. Regulation of the expression of the genes involved in alkane degradation is treated on a separate chapter in this handbook. The degradation of methane, which is a special case that is oxidized by a very specialized enzyme, is also covered on a separate chapter.

2 Uptake of *n*-Alkanes

The water solubility of *n*-alkanes decreases exponentially as their molecular weight increases (Eastcott et al., 1988; see *Table 1*). For alkanes having more than nine carbon atoms, solubility is negligible from the point of view of their uptake by microorganisms. This poses a problem for their biodegradation. The precise way in which alkanes enter the cell is unclear, but the mechanism probably differs depending on the bacterial species considered, the molecular weight of the alkane and the physico-chemical characteristics of the environment (Wentzel et al., 2007). The direct uptake of the alkane molecules from the water phase can only be considered as a relevant possibility for low molecular weight alkanes, which are still sufficiently soluble to assure a proper mass transfer to the cell. For medium- and long-chain *n*-alkanes, microorganisms may gain access to these compounds either by adhering to hydrocarbon droplets (which is facilitated by a hydrophobic cell surface) or by a surfactant-facilitated access.

Most bacteria able to degrade *n*-alkanes produce and secrete surfactants of diverse chemical nature that allow emulsification of the hydrocarbons (Hommel, 1990; Ron and Rosenberg,

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)

2002). Biosurfactants are believed to increase the surface area that hydrophobic compounds can expose to the water phase, thereby facilitating 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 alkanes such as hexadecane (Beal and Betts, 2000; Noordman and Janssen, 2002). However, in soils and other situations the usefulness of surfactants for the uptake of alkanes is less evident (Holden et al., 2002). It should be noted that efficient emulsification requires the production of relatively large amounts of the surfactant, which in turn requires high population densities of the surfactant-producing microorganism. This suggests that the role of surfactants at low cell densities could be different from emulsification. *P. aeruginosa* produces a rhamnolipid surfactant that stimulates the uptake of hexadecane through a process that requires energy (Beal and Betts, 2000; Noordman and Janssen, 2002). Uptake of the alkanes may also occur through their passive diffusion into the cell membrane.

Surfactants produced by microorganisms probably serve other biological functions in addition to emulsifying hydrocarbons to improve their uptake (Ron and Rosenberg, 2001). For example, surfactants facilitate adhesion to and detachment from surfaces of 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 alkane degrading bacteria that also behave as opportunistic pathogens, like *Pseudomonas aeruginosa*, these properties of biosurfactants can facilitate certain infections, so that they can also be considered as a virulence factor (Zulianello et al., 2006). In other words, bacteria can use surfactants for several purposes in different environmental conditions, the uptake of hydrocarbons being just one of the processes where the properties of surfactants can be useful.

3 Pathways for Degradation of *n*-Alkanes

In most cases described, aerobic degradation of *n*-alkanes 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 (Ashraf et al., 1994; Rehm and Reiff, 1981; van Hamme et al., 2003; Watkinson and Morgan, 1990; Wentzel et al., 2007). Subterminal oxidation has been reported as well in some microorganisms (*Fig. 1*; Britton, 1984; Kotani et al., 2003, 2006; Whyte et al., 1998). While oxidation of fatty alcohols and fatty acids is common among microorganisms, activation of the alkane molecule requires an enzyme system that is much less widespread.

4 Hydroxylation of *n*-Alkanes

In bacteria, the initial terminal hydroxylation of *n*-alkanes can be carried out by enzymes belonging to different families (*Table 2*; van Beilen and Funhoff, 2007; van Beilen et al., 2003). Microorganisms degrading short-chain-length alkanes (C_2–C_4, where the subindex indicates the number of carbon atoms of the alkane molecule) have enzymes related to methane monooxygenases. Strains degrading medium-chain-length alkanes (C_5–C_{11}), or long-chain-length alkanes ($>C_{12}$), frequently contain integral membrane non-heme iron monooxygenases related to the well-characterized *Pseudomonas putida* GPo1 AlkB alkane

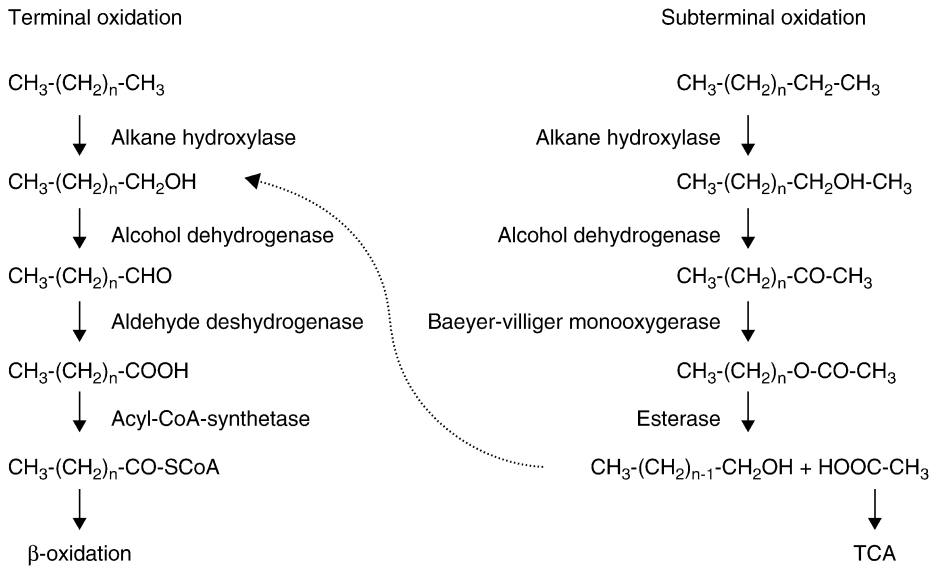

Figure 1

Most frequent pathways for the degradation of *n*-alkanes by terminal and subterminal oxidation.

Table 2

Enzyme classes oxidizing 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	C_5–C_{12}	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

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

hydroxylase. However, some strains contain alkane hydroxylating enzymes that belong to a family of soluble cytochrome P-450s and that are active against C_5–C_{11} alkanes. Finally, several strains assimilating alkanes of more than 18 carbon atoms contain alkane hydroxylases that seem to be unrelated to the former ones and that only recently have started to be characterized.

Several yeasts can assimilate alkanes as well. In those cases studied, the enzymes involved in the initial oxidation of the alkane molecule belong to the family of microsomal cytochrome P450 (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 considered, 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 alkanes, but not on methane (Ashraf et al., 1994). The enzymes that initially oxidize these 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 a narrow range of alkanes, while some methanotrophs additionally produce as well a soluble form of methane monooxygenase (sMMO) that is active on a broader range of substrates and oxidizes C_1–C_7 alkanes to the corresponding alcohols (Green and Dalton, 1989). *Pseudomonas butanovora*, which was later proposed to be a close relative to *Thauera sp.* on the basis of 16S DNA sequence (Anzai et al., 2000), can grow on C_2–C_4 alkanes by a pathway that sequentially oxidizes the terminal methyl group of the hydrocarbon (Arp, 1999). The first enzyme of the pathway, termed butane monooxygenase (BMO), is a non-heme iron monooxygenase similar to the sMMO and can hydroxylate C_2–C_9 alkanes (Sluis et al., 2002). The enzyme is composed of 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 has been proposed to require the assistance of a chaperonin-like protein, BmoG (Kurth et al., 2008).

Gordonia sp. TY-5, which can grow on propane as the carbon source, contains a propane monooxygenase that shows sequence similarity to sMMO, but has a very narrow substrate range; it can only oxidize propane and does so at the sub-terminal 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 encoding the *Gordonia sp.* TY-5 propane monooxygenase have been found as well in two propane-utilizing species, *Mycobacterium* sp. TY-6 and *Pseudonocardia* sp. TY-7 (Kotani et al., 2006). In *Mycobacterium* sp. TY-6, propane is oxidized at the terminal position. In *Pseudonocardia* sp. TY-7, however, both terminal and sub-terminal oxidation was observed. The butane monooxygenases of two other strains, *Mycobacterium vaccae* JOB5 and *Nocardioides* CF8, have been analyzed from a physiological point of view. In the absence of DNA sequence data, it seems that *M. vaccae* JOB5 butane monooxygenase shows properties similar to sMMO (Hamamura et al., 1999), while that of *Nocardioides* CF8 is a copper-containing enzyme similar to pMMO (Hamamura and Arp, 2000; Hamamura et al., 1999).

4.2 The AlkB Family of Alkane Hydroxylases

The most extensively characterized alkane degradation pathway is that encoded on the OCT plasmid of *P. putida* GPo1, formerly identified as *Pseudomonas oleovorans* GPo1. It was

originally characterized by Coon and coworkers (Baptist et al., 1963) and has later become a model system (van Beilen et al., 1994, 2001). The first enzyme of this pathway is an integral-membrane non-heme diiron monooxygenase, named AlkB, that hydroxylates alkanes at the terminal position. AlkB requires two soluble electron transfer proteins named rubredoxin (AlkG) and rubredoxin reductase (AlkT). Rubredoxin reductase, via its cofactor FAD, transfers electrons from NADH to the rubredoxin, which in turn transfers the electrons to AlkB (see *Fig. 2*). The biochemical properties of AlkB have been analyzed in detail. Although the crystal structure is not available, several approaches allowed to deduce that it has six transmembrane segments and a catalytic site that faces the cytoplasm. The active site includes four histidine-containing sequence motives that are conserved in other hydrocarbon monooxygenases and chelate two iron atoms (*Fig. 3*; Shanklin et al., 1994; van Beilen et al., 1992b). The diiron 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 atom is reduced to H_2O by electrons transferred by the rubredoxin. Oxidation is regio-and stereospecific (van Beilen et al., 1996).

The *P. putida* GPo1 AlkB alkane hydroxylase can oxidize propane, *n*-butane (Johnson and Hyman, 2006), as well as C_5–C_{13} alkanes (van Beilen et al., 2005b). All these alkanes can also support growth. Methane, ethane, or alkanes longer than C_{13}, are not oxidized. A mutagenesis approach allowed identifying a residue, Trp55, which appears to limit the size of the alkane molecule that AlkB can oxidize, since when replaced by Ser or Cys the substrate range increased to include C_{14} and C_{16} alkanes (van Beilen et al., 2005b). It was proposed that the AlkB active site might be a deep hydrophobic pocket formed by the proper alignment of the six transmembrane helices, and that the alkane molecule should slide 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 would

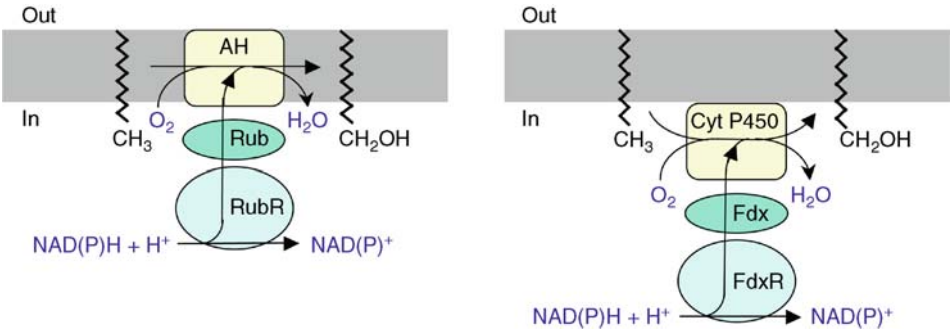

Figure 2

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

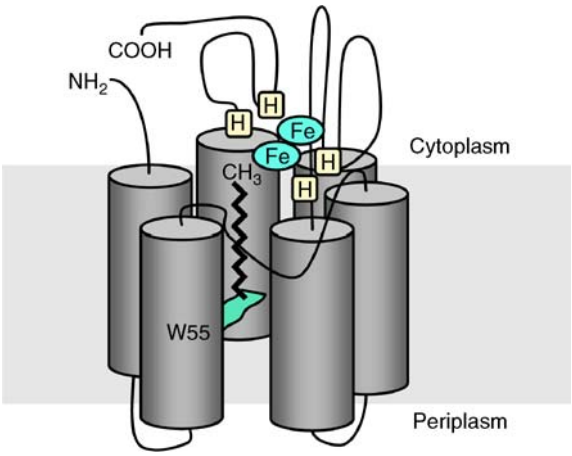

Figure 3

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

protrude into the hydrophobic pocket, impeding alkanes longer than C_{13} to enter deeper into the pocket, thereby 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 alkanes to fit in place into the hydrophobic pocket.

More than sixty AlkB homologs are known to date (Marín et al., 2001, 2003; Smits et al., 1999, 2002, 2003; van Beilen et al., 2002b, 2004). They have been found in both Gram-positive and Gram-negative microorganisms and show a high sequence diversity (van Beilen et al., 2003). Interestingly, only a few of these AlkB enzymes oxidize C_5–C_{13} alkanes, as does *P. putida* GPo1 AlkB, whereas most members of this family prefer alkanes larger 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, while 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 for *P. putida* GPo1 AlkG. Interestingly, they clustered in two groups. AlkG1-type rubredoxins could not transfer electrons to the alkane hydroxylase, while AlkG2-type enzymes were able to do so and could substitute for GPo1 AlkG (van Beilen et al., 2002a). AlkG1-type rubredoxins probably have other as yet unknown roles. In fact, rubredoxin-rubredoxin reductase systems are present in many other organisms that are unable to degrade alkanes, where they serve other functions. 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 solved in the case of *Pseudomonas aeruginosa*, seems to be highly optimized for rapid transport of reducing equivalents to the final receptor (Hagelueken et al., 2007).

4.3 Cytochrome P450 Alkane Hydroxylases

Cytochromes P450 are heme-thiolate proteins that catalyze the oxygenation of a large number of compounds. They are ubiquitous among all kingdoms of life and can be grouped in 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} alkanes contain alkane hydroxylases that belong to a distinct family of bacterial soluble cytochrome P450 monooxygenases. The first member characterized was CYP153A1 from *Acinetobacter* sp. EB104 (Maier et al., 2001), but similar enzymes have been found in diverse strains of mycobacteria, rhodococci and proteobacteria (Sekine et al., 2006; van Beilen et al., 2005a, 2006). These cytochromes P450 require the presence of a ferredoxin and of a ferredoxin reductase that transfer electrons from NAD(P)H to the cytochrome (*Fig. 2*). Complementation assays showed that many of these cytochrome P450 proteins can functionally substitute for *P. putida* GPo1 AlkB, showing that they are true alkane hydroxylases (van Beilen et al., 2006). The cytochrome P450 from *Mycobacterium* sp. HXN-1500 was purified and shown to hydroxylate C_6–C_{11} alkanes to 1-alkanols with high affinity and regioselectivity (Funhoff et al., 2006).

As stated above, several yeasts can assimilate alkanes. In those cases studied, 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). They receive electrons from NADPH via FAD- and FMN-containing reductases.

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

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

In *Acinetobacter* sp. M1, which can grow on C_{13}–C_{44} alkanes, several alkane oxidizing enzymes have been detected. Two of them, named 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+}, does not receive electrons from NADH and is therefore clearly unrelated to the AlkB family of hydroxylases (Maeng et al., 1996). It has been proposed to be a dioxygenase that oxidizes C_{10}–C_{30} alkanes generating *n*-alkyl hydroperoxides that render the corresponding aldehyde. A different *Acinetobacter* strain, named DSM 17874, also contains at least three alkane oxidizing enzymes. Two of them are AlkB paralogs similar to the AlkMa and AlkMb enzymes described above, and oxidize C_{10}–C_{20} alkanes (Throne-Holst et al., 2006). A third enzyme has been reported that oxidizes C_{20} to $>C_{32}$ alkanes. Its gene, designated *almA*, has been identified and codes for a flavin-binding monooxygenase (Throne-Holst et al., 2007). Genes homologous to *almA* were identified in several other long-chain *n*-alkane degrading strains, including *Acinetobacter* M1. Most notably, two genes similar to *almA* were also detected in the genome of *A. borkumensis* SK2.

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

Several bacterial strains can degrade $>C_{20}$ alkanes using enzyme systems that have still not been characterized. It is likely that new enzyme classes will be found in the near future responsible for the oxidation of these high molecular weight alkanes.

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, but not always, these alkane oxidation enzymes have different substrate ranges or different induction patterns. *A. borkumensis* SK2, which is specialized in assimilating alkanes, has two AlkB-related alkane hydroxylases and two genes encoding cytochrome P450s believed to be involved in alkane degradation, one of which is duplicated (Hara et al., 2004; Sabirova et al., 2006; Schneiker et al., 2006; van Beilen et al., 2004). As mentioned above, two genes that may encode alkane hydroxylases similar to AlmA were also detected in this bacterium (Throne-Holst et al., 2007). The presence of multiple alkane oxidation determinants in a single strain is not restricted to hydrocarbonoclastic bacteria, and can be observed as well in bacterial species that have a versatile metabolism. *P. aeruginosa* strains PAO1 and RR1 contain two AlkB-related alkane hydroxylases that are differentially regulated (Marín et al., 2001; Stover et al., 2000). *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} alkanes and a third one that oxidizes C_{32}–C_{36} alkanes (Throne-Holst et al., 2007). *Acinetobacter* sp. M1, besides the two AlkB-related hydroxylases, contains a dioxygenase that oxidizes long-chain-length alkanes (Maeng et al., 1996; Tani et al., 2001) and 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 alkanes of different size ranges (Hamamura et al., 2001; Kotani et al., 2006). *Rhodococcus* strains Q15 and NRRL B-16531 contain at least four AlkB-related alkane hydroxylases (Whyte et al., 2002) and, in the latter strain, two additional cytochrome P450s of the CYP153 family have 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 highly divergent alkane degradation genes in a single bacterial strain suggests that horizontal transfer has greatly facilitated the spread of these genes. 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). Similarly, the four AlkB-related proteins present in *Rhodococcus* strains Q15 and NRRL B-16531 are

as divergent as all hydroxylases analyzed from Gram-positive strains (Whyte et al., 2002). Some alkane degradation genes have been found on transposons (van Beilen et al., 2001) or 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 Alkanes

The primary fatty alcohols generated by terminal oxidation of alkanes are further oxidized to aldehydes by an alcohol dehydrogenase (ADH). There are several kinds of ADHs. Some use $NAD(P)^+$ as electron acceptor, while others do not depend on $NAD(P)^+$ and use electron acceptors such as cytochromes or ubiquinone. Most $NAD(P)^+$-independent ADHs contain pyrroloquinoline quinone (PQQ) as prosthetic group, and are commonly named quinoprotein ADHs.

Many bacteria contain several different ADHs that can be used for the assimilation of distinct alcohols. For example, *P. butanovora* 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 (Vangnai and Arp, 2001; Vangnai et al., 2002). BDH contains PQQ and heme *c* as prostetic 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 on C_2–C_8 primary alcohols and C_3–C_8 secondary alcohols (Vangnai et al., 2002). Growth of cells in butane leads to 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 assimilation of butane, although the simultaneous inactivation of both genes renders cells unable to grow on butane (Vangnai et al., 2002). When *P. butanovora* is grown on 2-butanol and lactate, two additional NAD^+-dependent secondary ADHs have been 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, although their precise role has not been reported (Vangnai et al., 2002). It is worth noting that BOH and BDH are active towards aldehydes (Vangnai and Arp, 2001; Vangnai et al., 2002).

Acinetobacter calcoaceticus HO1-N contains at least two ADHs. One of them requires NAD^+ and shows preference for decanol. The other one requires $NADP^+$ and has higher activity towards tetradecanol. An aldehyde dehydrogenase active towards 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 in the *P. putida* GPo1 OCT plasmid. The alcohol dehydrogenase, named AlkJ, is necessary for growth on *n*-alkanes only if the chromosomal AlcA alcohol dehydrogenase is inactivated by mutation (van Beilen et al., 1992a), indicating again a redundancy in these enzymes. Similarly, the plasmid encoded AlkH aldehyde dehydrogenase is not essential for growth on alkanes, which

agrees with the presence of several aldehyde dehydrogenases in the *P. putida* GPo1 chromosome (van Beilen et al., 1994).

In the case of the secondary alcohols generated by subterminal oxidation of alkanes, alcohol dehydrogenases transform them into ketones (Fig. 1). *Gordonia* sp. strain TY-5, a bacterium that can grow at the expense of propane and C_{13}–C_{22} 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 the three secondary ADHs, which are all expressed in propane-grown cells, ADH1 seemed to play the major role under the conditions analyzed. 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 oxidation of the aldehydes are further metabolized by β-oxidation, generating Acyl-CoA that enters the tricarboxylic acids cycle. However, when the carbon source is in excess relative to nitrogen, many bacteria derive 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 as granules (Alvarez and Steinbuchel, 2002; Prieto, 2007; Waltermann et al., 2005). These compounds can later serve as endogenous carbon and energy sources during starvation periods. Formation of storage lipids is frequent among hydrocarbon utilizing-marine bacteria. Alcanivorax strains, for example, can accumulate triacylglycerols and wax esters when growing at the expense of pyruvate or *n*-alkanes (Kalscheuer et al., 2007). On the other hand, *P. putida* GPo1, a soil bacterium, can form intracellular inclusions of poly-β-hydroxyoctanoate when grown on *n*-octane (de Smet et al., 1983), while *Acinetobacter* sp. M-1 forms wax esters when growing at the expense of 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), or much more complex compounds like pristane (reviewed in Britton, 1984; Watkinson and Morgan, 1990). *Alcanivorax* sp. 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 an ω- or β-oxidation of the hydrocarbon molecule (Watkinson and Morgan, 1990).

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 since they frequently oxidize not only their natural substrates, but other compounds as well, albeit with reduced efficiency. *P. putida* GPo1 AlkB can, for example, generate epoxides from alkenes and other chemicals with a terminal double bond, it oxidizes

alcohols to aldehydes and catalyzes 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 stereo-specific 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 with high enantiomeric excess. Optically active epoxides can be used to generate a number of chemicals that are useful precursors from which to derive several value-added products. The set-up of a cost-effective high-scale process based on this enzyme is complicated, however, due to practical issues such as substrate uptake, toxicity of the substrate and/or the product generated, uncoupling, oxygen mass transfer, low turnover with some compounds, or problems related to product recovery.

8 Research Needs

In spite of the extensive research on alkane degradation by bacteria performed during several decades, there are still aspects that remain poorly understood. One is how alkanes are incorporated or transported into the cell, which may differ for different alkanes and for different microorganisms. The enzymes for the degradation of low- and medium-chain length alkanes are rather well characterized, except for the paucity of structural data. However, some findings indicate that C_{20}–C_{50} alkanes are probably oxidized by enzymes that have still not been identified. It is also rather intriguing why bacterial strains frequently contain several different or related alkane hydroxylases that have very similar substrate specificities. It may be that these hydroxylases differ in aspects that are still unknown but that are important for cell biology. Finally, the use of alkane hydroxylases for biotransformations of industrial interest, which has a great potential, still has to solve several technical issues that limit the efficiency of the process.

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