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Degradation of Alkanes in contaminated sites

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Abstract

Petroleum hydrocarbons are important energy resources used by industry and in our daily life, whose production contributes highly to environmental pollution. To control such risk, bioremediation constitutes an environmentally friendly alternative technology that has been established and applied. It constitutes the primary mechanism for the elimination of hydrocarbons from contaminated sites by natural existing populations of microorganisms. Petroleum (or crude oil) is a complex mixture of hydrocarbons. Annually, millions of tons of crude petroleum oil enter the marine environment from either natural or anthropogenic sources. Hydrocarbon-degrading bacteria (HDB) are able to assimilate and metabolize hydrocarbons present in petroleum. Crude oil pollution constitutes a temporary condition of carbon excess coupled to a limited availability of nitrogen that prompts marine oil-degrading bacteria to accumulate storage compounds. This review focuses on the role and distribution in the environment of degradation bacteria and their (potential) applications in bioremediation and biocatalysis. Bacteria play an important role in the microbial degradation of oil, chlorinated hydrocarbons, fuel additives, and many other compounds. Environmental studies demonstrate the abundance of alkane degraders and have lead to the identification of many new species, including some that are (near) obligate alkanotrophs. Bioremediation is being increasingly seen as an affective, environmentally friendly treatment for contaminated shorelines from marine oil spills. Oil bioremediation is limited by the availability of nitrogen and phosphorous which are needed by the bacteria and are not present in sufficient amounts for the biodegradation of the spilled hydrocarbons.

Keywords: crud oil, hydrocarbon, bioremediation, alkan, bacteria.

Introduction

Petroleum hydrocarbons are the most common environmental pollutants, and oil spills pose a great hazard to terrestrial and marine ecosystems. Oil pollution may arise either accidentally or operationally whenever

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oil is produced, transported, stored, processed or used at sea or on land. Oil spills are a major menace to the environment because they severely damage the surrounding ecosystems (Hassanshahian et al. 2012a). Biodegradation by natural populations of microorganisms is the most basic and the most reliable mechanism by which thousands of xenobiotic pollutants, including crude oil, are eliminated from the environment(Cappello et al. 2007a). The effects of environmental conditions on the microbial degradation of hydrocarbons and the effects of hydrocarbon contamination on microbial communities are areas of great interest(Rahman et al. 2004). Bioremediation is a strategy to utilize biological activities to the greatest extent possible for the rapid elimination of environmental pollutants. Stimulation of the growth of indigenous microorganisms, biostimulation and inoculation with foreign oil-degrading bacteria are promising means of accelerating the detoxification of a polluted site with minimal impact on the ecological systems(Cappello et al. 2006). The growth of microorganisms on hydrocarbons presents particular problems because hydrocarbons are immiscible in water. Many bacteria are able to emulsify hydrocarbons in solution by producing surface active agents such as biosurfactants that increase the adhesion of cells to the substrate. Biosurfactants reduce the surface tension by accumulating at the interface of immiscible fluids, increasing the surface area of insoluble compounds, which leads to increased bioavailability and subsequent biodegradation of the hydrocarbons(Batista et al. 2006). Alkanes are major components of crude oil. Alkane hydroxylase is a key enzyme involved in alkane degradation. This enzyme, which introduces an oxygen atom derived from molecular oxygen into the alkane substrate, plays an important role in crude oil bioremediation(Van Beilen et al. 2003). Alkane hydroxylase genes are classified into three groups based on phylogenetic analysis. The group (I) alkane hydroxylases, encoded by alk-B genes, catalyze the degradation of short-chain n-alkanes (C_6–C_{12}). The group (II) alkane hydroxylases, encoded by alk-M genes, catalyze the degradation of medium-chain n-alkanes (C_8–C_{16}), and the group (III) alkane hydroxylases, encoded by alk-B genes, catalyze the degradation of long-chain n-alkanes (>C_{16})(Kohno et al. 2002). Examples of strains capable of growing with nalkanes as a sole carbon source include *Alcanivorax borkumensis SK2*, which shows growth on C_6 to C_{16} nalkanes(Hara et al. 2004); *Rhodocuccus sp. Q15*, which grows on C_8 to C_{32}(Whyte et al. 2002); and *Acinetobacter sp. M-1*, which can degrade C_{13} to C_{44} n-alkanes(Sakai et al. 1994). One well-studied system for aerobic n-alkane degradation is the alk system of *Pseudomonas putida GPo1*(Baptist et al. 1963; Eggink et al. 1987b), which is encoded by genes found on the octane (OCT) plasmid(Chakrabarty et al. 1973; van Beilen et al. 2001). The first step of n-alkane degradation by this system is catalyzed by AlkB, an integral membrane protein that carries out a terminal hydroxylation of the n-alkane(Kok et al. 1989). The electrons needed to carry out this step are delivered to AlkB via a rubredoxin reductase (AlkT) and two rubredoxins (AlkF and AlkG)(van Beilen et al. 2002b). The resulting alcohol is further converted to a fatty acid via a pathway involving an alcohol dehydrogenase (AlkJ), an aldehyde dehydrogenase (AlkH), and an acyl CoA synthetase (AlkK), after which, it enters the β-oxidation pathway(van Beilen et al. 2001). Enzyme systems homologous to the alk system of *P. putida* GPo1 have been found in several bacterial species(van Beilen et al. 2002b), and it has been shown that alkB homologues, generally named alkB or alkM, sometimes occur as two or more paralogues within the same strain(Whyte et al. 2002; van Beilen et al. 2004). For example, *A. borkumensis* carries two alkB homologues, alkB1 and alkB2, which have been shown to play a role in the degradation of C_6 to C_{12} n-alkanes(Hara et al. 2004). Recently, van Beilen et al. (2005) showed that the substrate range of AlkB from *P. putida GPo1* and AlkB1 from *A. borkumensis AP1* is determined by a specific amino acid in the respective proteins, W55 in the *P. putida* AlkB and W58 in the *A. borkumensis* AlkB1. They showed that if these tryptophanes were changed to a less bulky residue, e.g., serine or cysteine, the enzymes could catalyze the hydroxylation of n-alkanes with chain lengths of C_{14} and C_{16}, whereas the wild-type enzymes could only degrade nalkanes shorter than C_{13}(van Beilen et al. 2005).

C_1–C_4

Methane monooxygenases play a key role in the cycling of carbon in the biosphere, are useful biocatalysts, and play a major role in the degradation of xenobiotics in the environment(Murrell et al. 2000). Aerobic methane degraders typically contain membrane-bound particulate copper-containing enzymes (pMMO), whereas under copper limitation, some methanotrophs such as Methylococcus capsulatus (Bath), express soluble non-heme di-iron monooxygenases (sMMO). The latter enzyme system consists of a hydroxylase (MMOH, a 251-kDa α2β2γ2 heterodimer containing the carboxylate-bridged diiron center in the α- subunit), a regulatory component (MMOB), and an NADHdependent reductase(Merkx et al. 2001). As the soluble enzyme was much easier to handle than the membrane-bound enzyme, it initially received more attention, and a 3-D structure has been available since 1993(Lieberman et al. 2005). The sMMO enzyme belongs to a large family of soluble diiron monooxygenases, which have numerous applications in bioremediation and biocatalysis(Shennan 2006) and can be distinguished in five subgroups based on phylogenetic analyses and gene arrangements(Leahy et al. 2003; Coleman et al. 2006). Most of these enzymes act on small hydrophobic molecules, ranging from (halogenated)-alkanes and alkenes to aromatics such as phenol and tetrahydrofuran. The selectivity of sMMO for methane is controlled by the regulatory protein MMO-B; binding of this subunit to the MMO-H subunit, which contains the active site, appears to open a channel with the size of methane into the active site(Zhang et al. 2006). Until recently, structure, location, and mechanism of action of the active site of pMMO have remained elusive. However, Kitmitto and et al (2005), two independent groups has revealed the structure of the elusive pMMO(Kitmitto et al. 2005; Lieberman et al. 2005). It was shown to consist of a hydroxylase (pMMO-H, comprised of the three subunits PmoA, PmoB, and PmoC) and an additional component pMMO-R, formed by two polypeptides with molecular masses of 63 and 8 kDa. This component, which was first proposed to be the reductase, is now thought to be the methanol dehydrogenase, the subsequent enzyme in the methane–oxidation pathway(Myronova et al. 2006). The natural electron donor and electron–transfer pathway have still not been identified. The pMMO enzymes are more restricted in their substrate range than the sMMOs(Elliott et al. 1997), but they also act on longer alkanes as well as on several other hydrophobic compounds. It appears that enzymes involved in the degradation of ethane, propane, and butane belong to several distinct enzyme classes with similarity to sMMO and pMMO. A three component non-heme iron butane monooxygenase (BMO) in '*Pseudomonas butanovora*' was found to be similar to sMMO and hydroxylates C_2 to C_9 alkanes on the terminal position but appeared hardly to be active with methane as a substrate. The basis for this selectivity was studied by site-directed mutagenesis, revealing a striking sensitivity for inhibition of BMO by methanol. Due to geometric constraints of the active site, the product release is believed to be rate-limiting, and in a mutant, this restriction was partly abolished leading to a less methanol sensitive enzyme(Halsey et al. 2006). BMO expression is up-regulated by the oxidation products butyraldehyde and 1-butanol but repressed by propionate, resulting in a difference in the response to even versus odd-chain-length alkanes(Doughty et al. 2006). Kotani et al. (2003) have characterized the Gordonia sp. TY-5 propane monooxygenase, which is also similar to sMMO based on sequence comparisons, but unlike BMO, it appears to have a very narrow substrate range; it only oxidizes propane at the 2-position. The propane and butane degrader *Mycobacterium vaccae JOB5* is known for its ability to co-metabolically degrade trichloroethylene(Kotani et al. 2003) and the gasoline additive methyl-butyl-ether and inhibition patterns indicate that it contains a non-heme iron monooxygenase. *Nocardioides CF8* is likely to contain a copper-containing BMO similar to pMMO(Hamamura et al. 1999).

C_5–C_{16}

Many organisms were found to grow on or co-metabolically oxidize alkanes. Early biochemical studies pointed to the involvement of two main classes of enzymes; the first related to the ubiquitous cytochrome P450 enzymes (P450s) found in mammalian liver(Coon 2005; Bernhardt 2006). These alkane-hydroxylating P450s were found in yeasts and in few bacteria. In several *Pseudomonas* isolates, particulate alkane hydroxylases (pAHs) were detected, which were clearly unrelated to P450 enzymes. The alkane hydroxylase of one particular *Pseudomonas* isolate, now known as *P. putida GPo1*, was characterized in great detail. It was shown to be an integral-membrane non-heme diiron monooxygenase (AlkB), requiring two electron transfer proteins named rubredoxin and rubredoxin reductase. For a long time, this enzyme system remained as the only well-characterized enzyme of this class(van Beilen et al. 1994). However, short conserved sequence motifs discovered using additional pAH sequences from Acinetobacter sp. ADP1 (alkM) and *Mycobacterium tuberculosis H37Rv*(Smits et al. 1999), allowed the amplification of related genes from a wide range of bacteria from α-, β-, and γ-Proteobacteria and the *Actinomycetales*(Van Beilen et al. 2003).

C_{16+}

Alkanes longer than C_{16} support growth of many microorganisms. For example, many *Rhodococcus* isolates grow well on purified alkanes up to C_{32} by virtue of unknown enzyme systems(van Beilen et al. 2002a; van Beilen et al. 2002b), whereas an uncharacterized alkane oxygenase allow *P. fluorescens* to grow on C_{18}–C_{28} alkanes(Smits et al. 2002). Because these alkanes are solids, it has proven difficult to screen for mutants lacking this enzyme system. However, cloning of these enzyme systems by complementation or transposon mutagenesis should be possible. In 1996, Maeng et al. (1996) reported on the isolation of a flavin-containing alkane-dioxygenase in *Acinetobacter sp. M-1*, having a substrate range of C_{10}–C_{30}. Unfortunately, this enzyme has not been cloned yet. It should be noted that the same authors later reported the cloning and characterization of two integral membrane alkane hydroxylases from *Acinetobacter sp. M-1* that cover the same substrate range(Maeng et al. 1996; Tani et al. 2001) .

Microbial degradation of hydrocarbons

The impact of oil spill incidents on marine environments is enormous, and it has triggered research on cost-effective, environmentally benign cleanup strategies. Physical and chemical methods remove rapidly the majority of beached oil, but a significant part of the contaminants remains at the polluted site. In natural degradative processes that remove the remaining oil, bacteria are the predominant agents of hydrocarbon degradation in the environment(Röling et al. 2002). Petroleum oil is highly toxic to the majority of living organism. Its biodegradation in natural ecosystems is complex and depends on the nature of the oil, on the composition and physiological status of the indigenous microbial community, and on a variety of environmental factors which influence microbial activities, e.g., temperature, physical state of oil pollutants, nutrients, etc.(Atlas 1981). The utilization of hydrocarbons by microorganisms has already been reviewed almost 60 years ago by ZoBell (1946), who recognized that (1) many microorganisms are capable of utilizing hydrocarbons as sole carbon and energy sources, (2) they are widely distributed in nature, and (3) the microbial utilization of hydrocarbons is highly dependent on the chemical nature of the petroleum mixture and on environmental determinants(ZoBell 1946; Atlas 1981). A recent review pointed out that there are 79 bacterial genera that can use hydrocarbons as a sole source of carbon and energy, while nine cyanobacterial genera, 103 fungal genera, and 14 algal genera are known to also comprise members capable of degrading or transform hydrocarbons(Head et al. 2006). In the same way, 56 yeasts species are able to utilize hydrocarbons(Atlas 1981). In general, aliphatic paraffins are more readily degraded than aromatic hydrocarbons. Saturated compounds are degraded more readily than unsaturated compounds, and branched chains are decomposed less readily than straight chain

compounds. However, low molecular weight aromatics, such as benzene, toluene and xylene, which are among the toxic compounds found in petroleum, are also very readily degraded by marine microorganisms(Atlas 1995). Microbial hydrocarbon-degrading communities in marine environments of the diverse range of oil-degrading bacteria isolated to date, less than a quarter was isolated from marine environments(Yakimov et al. 2007). Biodegradation of petroleum in marine environments is principally carried out by diverse bacterial populations, including various *Pseudomonas* species(Atlas 1995), which are widely distributed in these environments. These strains had been isolated from diverse sites around the world, indicating that they are distributed ubiquitously(Atlas 1995; Head et al. 2006). In pristine waters, HDB comprise less than 1% of the total bacterial population(Atlas 1981), but they multiply and grow rapidly in oil-polluted waters, where they can constitute 80% to 90% of the microbial community(Harayama et al. 1999; Syutsubo et al. 2001; Kasai et al. 2002). The presence of hydrocarbons in the environment frequently brings about an in situ selective enrichment of hydrocarbon-utilizing microorganisms. There are two essential characteristics that define hydrocarbonutilizing microorganisms: (1) membrane-bound groupspecific oxygenases; and (2) mechanisms for optimizing contact between the microorganisms and the waterinsoluble hydrocarbon(Rosenberg et al. 1992). After an oil spill, a remarkable increase of the relative abundance of oil degraders is usually observed. Several culture-independent studies of oil-impacted marine environments have shown that some bacteria are rapidly and strongly selected when hydrocarbon degradation is stimulated by the addition of nutrients(Head et al. 2006). *Alcanivorax spp*. represent a good example of these bacteria, because they were shown to increase from being undetectable to constituting 70–90% of the bacterial population in oil-treated sea water within 1–2 weeks of nutrient amendment(Syutsubo et al. 2001). *Cycloclasticus pugetii* constitutes another example of "bacterial selection" after an oil spill accident(Maruyama et al. 2003). Marine oil degradation is a process involving a multitude of microorganisms, which are "specialists" in degradation of certain classes of hydrocarbons(Gertler et al. 2009). Obligate hydrocarbonoclastic marine bacteria an unusual group of marine HDB has been recognized and described over the past few years and has been shown to play an important role in the biological removal of petroleum hydrocarbons from contaminated sites. This group of marine bacteria, the obligate hydrocarbonoclastic marine bacteria (OHCB), exhibits a narrow substrate spectrum (obligate hydrocarbon utilization). Only a few species are able to metabolize substrates other than hydrocarbons(Yakimov et al. 2007). Within this group of *γ-proteobacteria*, a number of new genera and families have recently been discovered, comprising the genera *Alcanivorax*, *Cycloclasticus* , *Oleispira* ,*Oleiphilus* , and *Thalassolituus* (Yakimov et al. 2004). Together with species of the genus *Neptunomonas* and *Marinobacter*(Manilla-Pérez et al. 2010), these bacteria constitute an important group of marine hydrocarbon-degrading bacteria. From these, *Alcanivorax borkumensis SK2* became a model organism to study the OHCB because of its predominance and pivotal role in the degradation of hydrocarbons. *A. borkumensis SK2*, a slowly growing, oil-degrading marine bacterium The model strain of the OHCB, *A. borkumensis* strain SK2, is a slowly growing, alkanedegrading Gram negative bacterium, which is present only at low titers in pristine waters. After an oil spill, its population can increase dramatically, and it can become the most abundant organism in oil-polluted waters(Harayama et al. 1999; Syutsubo et al. 2001; Kasai et al. 2002). The predominance of A. borkumensis in early stages of petroleum degradation has also been reported in microcosms and mesocosms studies(Röling et al. 2002; Cappello et al. 2007a; Cappello et al. 2007b) as well as during a field-scale experiment (Röling et al. 2004). *Alcanivorax spp*. seem to be early colonizers after an oil spill event, because after an initial rapid increase in population size, *Alcanivorax spp*. decline to much lower numbers within a few weeks. This phenomenon is related to the depletion of saturated hydrocarbons, suggesting a specialized character of *Alcanivorax* strains to degrade these compounds(Head et al. 2006). Moreover, the importance of *A. borkumensis SK2* as an essential organism in the first steps of marine

hydrocarbon degradation has recently been shown(Gertler et al. 2009). Given the relevance that *A. borkumensis* got in the last years, the genome of this bacterium has been sequenced and annotated, and recent studies have shown the capacity of this strain for adaptation to different environmental stress factors such as UV-radiation, low temperature, or osmotic up-shift. Although genes coding for enzymes which are required for biosynthesis of bacterial storage compounds like polyhydroxyalkanoates (PHAs), triacylglycerols (TAGs), or wax esters (WEs), are present in the genome of A. borkumensis SK2, only TAGs and WEs seem to be the relevant carbon storage compounds produced by this strain. Besides *A. borkumensis SK2*, the genus *Alcanivorax* includes the recently isolated and described species *A. jadensis T9*, *Alcanivorax venustensis ISO4*, *Alcanivorax dieselolei B-5*, *Alcanivorax balearicus LMG 22508*, and *Alcanivorax hongdengensis A-11-3*. Except *A. balearicus*, which was isolated from a subterranean saline lake, all *Alcanivorax* strains were isolated from marine environments. In general, in addition to short-chain fatty acids and some alkanes, *Alcanivorax* strains grow on acetate, lactate, or pyruvate as sole carbon source and in presence of up to 12% (wt./vol.) NaCl. The occurrence of PHB is only reported for *A. venustensis* and A. jadensis, although a mutant of *A. borkumensis* has been recently reported, which produces PHAs extracellularly. Genome characteristics of HDB The genomes of several HDB have been sequenced. These include microorganisms which are able to degrade simple aromatic hydrocarbons such as *Pseudomonas putida KT2440* or *Azoarcus sp. EbN1*, or the aerobic saturated hydrocarbon-degrading bacterium *A. borkumensis* SK2(Manilla-Pérez et al. 2010). From the analysis of completed and partial genome sequences, it is evident that many organisms that are usually not considered to be hydrocarbon-degrading bacteria contain genes that are homologues to alkane hydroxylase genes, the enzymes involved in the first steps of alkane biodegradation(Van Beilen et al. 2003; Beilen et al. 2007). Despite of the intensive studies, several unknown factors remain concerning alkane-degrading enzyme systems. Of the five genera that include OHCB, only the genome of *A. borkumensis SK2* has been sequenced so far, thus allowing insights into genomic and proteomic basis of this metabolic specialization. *A. borkumensis SK2*, like other OHCB, is unable to grow on simple hexoses and other carbohydrates. Genomic analysis revealed that several key enzymes of the glycolytic, pentose phosphate shunt as well as the Entner Doudoroff pathway are lacking, for example glucokinase, glucose-6-phosphate dehydrogenase, aldose isomerase, 2-keto-3-deoxy-6-phosphogluconate aldolase as well as phosphogluconate dehydratase. Furthermore, no functional phosphoenol pyruvate-dependent phosphotransferase system or other types of sugar transporters like ABC transporters can be found in the genome of *A. borkumensis SK2*, despite its generally broad range of various transport proteins(Manilla-Pérez et al. 2010). However, all enzymes of the glycolytic pathway downstream of glucokinase are present. This is not too surprising since intermediates such as glyceraldehyde-3-phosphate or fructose-6-phosphate are essential for biosynthesis of nucleic acids, histidine, and other cell compounds via the pentose phosphate pathway, hence requiring a functional gluconeogenesis. Moreover, *A. borkumensis SK2* produces biosurfactants to make its main source of carbon and energy (alkanes) bioavailable. These biosurfactants consist of an anionic glucose lipid with a tetrameric oxyacyl side-chain (Abraham et al. 1998). In table 1, there are some genera of microorganisms that have been shown to metabolize aliphatic hydrocarbons(Van Beilen et al. 2003).

Table (1): some genera of microorganisms that have been shown to metabolize aliphatic hydrocarbons

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

Aerobic degradation of alkanes

During the past decades, research related to alkane degradation has focused on the identification and characterization of enzymes involved in the initial step of aerobic bacterial catabolic pathways. In most described cases, the nalkane is oxidized to the corresponding primary alcohol by substrate-specific terminal monooxygenases/hydroxylases. However, subterminal oxidation has also been described both for long-chain n-alkane substrates up to C_{16} and for n-alkanes of shorter chainlengths(Wentzel et al. 2007). The class of alkane hydroxylases involved in bacterial aerobic nalkane metabolism has recently been reviewed in detail by van Beilen (2007)(van Beilen et al. 2007). In that review, two unrelated classes of enzymes for long-chain n-alkane oxidation were proposed: (1) the class of cytochrome-P450-related enzymes in both yeasts and bacteria, e.g., bacterial CYP153 enzymes, and (2) the class of bacterial particulate alkane hydroxylases (pAHs). The latter class of integral membrane non-heme diiron monooxygenases of the AlkB-type allows a wide range of *Proteobacteria* and *Actinomycetales* to grow on nalkanes with carbon chain lengths from C_5 to C_{16}. AlkBtype enzymes function in complex with two electron transfer proteins, a dinuclear iron rubredoxin, and a mononuclear iron rubredoxin reductase channeling electrons from NADH to the active site of the alkane hydroxylase(Van Beilen et al. 2003). After initial oxidation of the n-alkane, the corresponding alcohol is subsequently oxidized further by alcohol dehydrogenase and aldehyde dehydrogenase to the corresponding aldehyde and carboxylic acid, respectively. The carboxylic acid then serves as a substrate for acyl-CoA synthetase, and the resulting acyl-CoA enters the β- oxidation pathway. The most extensively studied bacterial alkane degradation pathway of this sequence of enzymatic conversions is that of Pseudomonas putida GPo1 (formerly Pseudomonas oleovorans) encoded on the OCT plasmid(Baptist et al. 1963; Chakrabarty et al. 1973; van Beilen et al. 1994; van Beilen et al. 2001). For details and a comprehensive overview on the alk pathway, we refer to the review by van Hamme et al. (2003)(Van Hamme et al. 2003). For many n-alkane degraders, multiple alkane hydroxylases have been reported exhibiting overlapping substrate ranges, including both pAHs and cytochrome P450 enzymes. For example, *Rhodococcus erythropolis* contains up to five pAHs and two CYP153s(van Beilen et al. 2006), whereas *A. borkumensis* SK2 contains two pAHs

with overlapping substrate specificity (alkB1: C_5–C_{12}, alkB2: C_8–C_{16}) and three CYP153 enzymes with yet undetermined substrate range(Wentzel et al. 2007). Further enzyme candidates involved in long-chain nalkane oxidation up to C_{32} have been deduced from the *A. borkumensis* genome sequence, including putative monooxygenases and oxidoreductases. Also, for other bacterial isolates shown to metabolize long-chain n-alkanes with Cchains of C_{18} and longer, additional enzyme systems have been proposed. However, only a few yet uncharacterized enzymes have been suggested to catalyze oxidation of these long-chain n-alkanes. For example, for *P. fluorescence*, a yet uncharacterized long-chain n-alkane oxygenase was postulated, enabling this strain to grow on n-alkanes ranging from C_{18} to C_{28} (Smits et al. 2002). For *Acinetobacter sp. M-1*, a flavin-containing n-alkane dioxygenase with a proposed substrate range from C_{10} to C_{30} has been reported(Maeng et al. 1996). This strain was later shown to also harbor two integral membrane n-alkane hydroxylases covering the same substrate range(Tani et al. 2001). Using a novel high-throughput screening system for analyzing growth of transposon mutants of another *Acinetobacter* strain, *Acinetobacter sp. DSM 17874*, on solid nalkanes, several genes with potential functions specifically in long-chain n-alkane metabolism have lately been identified. This strain, growing on n-alkanes from C_{10} to C_{40}, had earlier been described to also harbor two AlkB-type pAHs with overlapping substrate preferences ranging from C_{10} to C_{18}. One of the novel genes identified, designated almA, encodes a putative monooxygenase of the flavinbinding family. This gene was analyzed in more detail and found to be involved in the utilization of n-alkanes with a chain length of C_{32} or longer. AlmA represents the first cloned gene encoding an enzyme specifically involved in the degradation of n-alkanes with carbon chains longer than C_{30}. Genes homologous to almA have been identified in and cloned from *Acinetobacter sp. RAG-1*, *Acinetobacter sp. M-1,* and *Acinetobacter baylyi ADP1*(Wentzel et al. 2007). In addition, sequence homology analysis has suggested the presence of similar enzymes also in *Marinobacter aquaeolei VT8, Oceanobacter sp. RED65, Ralstonia spp., Mycobacterium spp., Photorhabdus sp., Psychrobacter spp.,* and *Nocardia farcinica IFM10152*. Further investigations will be necessary to characterize AlmA with respect to its possible function in the oxidation of long-chain n-alkanes. Lately, genome and proteome analysis of *Geobacillus thermodenitrificans strain NG80-2* isolated from a deep-subsurface oil reservoir (Wang et al. 2006) revealed a plasmid-encoded novel thermophilic enzyme for terminal oxidation of n-alkanes, with no detectable similarity to other alkane oxidizing enzymes known to date. This enzyme, designated LadA, was found to be expressed in NG80-2 when grown on crude oil and hexadecane. By complementation of the AlkB functionality in an alkB knockout mutant strain of *Pseudomonas fluorescence CHA0* and in vitro analysis of the LadA protein purified after heterologous expression in *Escherichia coli*, this enzyme was characterized as a thermophilic soluble long-chain alkane monooxygenase for terminal oxidation of long-chain n-alkane substrates ranging from C_{15} to C_{36} (Feng et al. 2007).

Anaerobic degradation of alkanes

Anaerobic metabolism is a vital process with respect to petroleum hydrocarbon biodegradation and bioremediation and, given the unique biochemistry now being uncovered, is also vital with respect to biomimetic catalyst development. Anaerobic degradation of hydrocarbons under nitrate-reducing conditions has been regarded as an effective strategy for bioremediation of crude oil, fuel-contaminated aquifers and underground storage tanks(Rabus et al. 1996). The use of nitrate for in situ remediation of these contaminated environments has been driven not only by the favorable energetics of nitrate reduction but also by high water solubility and mobility of nitrate (92.1 g/100 ml water at 25 ^oC). Crude oil and its refined products constitute the major sources of alkanes in the environment(Ehrenreich et al. 2000). Due to their very low solubility in water, long-chain alkanes (>C_{12}) are generally not considered as toxic. However, at high concentrations, relatively short-chain alkanes (C_5-C_{12}) and cyclic alkanes have negative

effects on biological membranes(Gill et al. 1972; Sikkema et al. 1994; Sikkema et al. 1995). The fate of alkanes and the effectiveness of bioremediation strategies under such conditions require a better knowledge of the biochemical capacities of nitrate-reducing microorganisms. Anaerobic oxidation of hydrocarbons coupled to the reduction of nitrate was first demonstrated with pure isolates (strains ToN1, mXyN1, EbN1, and PbN1) for utilization of alkylbenzenes in crude oil(Rabus et al. 1996). Furthermore, a nitrate-reducing enrichment culture incubated with crude oil did not show any degradation of alkanes (C_5 to C_{12}) until alkylbenzenes (C_1 to C_3) were completely depleted. This indicated that the enrichment culture exhibited biphasic growth, in which alkanes were only partially consumed, and among them, n-hexane appeared to be the preferred substrate. Cell hybridization further revealed that the majority of the enriched denitrifiers affiliated with the b-subclass of the Proteobacteria (Rabus et al. 1999). Ecological distribution of anaerobic hydrocarbons degrading denitrifiers is not restricted to hydrocarbon-contaminated habitats since they have been commonly isolated from non-contaminated environments(Fries et al. 1994; Song et al. 1999; Zwolinski et al. 2000). The currently known pure isolates of alkane-degrading nitrate respiring bacteria (NRB) are affiliated with the b- and g-subclass of the *Proteobacteria*. At least six axenic cultures of alkane-degrading NRB have been documented so far. Strains OcN1 and HxN1were isolated with n-octane and n-hexane, respectively, from enrichment cultures prepared from ditch sediments. The two isolates are members of the family Rhodocyclaceae within the *b-Proteobacteria*. These strains are able to grow for complete oxidation of C_6-C_{12} n-alkanes to CO2 with transitory production of nitrite observed during the growth of strain HxN1(Ehrenreich et al. 2000). When grown anaerobically with crude oil, strain HxN1 has been shown to co-metabolize short-chain (C_4-C_5) and cyclic alkanes (cyclopentane and methylcyclo-pentane) in addition to the utilization of C_6 to C_8 n-alkanes as substrates(Wilkes et al. 2003). This is of great interest because n-butane, n-propane and cyclic alkanes as single substrate could not support the growth of strain HxN1, indicating that such co-metabolism of structurally diverse substrates is catalyzed by enzymes which are needed for the metabolism of the growth supporting substrates, but exert activities on co-substrates due to “relaxed substrate specificities” similar to that observed with anaerobic alkylbenzene-degrading bacteria(Beller et al. 1999; Wilkes et al. 2003). Anaerobic oxidation of long-chain n-alkanes (>C_{12}) coupled to the reduction of nitrate is apparently restricted to members of the g-Proteobacteria. Axenic cultures include strain HdN1 from activated sludge(Ehrenreich et al. 2000), *Marinobacter sp. BC36* and *BP42* from Berre lagoon microbial mats and the recently described denitrifying bacterium *Pseudomonas balearica strain BerOc6* from a brackish lagoon. These cultured members of the *g-Proteobacteria* are able to oxidize n-alkanes ranging from C_{14} to C_{20}. Phylogenetic analysis, based on 16S rRNA gene sequences, shows that strain HdN1 forms its own cluster and probably represent a new lineage within the *g-Proteobacteria*. Recently, molecular-based microbial community analysis of a nitrate-reducing enrichment amended with n-hexadecane as the sole source of carbon and energy revealed the dominant presence of several members of the *Proteobacteria.* One sequence retrieved from a DGGE profile was 82% similar to the anaerobic alkane-degrading nitrate reducer strain HdN1. Anaerobic oxidation of branched and cyclic alkanes has also been demonstrated to occur under nitrate-reducing conditions. Pristane (2,6,10,14-tetramethylpentadecane, C19H40), formerly regarded as recalcitrant under anaerobic conditions, has been shown to be biodegraded under nitrate-reducing conditions in enrichment cultures obtained from a diesel fuel-contaminated sediments. Within the cycloalkanes family, cyclohexane has always been the model substrate for biodegradation studies, however all these studies were conducted under aerobic conditions. As described above, the first direct evidence for the anaerobic oxidation of cycloalkanes, e.g., cyclopentane was found in the nitratereducing strain HxN1 grown anaerobically with n-alkanes from crude oil, the identity of the microorganisms capable of anaerobic degradation of cycloalkanes under nitrate-reducing conditions is still unknown. A recent study on anaerobic degradation of cyclohexane

under nitrate-reducing conditions resulted in the co-enrichment of members of the *d-Proteobacteria* and *Planctomycetales*. This enriched culture of freshwater denitrifying bacteria grown anaerobically with cyclohexane as the sole carbon and energy source was dominated by 16S rRNA gene sequence related to Geobacter spp. (~75% of all cells as detected by phylogenetic analysis and fluorescent whole-cell hybridization using typespecific probe), the likely cyclohexane-degrader. Subsequent nitrite produced during growth of the consortium was not consumed unless extra ammonium was added to the medium. These observations indicated that the Planctomycetales (anammox bacteria) as a partner, approximating 18% of all cells, was consuming nitrite via anaerobic ammonium oxidation(Mbadinga et al. 2011). Such symbiotic association between hydrocarbon-degraders and anammox bacteria can be extrapolated from environments such as petroleum reservoirs where anammox bacteria have also been identified as potential contributors to the nitrogen recyclers. It should be reminded that *Geobacter* species are often the predominant microorganisms found in hydrocarbon-contaminated environments in which Fe(III)-reduction occur and given that Fe(III)-reduction coupled to alkanes degradation has not yet been unequivocally demonstrated despite some experimental attempts(Zwolinski et al. 2000; Mbadinga et al. 2011). There are some anaerobic degrading bacteria of alkanes which have been shown in the table 2 (Wentzel et al. 2007).

Table (2): some bacteria that have been shown to metabolize aliphatic hydrocarbons

Phylum	**Species, strain**	**n-alkane substrate range**
β-Prot.	*Strain HxN1*	C6–C8
β-Prot.	*Strain HdN1*	C14–C20
β-Prot.	*Strain OcN1*	C8–C12
γ-Prot	*Marinobacter sp. BC36*	C18
γ-Prot	*Marinobacter sp. BC38*	C18
γ-Prot	*Marinobacter sp. BC42*	C18
δ-Prot.	*Strain TD3*	C6–C15
δ-Prot.	*Strain Hxd3*	C12–C20
δ-Prot.	*Strain Pnd3*	C14–C17
δ-Prot.	*Strain AK01*	C13–C18
δ-Prot.	*Desulfatibacillum aliphaticivorans CV2803*	C13–C18
δ-Prot.	*Clone B1–B3*	C16

Conclusion

Alkanes are ubiquitous in the environment due to biogenic production and oil pollution, and many aerobic microorganisms are able to use these highly reduced hydrocarbons as a sole carbon and energy source. The oxygenases that are required for the initial activation of alkanes belong to several different enzyme classes, some of which only oxidize shortchain alkanes, while others oxidize medium and long-chain alkanes. Membrane bound alkane hydroxylases related to AlkB of *P. putida GPo1* have been found in many bacteria able to grow on C_5-C_{16} alkanes, and have also been detected in environments such as soils and aquifers. Other enzyme systems involved in alkane assimilation have been studied only in a limited number of strains (often just 1 or 2). Future research efforts are likely to yield additional and perhaps completely novel alkane hydroxylase systems. A major challenge will be to explain the parallel evolution of such diverse families of alkane hydroxylases with essentially the same function. Despite the growing acceptance of bioremediation as a means to treat spilled oil in sites, the mechanisms that promote the

process under field conditions remain poorly constrained. Although general statements can be made regarding the enhancement of biodegradation by nutrient amendment, there is no consensus on how to best optimize nutrient additions. Subsequently, oil spill treatment strategies are largely developed empirically from previous experience or laboratory feasibility studies. Anaerobic hydrocarbon degradation in environments has only recently been widely accepted and there is a need to determine both how widespread an occurrence this is and in what circumstances it will have a significant impact on the dissipation of crude oil contamination. The environmental factors that promote the process must also be identified if it is to be exploited for the treatment of spilled oil. Potential applications in bioremediation and biocatalysis are a strong incentive to study cometabolic oxidations by alkane hydroxylase systems in much greater detail. Studies of alkane hydroxylase gene diversity, coupled with information on substrate range, induction, enzyme kinetics and host properties, should help to optimize the biodegradative activity of indigenous hydrocarbon degrading strains, benefit biocatalytic applications, and promote fundamental research on the activation of oxygen by enzymes and biomimetic catalysts.

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