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Minireview

Degradation of alkanes by bacteria

Fernando Rojo*
Departamento de Biotecnología Microbiana, Centro Nacional de Biotecnología, CSIC, Campus de la Universidad Autónoma de Madrid, Cantoblanco, 28049 – Madrid, Spain.

Summary

Pollution of soil and water environments by crude oil has been, and is still today, an important problem. Crude oil is a complex mixture of thousands of compounds. Among them, alkanes constitute the major fraction. Alkanes are saturated hydrocarbons of different sizes and structures. Although they are chemically very inert, most of them can be efficiently degraded by several microorganisms. This review summarizes current knowledge on how microorganisms degrade alkanes, focusing on the biochemical pathways used and on how the expression of pathway genes is regulated and integrated within cell physiology.

Introduction

Alkanes are saturated hydrocarbons, formed exclusively by carbon and hydrogen atoms. They can be linear (*n*-alkanes), cyclic (*cyclo*-alkanes) or branched (*iso*-alkanes). Those having between one and four carbon atoms (methane to butane) are gaseous at ambient temperature. Larger molecules are liquid or solid. Alkanes can constitute up to 50% of crude oil, depending on the oil source, but are also produced by many living organisms such as plants, green algae, bacteria or animals. This probably explains why alkanes are present at low concentrations in most soil and water environments. As alkanes are apolar molecules that are chemically very inert (Labinger and Bercaw, 2002), their metabolism by microorganisms poses challenges related to their low water solubility, their tendency to accumulate in cell membranes, and the energy needed to activate the molecule. However, several microorganisms, both aerobic and anaerobic, can use diverse alkanes as a source of carbon and energy. Several reviews have covered different aspects of the physiology, enzymes and pathways responsible for the degradation of alkanes (Watkinson and Morgan, 1990; Ashraf *et al*., 1994; van Beilen *et al*., 2003; van Hamme *et al*., 2003; Coon, 2005; van Beilen and Funhoff, 2007; Wentzel *et al*., 2007), so that this review is devoted to stress recent findings and how the expression of the alkane-degradation genes is regulated.

Received 23 February 2009; accepted 3 April 2009. *For correspondence. E-mail frojo@cnb.csic.es; Tel. (+34) 91 585 45 39; Fax (+34) 91 585 45 06.

Alkane-degrading bacteria: specialized and non-specialized species

Many microorganisms (bacteria, filamentous fungi and yeasts) can degrade alkanes, using them as the carbon source (van Beilen *et al*., 2003; Wentzel *et al*., 2007). A typical soil, sand or ocean sediment contains significant amounts of hydrocarbon-degrading microorganisms, and their numbers increase considerably in oil-polluted sites (Bragg *et al*., 1994; Harayama *et al*., 2004; Head *et al*., 2006). Various alkane degraders are bacteria that have a very versatile metabolism, so that they can use as carbon source many other compounds in addition to alkanes (Margesin *et al*., 2003; Harayama *et al*., 2004). Most frequently, alkanes are not preferred growth substrates for these bacteria, which will rather utilize other compounds before turning to alkanes. On the other hand, some bacterial species 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). Particular attention has been paid to *Alcanivorax borkumensis*, a marine bacterium that can assimilate linear and branched alkanes, but which is unable to metabolize aromatic hydrocarbons, sugars, amino acids, fatty acids and most other common carbon sources (Yakimov *et al*., 1998; Schneiker *et al*., 2006). *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 (Kasai *et al*., 2002; Hara *et al*., 2003; Harayama *et al*., 2004; Yakimov *et al*., 2007; McKew *et al*., 2007a,b). Hydrocarbonoclastic alkane-degrading bacteria of the genera *Thalassolituus* (Yakimov *et al*. 2004), *Oleiphilus* (Golyshin *et al*. 2002) and *Oleispira* (Yakimov *et al*. 2003) also participate in the biodegradation of oil spills in several environments (McKew *et al*., 2007a,b; Coulon *et al*., 2007).

Uptake of *n*-alkanes

Alkanes are very insoluble in water, the solubility decreasing as the molecular weight increases (Eastcott *et al*., 1988). Except for alkanes of very low molecular weight, solubility values are well below the micromolar range (1.4×10^{-4} M for hexane, and 2×10^{-10} M for hexadecane). This hampers the uptake of alkanes by microorganisms. It is unclear how alkanes enter the cell. The mechanism may differ depending on the bacterial species considered, the molecular weight of the alkane and the physico-chemical characteristics of the environment (Wentzel *et al*., 2007). Low-molecular-weight alkanes are still soluble enough so that a direct uptake of the alkane from water can assure a sufficient mass-transfer to the cell. For medium- and long-chain-length *n*-alkanes, microorganisms may gain access to them either by adhering to hydrocarbon droplets or by a surfactant-facilitated process. Most alkane-degrading bacteria secrete diverse surfactants that facilitate emulsification of the hydrocarbons (Hommel, 1990; Ron and Rosenberg, 2002). Surfactants have been reported to increase the uptake and assimilation of alkanes such as hexadecane in liquid cultures (Beal and Betts, 2000; Noordman and Janssen, 2002), but their usefulness in soils and other situations is less evident (Holden *et al*., 2002). Surfactants produced by microorganisms probably have other roles as well, such as facilitating cell motility on solid surfaces (Köhler *et al*., 2000; Caiazza *et al*., 2005), or the adhesion/detachment to surfaces or biofilms (Neu, 1996; Boles *et al*., 2005).

Aerobic degradation of alkanes

Aerobic alkane degraders use O_2 as a reactant for the activation of the alkane molecule. The alkane-activating enzymes, which are monooxygenases, overcome the low chemical reactivity of the hydrocabon by generating reactive oxygen species. Oxidation of methane, which is a special case, renders methanol that is subsequently transformed to formaldehyde and then to formic acid (Fig. 1). This compound can be converted to CO_2, or assimilated for biosynthesis of multicarbon compounds either by the ribulose monophosphate pathway, or by the serine pathway, depending on the microorganism considered (reviewed in Lieberman and Rosenzweig, 2004). In the case of *n*-alkanes containing two or more carbon atoms, aerobic degradation usually starts by the oxidation of a terminal methyl group to render a primary alcohol,

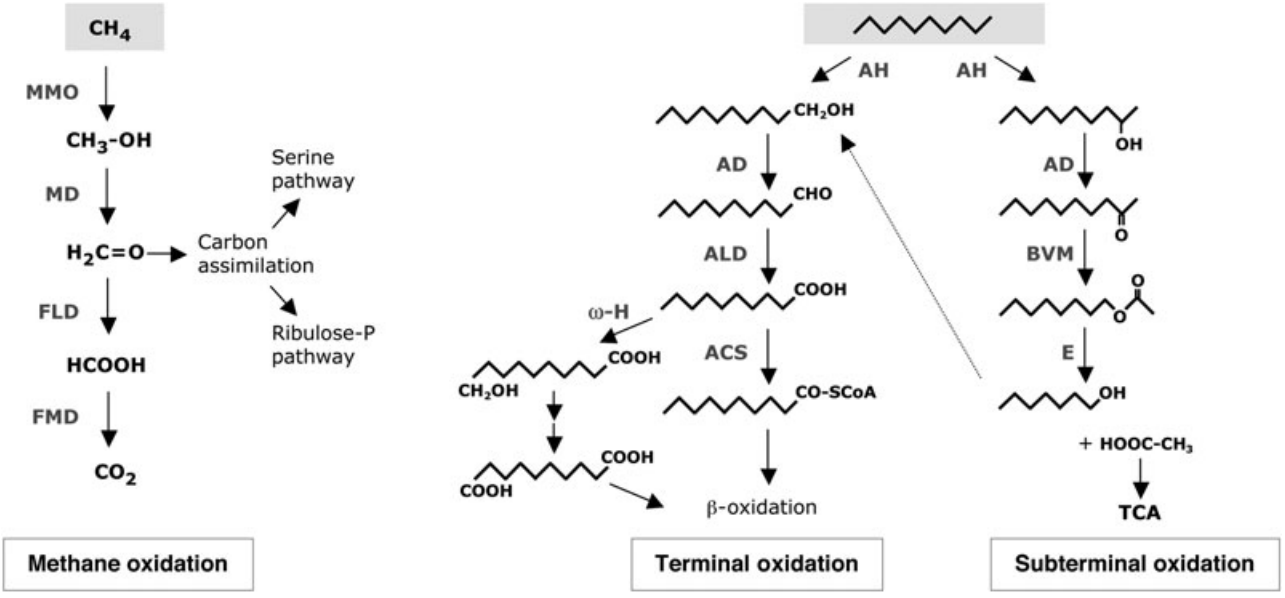

Fig. 1. Aerobic pathways for the degradation of methane (left), and of larger *n*-alkanes by terminal and subterminal oxidation (right). Initial activation of the alkane molecule requires O_2 as a reactant. MMO, methane monooxygenase; MD, methanol dehydrogenase, FLD, formaldehyde dehydrogenase; FMD, formate dehydrogenase. AH, alkane hydroxylase; AD, alcohol dehydrogenase; ALD, aldehyde dehydrogenase; ACS, acyl-CoA synthetase; ω-H, ω-hydroxylase; BVM, Baeyer–Villiger monooxygenase; E, esterase; TCA, tricarboxylic acids cycle.

which is further oxidized to the corresponding aldehyde, and finally converted into a fatty acid (Fig. 1). Fatty acids are conjugated to CoA and further processed by β-oxidation to generate acetyl-CoA (Watkinson and Morgan, 1990; Ashraf *et al*., 1994; van Hamme *et al*., 2003; Wentzel *et al*., 2007). In some cases, both ends of the alkane molecule are oxidized through ω-hydroxylation of fatty acids at the terminal methyl group (the ω position), rendering an ω-hydroxy fatty acid that is further converted into a dicarboxylic acid and processed by β-oxidation (Watkinson and Morgan, 1990; Coon, 2005).

Subterminal oxidation of *n*-alkanes has also been reported (Fig. 1; Whyte *et al*., 1998; Kotani *et al*., 2006; 2007). The product generated, a secondary alcohol, is converted to the corresponding ketone (Fig. 1), and then oxidized by a Baeyer–Villiger monooxygenase to render an ester. The ester is hydrolysed by an esterase, generating an alcohol and a fatty acid. Both terminal and subterminal oxidation can coexist in some microorganisms.

While oxidation of fatty alcohols and fatty acids is common among microorganisms, activation of the alkane molecule requires enzymes that are much less widespread, and that can belong to different families. Microorganisms that use methane as carbon and energy source, named methanotrophs, oxidize methane by means of a methane monooxygenase. Those degrading short-chain-length alkanes (C_2–C_4, where the sub-index indicates the number of carbon atoms of the alkane molecule) have enzymes related to methane monooxygenases (Hamamura *et al*., 1999; Dubbels *et al*., 2007). Strains degrading medium-chain-length alkanes (C_5–C_{11}), or long-chain-length alkanes ($> C_{12}$), frequently contain integral membrane non-haem iron monooxygenases related to the well-characterized *Pseudomonas putida* GPo1 AlkB alkane hydroxylase. However, some bacteria contain enzymes that belong to a family of soluble cytochrome P450 that hydroxylate C_5–C_{11} alkanes. Finally, some strains assimilating alkanes of more than 18 carbon atoms contain alkane hydroxylases unrelated to the former ones (reviewed in van Beilen *et al*., 2003; van Beilen and Funhoff, 2007).

Methane monooxygenases and related alkane hydroxylases

With one exception, all known methanotrophs produce a membrane-bound particulate methane monooxygenase (pMMO), and a few of them contain also a soluble methane monooxygenase (sMMO). In strains containing both pMMO and sMMO, expression of the sMMO occurs only under conditions of low copper availability (reviewed in Lieberman and Rosenzweig, 2004; Hakemian and Rosenzweig, 2007). Soluble methane monooxygenase has been studied in detail; it has three components: a hydroxylase composed by three polypeptides arranged as a $\alpha_2\beta_2\gamma_2$ dimer, a reductase that shuttles electrons from NADH to the hydroxylase, and a regulatory protein that is required for activity. Soluble methane monooxygenase contains a diiron active site. Particulate methane monooxygenase is much more prevalent than sMMO, but is less understood. It is composed of three subunits, α, β and γ, also known as PmoA, PmoB and PmoC, and is believed to contain copper in the active site. The biochemistry of sMMO and pMMO has been reviewed recently (Sazinsky and Lippard, 2006; Hakemian and Rosenzweig, 2007).

Several bacterial strains can grow on C_2–C_4 gaseous alkanes, but not on methane (Ashraf *et al*., 1994). *Pseudomonas butanovora*, which is the best-studied example, can assimilate C_2–C_4 alkanes by a sequential oxidation of the terminal methyl group of the hydrocarbon (Arp, 1999). The first enzyme of the pathway, termed butane monooxygenase (BMO), is a non-haem iron monooxygenase similar to the sMMO and can hydroxylate C_2–C_9 alkanes (Sluis *et al*., 2002; 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 can use propane as carbon source using a narrow-substrate-range propane monooxygenase similar to sMMO that oxidizes propane at the subterminal position, generating 2-propanol (Kotani *et al*., 2003). This secondary alcohol is oxidized to acetone, which is further transformed into methylacetate and, finally, into acetic acid and methanol (Kotani *et al*., 2007). Similar propane monooxygenases have been found in the propane-utilizing species *Mycobacterium* sp. TY-6 and *Pseudonocardia* sp. TY-7 (Kotani *et al*., 2006). While strain TY-6 oxidizes propane at the terminal position, in strain TY-7 both terminal and subterminal oxidation was observed. The BMOs of *Mycobacterium vaccae* JOB5 and *Nocardioides* CF8 have been analysed from a physiological point of view. While the former one shows properties similar to sMMO (Hamamura *et al*., 1999), that of *Nocardioides* CF8 is a copper-containing enzyme similar to pMMO (Hamamura *et al*., 1999; Hamamura and Arp, 2000).

The AlkB family of alkane hydroxylases

The best-characterized alkane-degradation pathway is that encoded by the OCT plasmid of *P. putida* GPo1 (formerly *Pseudomonas oleovorans* GPo1; Baptist *et al*., 1963; van Beilen *et al*., 2001). The first enzyme of this pathway is an integral-membrane non-haem 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. Although the crystal structure of AlkB is not available, it is believed to have six transmembrane segments and a catalytic site that faces the cytoplasm. The active site includes four His-containing sequence motives that are conserved in other hydrocarbon monooxygenases, and which chelate two iron atoms (van Beilen *et al*., 1992a; Shanklin *et al*., 1994). The diiron cluster allows the O_2-dependent activation of the alkane through a substrate radical intermediate (Shanklin *et al*., 1997; Austin *et al*., 2000; Bertrand *et al*., 2005). One of the O_2 atoms is transferred to the terminal methyl group of the alkane, rendering an alcohol, while the other one 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 to 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. AlkB has been proposed to contain a deep hydrophobic pocket formed by the six transmembrane helices; the alkane molecule should slide into this pocket until the terminal methyl group is correctly positioned relative to the His residues that chelate the iron atoms (van Beilen *et al*., 2005b). Amino acids with bulky side-chains protruding into the hydrophobic pocket can impose a limit to the size of the alkane molecule that can slide into the pocket and still allow a proper alignment of the terminal methyl group with the catalytic His residues. Substitution of these amino acids by residues with less bulky side-chains allows larger alkanes to fit in place into the hydrophobic pocket.

More than 60 AlkB homologues are known to date; they have been found in both Gram-positive and Gram-negative microorganisms and show a high sequence diversity (Smits *et al*., 1999; 2002; 2003; Marín *et al*., 2001; van Beilen *et al*., 2002b; 2003; 2004; Kuhn *et al*., 2009).

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 from alkane-degrading bacteria have been cloned and analysed in complementation assays for their ability to substitute *P. putida* GPo1 AlkG. They clustered into two groups. AlkG1-type rubredoxins cannot transfer electrons to the alkane hydroxylase, while AlkG2-type enzymes can do so and can substitute for 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 organisms that are unable to degrade alkanes, where they serve other functions. For example, they participate in oxidative stress responses in anaerobic bacteria, 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 *Pseudomonas aeruginosa* rubredoxin–rubredoxin reductase complex, which has been solved, shows a protein highly optimized for rapid transport of reducing equivalents to the final receptor (Hagelueken *et al*., 2007).

Cytochrome P450 alkane hydroxylases

Cytochromes P450 are hemoproteins that hydroxylate 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. Several bacterial strains that degrade C_5–C_{10} alkanes contain alkane hydroxylases that belong to a distinct family of 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 (van Beilen *et al*., 2005a; 2006; Sekine *et al*., 2006). These cytochromes P450 require a ferredoxin and a ferredoxin reductase that transfer electrons from NAD(P)H to the cytochrome. 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).

It is worth noting that several yeasts can assimilate alkanes and, at least in some environments, they may have an important role in the biodegradation of alkanes in oil-contaminated sites (Schmitz *et al*., 2000). In those cases studied, the enzymes involved in the initial oxidation of the alkane molecule are microsomal cytochromes P450 (Zimmer *et al*., 1996; Ohkuma *et al*., 1998; Iida *et al*., 2000).

Alkane hydroxylases for long-chain n*-alkanes*

Several bacterial strains can assimilate alkanes larger than C_{20}. These strains usually contain several alkane hydroxylases. Those active on C_{10}–C_{20} alkanes are usually related to *P. putida* GPo1 AlkB or to *Acinetobacter* sp. EB104 cytochrome P450. However, the enzymes that oxidize alkanes larger than C_{20} seem to be totally different. For example, *Acinetobacter* sp. M1, which can grow on C_{13}–C_{44} alkanes, contains a soluble, Cu^{2+}-dependent alkane hydroxylase that is active on C_{10}–C_{30} alkanes; it

has been proposed to be a dioxygenase that generates *n*-alkyl hydroperoxides to render the corresponding aldehydes (Maeng *et al*., 1996; Tani *et al*., 2001). A different *Acinetobacter* strain, DSM 17874, has been found to contain a flavin-binding monooxygenase, named AlmA, which oxidizes C_{20} to > C_{32} 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 *A. borkumensis* SK2. A different long-chain alkane hydroxylase, named LadA, has been characterized in *Geobacillus thermodenitrificans* NG80-2 (Feng *et al*., 2007). It oxidizes C_{15}–C_{36} alkanes, generating primary alcohols. Its crystal structure has shown that it is a two-component flavin-dependent oxygenase belonging to the bacterial luciferase family of proteins (Li *et al*., 2008). Several bacterial strains can degrade > C_{20} alkanes using enzyme systems that have still not been characterized and that may include new proteins unrelated to those currently known.

Metabolism of the alcohols and aldehydes derived from the oxidation of alkanes

The terminal oxidation of alkanes by alkane hydroxylases generates primary fatty alcohols, which 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 transfer electrons to cytochromes or to ubiquinone. Most $NAD(P)^+$-independent ADHs contain pyrroloquinoline quinone as prosthetic group, and are named quinoprotein ADHs. Some bacterial species contain several different ADHs that can be used for the assimilation of distinct alcohols. For example, *P. butanovora* has at least four different ADHs with different specificities towards primary and secondary alcohols (Vangnai and Arp, 2001; Vangnai *et al*., 2002). *Acinetobacter calcoaceticus* HO1-N contains at least two ADHs; one shows preference for decanol while the other one has higher activity towards tetradecanol. Genes coding for alcohol and aldehyde dehydrogenases are also present in the *P. putida* GPo1 OCT plasmid. However, they are not essential for growth on alkanes because of the presence of similar enzymes in the *P. putida* GPo1 chromosome (van Beilen *et al*., 1992b; 1994).

The secondary alcohols generated by subterminal oxidation of alkanes are oxidized to ketones by ADHs (Fig. 1). *Gordonia* sp. strain TY-5, which can grow at the expense of propane or of C_{13}–C_{22} alkanes, metabolizes propane via 2-propanol and contains three NAD^+-dependent secondary ADHs, all of which are expressed in propane-grown cells (Kotani *et al*., 2003). NAD^+-dependent secondary ADHs have been identified in other bacteria such as *Rhodococcus rhodochrous* PNKb1 (Ashraf and Murrell, 1990), *M. vaccae* JOB5 (Coleman and Perry, 1985) and *Pseudomonas fluorescens* NRRL B-1244 (Hou *et al*., 1983).

Degradation of branched-chain alkanes

Branched-chain alkanes are more difficult to degrade than linear *n*-alkanes (Pirnik *et al*., 1974). However, several bacterial strains can degrade branched-chain alkanes such as isooctane (Solano-Serena *et al*., 2004) or 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).

Anaerobic degradation of alkanes

Under strictly anaerobic conditions alkanes have to be activated through a mechanism that does not rely on O_2. Several bacterial strains able to use alkanes as carbon source in the absence of O_2 have been described in the last few years (reviewed in Widdel and Rabus, 2001). These microorganisms use nitrate or sulfate as electron acceptor. Growth is significantly slower than that of aerobic alkane degraders. However, anaerobic degradation of alkanes plays an important role in the recycling of hydrocarbons in the environment. The strains analysed normally use a narrow range of alkanes as substrate. For example, strain BuS5, a sulfate-reducing bacteria that belongs to the *Desulfosarcina*/*Desulfococcus* cluster, assimilates only propane and butane (Kniemeyer *et al*., 2007); *Azoarcus* sp. HxN1, a denitrifying bacteria, uses C_6–C_8 alkanes, while *Desulfobacterium* Hdx3 metabolizes C_{12}–C_{20} alkanes (reviewed in Widdel and Rabus, 2001). The metabolic pathways used have been investigated for some strains. Two general strategies appear to be used (Fig. 2). One involves activation of the alkane at a subterminal position by addition of a fumarate molecule to the alkane, yielding an alkylsuccinate derivative. This reaction is believed to occur through generation of an organic radical intermediate, most likely a glycyl radical (Rabus *et al*., 2001). The reaction product is subsequently linked to CoA and converted into an acyl-CoA that can be further metabolized by β-oxidation. In the second reaction mechanism, which has been described only for propane, the fumarate molecule is added to one of the terminal carbon atoms of the alkane (Kniemeyer *et al*., 2007).

To date, individual microorganisms that can oxidize methane with electron acceptors other than O_2 have not

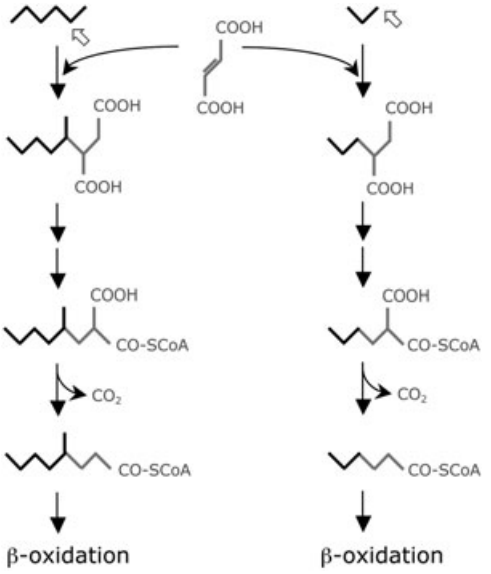

Fig. 2. Anaerobic degradation of alkanes. Initial activation of the alkane (in black) occurs by addition of a fumarate molecule (in grey), which is later regenerated. Addition (arrow) usually occurs at a subterminal position, but terminal addition has also been proposed in one case (see text for details). Further degradation requires a C-skeleton rearrangement; the products are most likely processed by β-oxidation.

been isolated. However, several multispecies consortia have been identified that cycle methane and single-carbon compounds under sulfate-reducing or nitrate-reducing conditions (reviewed in Caldwell *et al*., 2008). The enzymology of the process is not well understood.

Organization of alkane-degradation genes

The organization of the genes involved in alkane oxidation differs significantly among alkane-degrading bacteria (for a review, see van Beilen *et al*., 2003). The alkane-degradation genes encoded by the OCT plasmid of *P. putida* GPo1 are clustered in two operons, and this pathway has clearly been transferred horizontally to many bacteria (van Beilen *et al*., 2001). However, linkage has been lost to different degrees in several strains. In many cases, the gene coding for the alkane hydroxylase maps separately from those of the auxiliary proteins rubredoxin and rubredoxin reductase, and the genes coding for alcohol and aldehyde dehydrogenases map separately as well. When several alkane hydroxylases coexist in a single strain, they are normally located at different sites in the chromosome. Moreover, the regulators that control the expression of alkane-degradation genes may or may not map adjacent to the genes they regulate. Therefore, the degree of clustering of alkane-degradation genes is highly variable among bacterial strains.

Regulation of alkane-degradation pathways

Where analysed, and with some exceptions, expression of the genes involved in the initial oxidation of alkanes is tightly controlled. A specific regulator assures that the pathway genes are expressed only in the presence of the appropriate alkanes. In addition, superimposed to this specific regulation there are several mechanisms that modulate the induction of the pathway genes according to cell needs.

The known specific regulators that induce alkane-degradation genes in response to alkanes belong to different families, such as the LuxR/MalT, the AraC/XylS, the GntR or other non-related families of regulators. For some of them there is evidence supporting that *n*-alkanes or *n*-alkanols act as effectors. As alkanes are apolar molecules that most likely accumulate into the cytoplasmic membrane, while transcriptional regulators are normally cytoplasmic proteins, the question arises as to how regulators interact with the alkanes. The *A. borkumensis* AlkS transcriptional regulator is believed to activate expression of the gene coding for the AlkB1 alkane hydroxylase and of downstream genes in response to alkanes (van Beilen *et al*., 2004; Schneiker *et al*., 2006). In a proteomic study this regulator appeared associated to the membrane fraction, rather than to the cytoplasmic fraction (Sabirova *et al*., 2006). Although AlkS does not show the characteristics expected for a membrane protein, it may have affinity for the inner side of the cytoplasmic membrane, where it has an easy access to the alkanes acting as effectors. After binding the alkane, AlkS should move and find its binding site on the DNA. The membrane affinity of other alkane-responsive regulators has not been analysed.

The induction of the alkane-degradation genes can be modulated in several ways to accommodate it to the existing environmental or physiological conditions. Three examples will be considered here: differential regulation, product repression and catabolite repression.

Differential regulation of multiple alkane hydroxylases

Some bacterial strains contain only one alkane hydroxylase, as is the case for the well-characterized alkane degrader *P. putida* GPo1. However, many other strains have several alkane-degradation systems, each one being active on alkanes of a certain chain-length or being expressed under specific physiological conditions. Differential regulation is directed by specific regulators that respond to a particular range of effectors, or by global regulators that respond to a physiological signal. For example, *Acinetobacter* sp. strain M-1 contains two AlkB-related alkane hydroxylases, named AlkMa and AlkMb, which are differentially regulated depending on the alkane present in the medium. The enzymatic differences

between the two proteins are not clear to date. Expression of AlkMa, which is controlled by the AlkRa regulator, is induced by alkanes having a very long chain length ($> C_{22}$), while that of AlkMb is induced by AlkRb in the presence of C_{16}–C_{22} alkanes (Tani *et al*., 2001).

Pseudomonas aeruginosa strains RR1 and PAO1 contain two alkane hydroxylases, AlkB1 and AlkB2. At least in strain PAO1, the substrate range of these two enzymes overlaps significantly, because AlkB1 oxidizes C_{16}–C_{24} *n*-alkanes while AlkB2 is active on C_{12}–C_{20} *n*-alkanes. The regulation of the genes coding for AlkB1 and AlkB2 has been studied in strain RR1 (Marín *et al*., 2003). Both genes are induced by C_{10}–C_{22} alkanes, although expression of *alkB1* is almost twice as efficient as that of *alkB2*. The *alkB2* gene is induced preferentially during the early exponential phase of growth, while *alkB1* is induced in the late-exponential phase of growth. The expression of both genes declines in stationary phase. The regulators responsible for this differential regulation have not been identified.

Some bacterial strains have three or more alkane oxidation systems; this has been found both in hydrocarbonoclastic bacteria and in species having a versatile metabolism. For example, *A. borkumensis* has two AlkB-like alkane hydroxylases and three genes coding for cytochromes P450 believed to be involved in alkane oxidation (van Beilen *et al*., 2004; Hara *et al*., 2004; Schneiker *et al*., 2006). In addition, *A. borkumensis* seems to have other uncharacterized genes involved in oxidation of branched alkanes and phytane (Schneiker *et al*., 2006). Finally, a gene similar to *Acinetobacter* sp. DSM 17874 *almA*, which oxidizes alkanes of very long chain length, has been predicted in *A. borkumensis* SK2 (Throne-Holst *et al*., 2007). Expression of all these alkane oxidation genes should be differentially induced according to the substrate present under each circumstance, although the regulators involved and/or the signals involved are poorly characterized.

The substrate range of the *A. borkumensis* AlkB-like alkane hydroxylases partially overlaps. AlkB1 oxidizes C_5–C_{12} *n*-alkanes, while AlkB2 is active on C_8–C_{16} *n*-alkanes (van Beilen *et al*., 2004). They probably share the auxiliary proteins rubredoxin and rubredoxin reductase, which are encoded by genes that map separately from *alkB1* and *alkB2*. The expression of *alkB1* and *alkB2* is very low when cells grow using pyruvate as the carbon source, but is strongly induced when C_{10}–C_{16} alkanes are metabolized; expression decreases considerably upon entry into stationary phase (van Beilen *et al*., 2004; Sabirova *et al*., 2006; Schneiker *et al*., 2006). A gene coding for a protein showing similarity to the *P. putida* GPo1 AlkS transcriptional activator maps adjacent to *alkB1*. Contrary to what was observed for *P. putida* GPo1 *alkS,* transcription of *A. borkumensis* AP1 *alkS* seems to be constitutive (van Beilen *et al*., 2004). A proteomic analysis detected higher AlkS levels in hexadecane-grown cells than in pyruvate-grown cells (Sabirova *et al*., 2006), although it is unknown whether this is due to increased *alkS* transcription, to a higher stability of the AlkS protein in the presence of alkanes, or to other reasons. The promoter for the *A. borkumensis alkB1* gene contains an *alkS*-binding site immediately upstream of the −35 promoter element and *P. putida* GPo1 AlkS could substitute for *A. borkumensis* AP1 at activating expression of the *alkB1* gene (van Beilen *et al*., 2004). Therefore, *A. borkumensis* AlkS probably activates expression of the *alkB1* alkane hydroxylase in response to alkanes. However, AlkS is unlikely to regulate expression of *alkB2*. A gene coding for a transcriptional regulator of the GntR family is located just upstream of *alkB2*, although its role has not been reported.

The three *A. borkumensis* genes coding for similar cytochromes P450 of the CYP153 family are believed to participate in alkane-degradation (Schneiker *et al*., 2006). Cytochrome P450-1 maps adjacent to other genes involved in the oxidation of alkanes. Cytochrome P450-2 is identical to P450-1, and highly homologous to P450-3. Proteomic profiling analyses revealed that P450-1 and/or P450-2, which cannot be differentiated with this technique, are expressed in cells grown with either pyruvate or hexadecane as the carbon source, although expression was higher in alkane-grown cells (Sabirova *et al*., 2006). As P450-1 is probably cotranscribed with other adjacent genes that are upregulated by hexadecane, it is likely that expression of P450-1 is induced by hexadecane but that of P450-2 and P450-3 is not. A gene coding for a transcriptional regulator of the AraC family maps close to P450-1, but its role has not been reported.

Product repression

In some cases the activation of the pathway genes is inhibited when the concentration of the alkane-degradation products increases over a certain threshold, a phenomenon called ‘product repression’. In *P. butanovora*, for example, expression of the genes coding for BMO is activated by BmoR, a σ^{54}-dependent transcriptional regulator that recognizes as effectors the alcohols and aldehydes derived from the alkanes oxidized by BMO; alkanes are not recognized as effectors (Fig. 3; Kurth *et al*., 2008). This avoids induction by compounds that are not substrates for BMO. Propionate, the final product of propane oxidation, acts as a potent repressor of BMO operon transcription (Doughty *et al*., 2006) and as a direct inhibitor of BMO activity (Doughty *et al*., 2007), an effect that persists until propionate catabolism is induced. Propionate catabolism is inactive during growth on

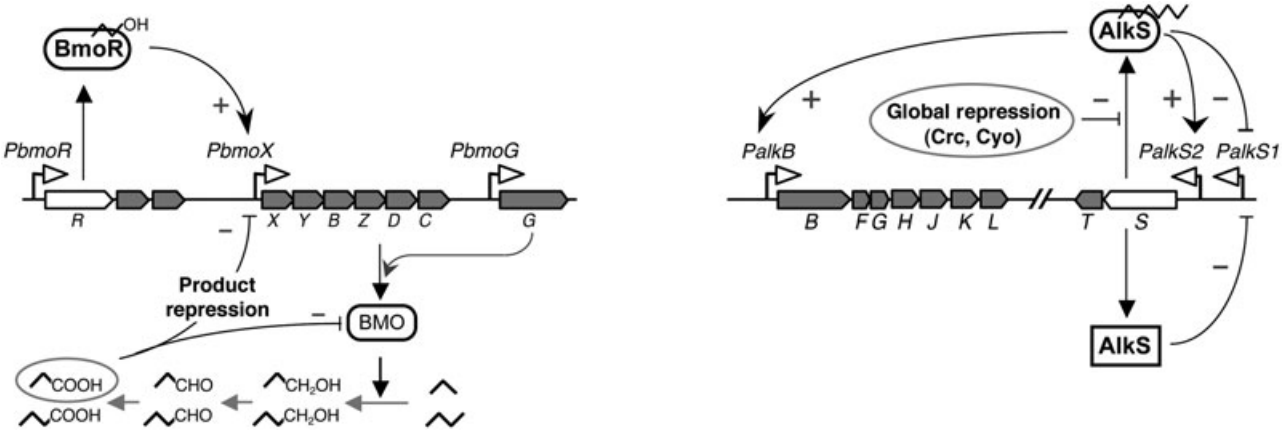

Fig. 3. Regulation of the genes coding for *P. butanovora* BMO (left), and for the *P. putida* GPo1 alkane-degradation pathway (right). Promoters are indicated by open arrows. See text for details.

butane, but is activated by propionate or upon growth on propane or pentane.

There are other examples of product repression in pathways for medium-chain-length alkanes (Ratajczak *et al*., 1998; Marín *et al*., 2001; 2003; Doughty *et al*., 2006). This regulatory mechanism may be a way to coordinate the generation of fatty alcohols and fatty acids from alkanes with their further metabolism, because these compounds tend to accumulate in the cell membrane, increasing the levels of *trans*-unsaturated fatty acids and modifying the composition of the membrane with deleterious consequences to cell physiology (Chen *et al*., 1995; 1996).

Catabolite repression control

Bacterial species that have a versatile metabolism usually possess global regulation systems that assure a hierarchical assimilation of the individual carbon sources, thereby favouring the use of some compounds over other non-preferred substrates. This process is termed catabolite repression. Hydrocarbons are typically non-preferred growth substrates. The expression of alkane-degradation pathways is inhibited by the presence of many other carbon sources in several strains of *P. putida*, *P. aeruginosa*, *B. cepacia*, *P. butanovora* or *Acinetobacter* sp. (Ratajczak *et al*., 1998; Yuste *et al*., 1998; Staijen *et al*., 1999; Marín *et al*., 2001; 2003; Doughty *et al*., 2006). The preferred compounds, and the mechanisms used to modulate the expression of catabolic pathways, vary depending on the bacterial species considered (Rojo *et al*., 2004; Deutscher, 2008; Görke and Stülke, 2008). Catabolite repression probably arises from a number of global regulatory mechanisms directed to optimize carbon metabolism and energy generation in response to different signals. It is not clear what these signals are, but they may be related to the concentration of key metabolites or molecules in the cell that in turn depend on the efficiency of the different catabolic pathways in terms of energy gain.

Catabolite repression has been studied in detail for the alkane-degradation pathway encoded by the *P. putida* GPo1 OCT plasmid, which encodes all genes required for the assimilation of C_3–C_{13} alkanes. The genes of this pathway are grouped in two clusters, *alkBFGHJKL* and *alkST* (van Beilen *et al*., 1994; 2001; see Fig. 3). In the presence of alkanes, the AlkS transcriptional regulator activates the expression of its own gene, and that of *alkT*, from a promoter named *PalkS2*. This allows achieving AlkS levels that are high enough to activate the expression of the *alkBFGHJKL* operon from the *PalkB* promoter (Kok *et al*., 1989; Canosa *et al*., 1999; 2000; Panke *et al*., 1999). AlkS recognizes C_5–C_{10} *n*-alkanes as effectors, but does not respond to shorter or larger alkanes (Sticher *et al*., 1997). *Pseudomonas putida* GPo1 grows optimally on C_5–C_{10} alkanes, but can use C_3–C_4 and C_{11}–C_{13} alkanes as well, although growth is much slower and shows a long lag time (van Beilen *et al*., 2005b; Johnson and Hyman, 2006), probably because these alkanes are poor inducers of the pathway genes. When using an efficient effector such as octane, AlkS can respond to alkane concentrations as low as 25 nM (Sticher *et al*., 1997).

Activation of promoters *PalkB* and *PalkS2* by AlkS is negatively modulated by a dominant global control when cells grow in a complete medium containing alkanes, or in a minimal salts medium containing alkanes and other alternative carbon sources such as amino acids, succinate or lactate (Yuste *et al*., 1998; Staijen *et al*., 1999; Canosa *et al*., 2000). Compounds such as citrate, pyruvate or glycerol, which are also metabolized, do not exert this inhibitory effect. Repression is particularly strong (about 70-fold as measured with a *PalkB–lacZ* transcriptional fusion) during exponential growth in a complete medium, where amino acids are the carbon source used, and rapidly fades away when cells enter into stationary

phase. The repression exerted by succinate or lactate in a minimal salts medium is milder, in the range of 4- to 5-fold (Yuste *et al*., 1998). Repression is not related to the growth rate that each carbon source supports. Continuous cultures in which the source of nitrogen was limited to attain a slow growth rate, but that contained succinate in excess as the carbon source, showed a clear catabolite repression effect. However, repression was not observed when the same growth rate was obtained by limiting the availability of succinate, keeping all other nutrients in excess (Dinamarca *et al*., 2003). Therefore, it is the presence of an excess of succinate what inhibits expression of the alkane-degradation pathway, not the growth rate *per se*.

When cells grow in a complete medium, the negative control inhibiting expression of the alkane-degradation genes depends on the additive effects of two global regulation systems. One of them relies on the global regulatory protein Crc (Yuste and Rojo, 2001), while the other one apparently receives information from the cytochrome *o* ubiquinol oxidase (Cyo), a component of the electron transport chain (Dinamarca *et al*., 2002; 2003). However, when cells grow in a minimal salts medium containing succinate as the carbon source, the role of Crc is small and the inhibitory effect derives mainly from the Cyo terminal oxidase (Yuste and Rojo, 2001; Dinamarca *et al*., 2003).

At least in a complete medium, the inhibition process generates a strong decrease in the levels of the AlkS transcriptional activator, an unstable protein present in limiting amounts even under inducing conditions (Yuste and Rojo, 2001). Keeping AlkS levels below those required for maximal induction of the pathway simultaneously inhibits transcription of the *alkST* and *alkBFGHJKL* operons. Crc is an RNA-binding protein that interacts with the 5′-end of *alkS* mRNA, inhibiting its translation (Moreno *et al*., 2007). Decreasing translation of *alkS* indirectly reduces *alkS* transcription because AlkS activates the expression of its own gene from promoter *PalkS2*. Crc also inhibits expression of many other catabolic pathways for several non-preferred compounds in *Pseudomonads* (MacGregor *et al*., 1996; Hester *et al*., 2000a,b; Morales *et al*., 2004; Aranda-Olmedo *et al*., 2005; Moreno and Rojo, 2008; Moreno *et al*., 2009). The levels and activity of Crc vary depending on growth conditions (Ruiz-Manzano *et al*., 2005), but the underlying signals are not known.

The mechanism through which Cyo inhibits the expression of the alkane-degradation pathway is unclear. Cyo is one of the five terminal oxidases characterized in *P. putida*. The differential expression of these terminal oxidases is carefully regulated to optimize energy production under the prevailing environmental conditions (Williams *et al*., 2007; Ugidos *et al*., 2008). Inactivation of Cyo partially relieves the repression exerted on the alkane-degradation pathway under several conditions, while inactivation of any of the other four terminal oxidases does not (Dinamarca *et al*., 2002; Morales *et al*., 2006). The expression of the Cyo terminal oxidase varies according to oxygen levels and the carbon source being used, and there is a clear correlation between Cyo levels and the extent of repression of the alkane-degradation pathway (Dinamarca *et al*., 2003). The absence of Cyo affects the expression of many other genes, which led to the proposal that Cyo may be a component of a global regulation network that transmits information on the activity of the electron transport chain to coordinate respiration and carbon metabolism (Petruschka *et al*., 2001; Dinamarca *et al*., 2002; Morales *et al*., 2006). Cyo may perhaps transmit a signal to a still uncharacterized factor that, directly or indirectly, would affect the expression of diverse genes. The molecular details, however, are still unknown.

Converting excess carbon into storage materials

When the carbon source is in excess relative to nitrogen, many bacteria transform part of the carbon into storage materials such as triacylglycerols, wax esters, poly(hydroxybutyrate) or poly(3-hydroxyalkanoates), which accumulate as lipid bodies or as granules (Alvarez and Steinbüchel, 2002; Waltermann *et al*., 2005; Prieto, 2007). 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). *Pseudomonas 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 from hexadecane (Ishige *et al*., 2000; 2002).

Concluding remarks

Research performed in the last few years has resulted in many new insights on how microorganisms degrade alkanes. However, several aspects 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, new and different enzymes have recently been found that oxidize high-molecular-weight (C_{20}–C_{50}) alkanes. There are several hints indicating that additional new alkane hydroxylases do exist that have still not been identified. It is also intriguing why bacterial

strains frequently contain several alkane hydroxylases that have very similar substrate specificities. Perhaps these hydroxylases differ in aspects that are still unknown but that are important for cell biology.

Regulation of the genes coding for the alkane-degradation pathways still has many unresolved issues. It has been important to realize that the expression of the alkane-degradation pathways is frequently coordinated with other aspects of cell metabolism. Most efforts have been directed to elucidate the mechanisms responsible for the catabolite repression control, but there are other global regulation phenomena that modulate the expression of alkane-degradation genes. Elucidation of these mechanisms is important to understand how alkanes are degraded in Nature and to design bioremediation strategies that are efficient at stimulating the degradation of alkanes in contaminated sites. The picture is far from clear and requires much more efforts in different microorganisms, because the molecular mechanisms will likely be different in each case.

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