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Microbial Hydrocarbon Degradation: Efforts to Understand Biodegradation in Petroleum Reservoirs

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1. Introduction

The understanding of the phylogenetic diversity, metabolic capabilities, ecological roles, and community dynamics taking place in oil reservoir microbial communities is far from complete. The interest in studying microbial diversity and metabolism in petroleum reservoirs lies mainly but not only on providing a better comprehension of biodegradation of crude oils, since it represents a worldwide problem for petroleum industry. Generally, biodegradation of oil affects physical and chemical properties of the petroleum, resulting in a decrease of its hydrocarbon content and an increase in oil density, sulphur content, acidity and viscosity, leading to a negative economic consequence for oil production and refining operations [1,2]. Another important point for studying biodegradation lies on its important role in the global carbon cycle and the direct impact on bioremediation of polluted ecosystems. Furthermore, many of the enzymes involved in the degradation pathways are considered key catalysts in industrial biotechnology [3].

Despite these motivations and long recognition of petroleum as a the most important "primary energy" source, at present, microorganisms and factors involved in biodegradation of crude oil hydrocarbons in petroleum reservoirs are still not fully understood. The inaccessibility and complex microbiological sampling of petroleum reservoirs as well as the inherent limitations of the traditional culturing methods conventionally employed can explain this fact. Culture-based techniques have traditionally been the primary tools utilized for studying the microbiology of terrestrial and subsurface environments [4], which allowed the recovery and documentation of a large collection of bacteria capable of hydrocarbon utilization. Studies of numerous aerobic and anaerobic bacterial isolates have revealed mechanisms, which allow them to degrade specific classes of the highly diverse range of hydrocarbon compounds.

Therefore, all we know about the degradation of petroleum compounds has come from studying isolated microorganisms. Here, we provide an overview of what is currently known about the mechanisms of aerobic and anaerobic degradation of hydrocarbons, as a result from biochemical and genomic approaches, we give a perspective of the petroleum microbial diversity unraveled so far, and finally we discuss the common oil reservoir characteristics that can be used to predict the most probable mechanism of degradation into deep petroleum reservoirs.

It is well known that microbial diversity in environment is several orders of magnitude higher than the one assumed based on previous cultivation methods [5]. A particularly large number of novel techniques have been developed, which now allow the determination of the *in situ* microbial diversity and activity on a particular site, screening for a particular gene or activity of interest, gene quantification, and DNA and mRNA sequencing and analysis from total communities. This book chapter will address how the implementation of such culture-independent molecular methods allow the access to the microbial diversity and metabolic potential of microorganisms and bring novel information about microbial diversity and new pathways involved in biodegradation processes taking place in petroleum reservoirs. This information will certainly contribute to a broader perspective of the biodegradation processes and corroborate with previous findings that degradation of pollutants in many cases is carried out by microbial consortia rather than a single species [6], where key species and catabolic genes are often not identical to those that have been isolated and described in the laboratory [7, 8].

2. Microbial diversity in oil reservoirs

Recognition of indigenous microbiota harbored by oil reservoirs has been discussed for a long time. Actually, determining the nature of isolated microorganisms from oil reservoirs (indigenous or nonindigenous) is a difficult issue concerning petroleum microbiologists. The reasons for this controversy rely mainly on the difficulty of aseptic sampling in deep oil reservoirs. This means that microorganisms observed in oil field fluids conceivably could be contaminants introduced during drilling operations and/or during sample retrieval, or could be material sloughed from biofilms growing in installed pipes. Another reason for skepticism is the commonplace practice of ''water- flooding'' (injection of surface waters or re-injection of natural formation waters to maintain reservoir pressure for oil production); since in this case microbes would be introduced during injection and therefore would not necessarily represent indigenous species [9].

In addition to this controversy, there is the fact that petroleum reservoirs are considered extreme environments where *in situ* conditions, like high pressure, temperature, salinity and anaerobic conditions, are considered as inhospitable to microbial activity. In fact, perception of deep subsurface as a sterile environment has only changed during the past two decades with the increasing awareness of the ability of microbes to colonize extreme environments. Actually, with the use of more sophisticated and appropriate sampling and cultivation

techniques, as well as the application of molecular biological techniques to oil field fluids, the dogma of the sterile deep subsurface has been dispelled [9]. Rather, it has become clear that many oil reservoirs do harbor indigenous microbes (*e.g.* the genera *Geotoga* and *Petrotoga* isolated only from oil reservoirs) [10]. Nowadays it is clear that worldwide petroleum reserves are dominated by deposits that have been microbially degraded over geological time and biodegraded petroleum reservoirs represent the most dramatic manifestation of the deep biosphere [11]

In spite of the polemics on which micro-organisms would actually be native and which would be contaminants in oil reservoirs, a wide range of microbial taxonomic groups have been identified in oil reservoirs geographically distant using traditional techniques adapted to *in situ* conditions, as described by L'Haridon et al. [12], Grassia et al. [13] and reviewed by Magot et al [14], or combined with cultivation-independent molecular methods, as reported by Orphan et al. [15]. Table 1 summarizes the various physiological and taxonomical groups and species that have been isolated from oil reservoirs.

3. Aspects from oil reservoir determining microbial degradation

For a long time, the mechanism considered to be prevalent for oil degradation in petroleum reservoirs was the well documented aerobic microbial metabolism and it has long been thought that the flow of oxygen through meteoric waters was necessary for in-reservoir petroleum biodegradation [16]. This mechanism has been widely accepted despite the fact that oxygen would likely be consumed by oxidation of organic matter in near surface sediments and therefore, would be very unlikely for oxygen to reach deep petroleum reservoirs [11].

Recently, the discovery of the ability of microorganisms to degrade anaerobically hydrocarbon oil components and the detection of metabolites characteristic of anaerobic hydrocarbon degradation in oil samples from biodegraded reservoirs, but not in non-degraded reservoirs or aerobically degraded oils [11], have provided valuable information to determine the processes involved in the degradation of oil reservoirs. Nowadays, evidences of such degradation through anaerobic rather than aerobic processes are becoming more substantial and compelling [17].

It is known that microorganisms in anaerobic conditions can use a variety of final electron acceptors, including nitrate, iron, sulfate, manganese and, more recently, chlorate. Anaerobic degradation has also been coupled to methanogenesis, fermentation and phototrophic metabolism but growth of these microorganisms and, therefore, biodegradation rates are significantly lower compared to aerobic degraders. These anaerobic processes have been demonstrated in surface sediments and pure cultures or enrichments in laboratories [18] and all of them potentially play a role in oil biodegradation in anoxic petroleum reservoirs [11]. However, nitrate, like oxygen, is highly reactive and would likely be completely consumed before it could reach the oil reservoir [17]. In deep reservoirs, the supply of large amounts of Fe(III) or manganese(IV) via meteoric water influx are unlikely due to poor solubility and slow water recharge rates in subterranean cycles. Therefore, iron and manganese, which could be

used as electro acceptors for oil oxidation, are unlikely to be responsible for significant compositional changes in the oil, considering their limited availability in the reservoir. Accordingly, oil degradation linked to sulfate reduction and methanogenic would therefore explain the consistent hydrocarbon compositional patterns seen in degraded oils worldwide [17]. Sulfate arises from geological sources, such as evaporitic sediments and limestone, or from the injection of seawater for pressure stabilization, and may lead to significant oil degradation and increased residual-oil sulfur content. Methanogenic oil degradation, on the other hand, does not require external electron acceptors and leads to less overall souring of the oil reservoir. Several studies have described *in vitro* methanogenic degradation of crude oil related compounds [19, 20] Jones et al., 2008), including n-alkanes [21, 20] and aromatic hydrocarbons [17].

Organism	Taxonomical group	Metabolism	Origin	Reference
Thermodesulforhabdus norvegicus	Deltaproteobacteria	Sulfate-reducer	Oil field in Norway	[22]
Desulfacinum infernum	Deltaproteobacteria	Sulfate-reducer	North see petroleum reservoir near Scotland	[23]
Desulfomicrobium norvegicum	Deltaproteobacteria	Sulfate reducer	Petroleum reservoir in Canada	[24]
Desulfovibrio sp.	Deltaproteobacteria	Sulfate reducer	Petroleum reservoir in Canada	[24]
Dethiosulfovibrio peptidovorans	Bacteria, Synergistetes	Sulfate reducer	Oil well in the Emeraude oilfield in Congo, Central Africa,	[25]
Desulfotomaculum thermocisternum	Bacteria, Firmicutes	Sulfate reducer	Oil reservoir in the North sea	[26]
Deferribacter sp.	Bacteria, Deferribacteres	Sulfate reducer	California oil fields	[15]
Halanaerobium congolense	Bacteria, Firmicutes	Thiosulfate- and sulfur-reducing bacterium	African oil field	[27]
Thauera phenylacetica	Betaproteobacteria	Nitrate reducer	Petroleum reservoir in Canada	[24]
Pseudomonas stutzeri	Gammaproteobacteria	Nitrate reducer	Petroleum reservoir in Canada	[24]
Garciella nitratireducens	Bacteria, Firmicutes	Nitrate reducer	Oil field in Tabasco, Gulf of Mexico	[28]
Geobacillus subterraneus, Geobacillus uzenensis	Bacteria, Firmicutes	Nitrate reducer	Petroleum reservoir in China	[29]

Organism	Taxonomical group	Metabolism	Origin	Reference
Lactosphaera pasteurii	Bacteria, Firmicutes	Fermentative	Petroleum reservoir in Canada	[24]
Propionicimonas paludicola	Bacteria, Firmicutes	Fermentative	Petroleum reservoir in Canada	[24]
Anaerobaculum	Bacteria, Synergistetes	Fermentative	California oil fields	[15]
Thermococcus sp.	Archaea, Euryarchaeota	Fermentative	California oil fields	[15]
Thermococcus sibericus	Archaea, Euryarchaeota	Fermentative	Petroleum reservoir in Western Siberia	[30]
Petrotoga sp.	Bacteria, Thermotogae	Fermentative	California oil fields	[15]
Petrotoga olearia; P. siberica	Bacteria, Thermotogae	Fermentative	Petroleum reservoir in Western Siberia	[12]
Thermoanaerobacter	Bacteria, Firmicutes	Fermentative	California oil fields	[15]
Thermotoga sp.	Bacteria, Thermotogae	Fermentative	California oil fields	[15]
Thermosipho geolei	Bacteria, Thermotogae	Fermentative	Petroleum reservoir in Western Siberia	[12]
Anaerobaculum thermoterrenum	Bacteria, Synergistetes	Fermentative	Oil well in Utah	[23]
Fusibacter paucivorans	Bacteria, Firmicutes	Fermentative	Oil well in the Emeraude oilfield in Congo, Central Africa	[31]
Thermovirga lienii	Bacteria, Synergistetes	Fermentative	Oil reservoir in the North sea	[32]
Methanococcus	Archaea, Euryarchaeota	Methanogen	California oil fields	[15]
Methanococcus thermolithotrophicus	Archaea, Euryarchaeota	Methanogen	North sea old field in Norway	[33]
Methanoculleus	Archaea, Euryarchaeota	Methanogen	California oil fields	[15]
Methanobacterium	Archaea, Euryarchaeota	Methanogen	California oil fields	[15]

Table 1. Summary of bacteria isolated from oil reservoirs worldwide.

Deep subsurface environments such as petroleum reservoirs are logistically much more difficult to study than contaminated shallow subsurface environments [17]. Since in many biodegraded petroleum reservoirs most biodegradation occurs close to the oil water transition zone, it has been proposed that the oil–water transition zone (OWTZ) provides suitable physical and chemical conditions for microbial activity [17].

There are other physical and chemical parameters influencing *in situ* biodegradation. Temperature is one of the main factors which limits oil degradation in reservoir, and, empirically,

it has been repeatedly observed that biodegradation does not occur in oil reservoirs with *in situ* temperatures >80-90°C [34]. Salinity is another factor that affects in-reservoir oil biodegradation, especially in combination with temperature [13]. Typically, reservoirs with highly saline waters show limited oil biodegradation [11]. This is consistent with the observations that it has not been possible to cultivate microorganisms from reservoir waters with salinity greater than 100 g/L [13]. Pressure seems to be a less limiting factor, except that it may select for certain physiological types and influences the pH of pore waters by increasing dissolution of CO_2 [9]. The availability of electron donors and acceptors governs the type of bacterial metabolic activities within oil field environments [14]. The potential electron donors include CO_2, hydrocarbons, H_2 and numerous organic molecules. Availability of fixed nitrogen is unlikely to limit microbial activity in reservoirs. However, the availability of water-soluble nutrients, like phosphorus and/ or oxidants (terminal electron acceptors such as ferrous iron, sulfate or CO2), is more likely to limit *in situ* microbial activity [9]. Nonetheless, physiological characteristics of microorganisms indigenous to petroleum reservoirs shed light on the conditions under which petroleum degradation may occur and the potential degradation mechanisms.

4. Hydrocarbon degradation

Hydrocarbons are understood as the compounds that consist exclusively of carbon and hydrogen. Because of the lack of functional groups, hydrocarbons are largely apolar and exhibit low chemical reactivity at room temperature. Differences in their reactivities are primarily determined by the occurrence, type and arrangement of unsaturated bonds. Therefore, in this chapter, we will use the common way to classify hydrocarbons according to their bonding features: i) aliphatic group, which includes straight-chain (n-alkanes), branched-chain and cyclic compounds and ii) aromatic group which includes mono or polycyclic hydrocarbons an many important compounds which also contain aliphatic hydrocarbon chains (*e. g.*, alkylbenzenes).

Already a century ago, bacterial isolates had been reported to use aliphatic and aromatic hydrocarbons as sole carbon and energy sources [35]. Since then, numerous aerobic, and also anaerobic, bacterial isolates have been studied in order to understand the mechanisms which allow them to degrade specific members of the highly diverse aliphatic and aromatic compounds. Degradation by such isolates has been investigated thoroughly and results have revealed that they can completely degrade most classes of hydrocarbons, including alkanes, alkenes, alkynes and aromatic compounds. Such degradation can occur aerobically, with oxygen, or anaerobically, with nitrate, ferric iron, sulfate or other electron acceptors [36].

Efforts to overview the metabolism of hydrocarbons in microorganisms are confronted with the chemical diversity of such compounds and their reactivities, as well as with various microbial life styles [36]. The study of biodegradation is conventionally treated in separate areas: aliphatic vs. aromatic hydrocarbons, aerobic vs. anaerobic degradation pathways, physiology and overall metabolic pathways vs. enzymatic mechanisms and structures, often

with limited knowledge and data exchange. Nonetheless, each of these study areas deals with the same central point that is the ''metabolic challenge'' to guide an apolar, unreactive compound composed only of carbon and hydrogen into the metabolism [36]. The hydrocarbon must be first functionalized and currently it has been recognized that there is a surprisingly diversity of reactions of activation that had evolved in microorganisms (Table 2).

Mechanisms for hydrocarbon activation		
	Aerobic	**Anaerobic**
Short-Chain non-methane alkanes C2-C10	• Non-heme iron monooxygenase similar to sMMO (C2-C9) • Copper-containing monooxygenase similar to pMMO (C2-C9) • Heme-iron monooxygenases (also refered as soluble Cytochrome P450 (C5-C12)	• Fumarate addition
Long-Chain alkanes >C10	• Heme-Monooxygenase (P450 type) • [Fe2]-Monooxygenase • Non-heme iron monooxygenase (AlkB-related) (C3-C13 or C10-C20) • Flavin-binding monooxygenase (AlmA) (C20- C36) • Thermophilic flavin-dependent monooxygenase (LadA) (C10-C30)	• Fumarate addition • Carboxylation
Aromatic hydrocarbons	• [Fe]-Dioxygenase • [Fe2]-Monooxygenase • [Flavin]-Monooxygenase	• Fumarate addition • Hydroxylation • Carboxylation

Table 2. Overview of aerobic and anaerobic mechanisms for hydrocarbon activation in bacteria.

Mechanisms for hydrocarbon activation are basically different in aerobic and anaerobic microorganisms. Under oxic conditions, hydrocarbon metabolism is always initiated using molecular oxygen as a co-substrate in mono- or dioxygenase reactions that enable the terminal or sub-terminal hydroxylation of aliphatic alkane chains or the mono or dihydroxylation of aromatic rings [37]. In the hydrocarbon activation under anoxic conditions, some proposed reactions comprise: (1) addition to fumarate by glycyl-radical enzymes, (2) methylation of unsubstituted aromatics, (3) hydroxylation with water by molybdenum cofactor containing enzymes of an alkyl substituent via dehydrogenase, and (4) carboxylation catalyzed by yet-uncharacterized enzymes which may actually represent a combination of reaction (2) followed by reaction (1) [38; 37]. Although all these mechanisms of hydrocarbon anaerobic activation have been proposed, the required signature metabolites and enzymes involved have been characterized only for (1) addition to fumarate (demonstrated for toluene, xylene, ethylben-

zene, methylnaphthalene, alkanes and alicyclic alkanes); for (3) hydroxylation (demonstrated for ethylbenzene); and for (4) carboxylation (demonstrated for benzene and naphtalene) [39].

5. Biochemical and genetic pathways of microbial hydrocarbon degradation
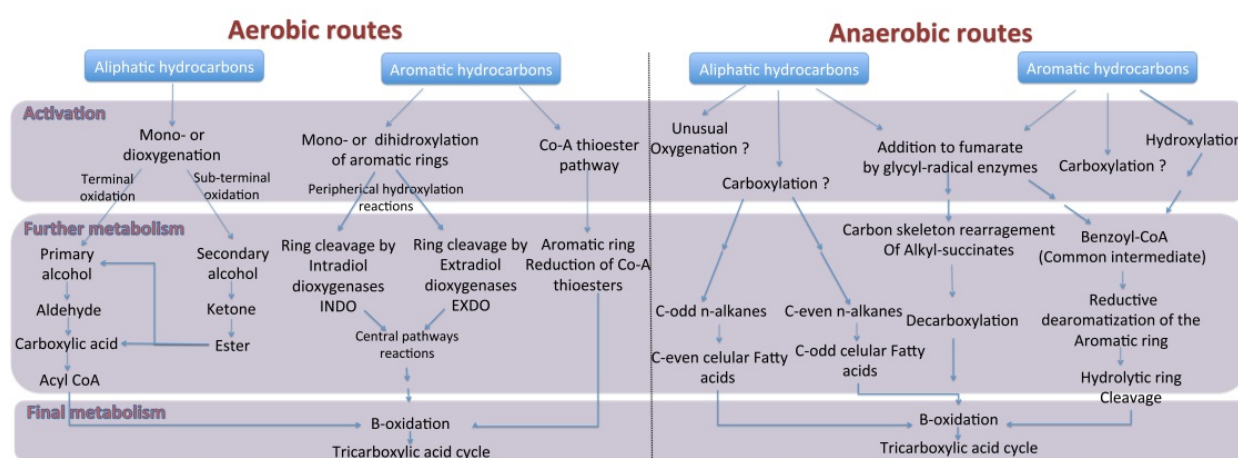
The enzymatic reactions involved in the aerobic degradation of hydrocarbons by bacteria have been extensively studied for several decades [37]. Genes encoding enzymes for degradation are relatively well understood for aerobic and easily cultivable microorganisms, particularly for a *Pseudomonas* strain, known as *P. putida* GPo1, as well as for the strains *Acinetobacter* sp. ADP1 and *Mycobacterium tuberculosis* H37Rv [39, 40]. On the other hand, the anaerobic hydrocarbon degradation has gained more attention since is supposed to be the predominant mechanism occurring in several polluted environments and oil reservoirs. However, its study is an incipient area because of the peculiarities of the reservoir environment and difficulties that arise from attempts to characterize these communities. Nevertheless, several bacteria from other environments able to use alkanes as carbon source in the absence of oxygen have been described in the last few years [41], but anaerobic bacteria able to degrade hydrocarbons under conditions found in deep petroleum reservoirs have not been isolated so far [2]. Figure 1 represents an overview of the main mechanisms and pathways used by microorganisms to degrade hydrocarbon compounds under aerobic and anaerobic conditions.

5.1. Aerobic degradation

5.1.1. Aliphatic hydrocarbons

In most degradation pathways described, the substrate n-alkane is oxidized to the corresponding alcohol by substrate-specific terminal monooxygenases/hydroxylases. The alcohol is then oxidized to the corresponding aldehyde, and finally converted into a fatty acid. Fatty acids are conjugated to CoA and subsequently processed by β – oxidation to generate acetyl-CoA [42, 40]. Subterminal oxidation has also been described for both short and long-chain alkanes [40]. Both terminal and sub-terminal oxidation can coexist in some microorganisms [41]. Initial terminal hydroxylation of n-alkanes in bacteria can be carried out by enzymes belonging to different classes, named: (1) propane monooxygenase (C3), (2) different classes of butane monooxygenase (C2-C9), (3) CYP153 monooxygenases (C5-C12), (4) AlkB-related non-heme iron monooxigenase (C3-C10 or C10-C20), (5) flavin-binding monooxigenase AlmA (C20-C36), (6) flavin-dependent monooxygenase LadA (C10-C30), (7) copper flavin-dependent dioxygenase (C10-C30) [43].

Among all the alkane activating enzymes, the integral membrane non-heme iron monooxygenase (AlkB) is the best characterized one. Microorganisms degrading medium (C5-C11) and long (>C12)-length alkanes have been frequently related to the presence of *alk*B genes and that is why the presence of such genes have been widely used as functional biomarker for the characterization of aerobic alkane-degrading bacterial populations in several environmental

Figure 1. Pathways for aerobic and anaerobic bacterial degradation of hydrocarbon compounds. Two arrows represent more than one reaction.

samples [44, 45] and in bioremediation experiments [46, 47]. The degradation pathway of the *alk* system was first described in *Pseudomonas putida* GPo1 (formerly identified as *P. oleovorans* GPo1), where it is located on the OCT plasmid. In this model system, OCT plasmid contains two operons: *alk*BFGHJKL and *alk*ST [48]. The first operon encodes two components of the *alk* system, a particulate non-heme integral membrane alkane monooxygenase (AlkB) and the soluble protein rubredoxin (AlkG), as well as other enzymes involved in further steps. The second operon encodes for a rubredoxin reductase (AlkT and AlkS), which regulates the expression of the *alk*BFGHJKL operon [48, 49]. Since this system was described, AlkB homologous have been found in many alkane-degrading α- β – and γ –Proteobacteria and high G + C content Gram-positive bacteria (Actinobacteria) [39] and an increasing collection of alkane hydroxylase gene sequences has allowed the diversity analysis of hydrocarbon-degrading microbial populations in different ecosystems. However, comparisons of cloned *alk*B genes or gene fragments have showed that sequence diversity is very high, even among *alk*B genes within the same species [50].

In despite of the relevance of *alk*B genes as a functional biomarker of alkane-degrading bacterial communities, knowledge on the presence and diversity of *alk*B genes in oil reservoirs is scarce. Tourova et al. [51] analysed *alk*B diversity in thermophilic bacterial strains of the genus *Geobacillus* isolated from oil reservoirs or hot springs. They detected, for the first time, sets of *alk*B gene homologous in thermophilic bacteria, and some strains showed different homologous within the same genome. This fact was explained by the occurrence of horizontal gene transfer among these bacteria. Recently, Li et al. [52] aimed to evaluate *alk*B gene diversity and distribution in production water from 3 oilfields in China through a specific PCR-DGGE method. Results showed that sequences found in the water samples were similar to *alk*B genes from other corresponding alkane-degrading strains. But at the same time, they showed the presence of a considerable genetic diversity of alkB genes in the wastewater as evidenced by a total of 13 unique DNA bands detected. Studies on the degradation of alkanes in oil reservoirs are currently in a start point, but in the future they certainly will help to understand the process of degradation in oil reservoir.

In comparison to the few efforts in studying *alk*B system in oil reservoirs, much less is known about the presence of the other enzymatic systems previously listed, which have been described for aerobic degradation of n-alkanes in isolated bacteria or laboratory microcosms. For the most recent elucidated systems for alkane oxidation, named *almA* and *ladA* genes, nothing is known about the environmental distribution of these type of genes in petroleum contaminated sites [53] or oil fields, although the LadA complete degradation pathway has been characterized through genome and proteome analysis of *Geobacillus thermodenitrificans* NG80-2, a thermophilic strain isolated from a deep oil reservoir in Northern China [54]. Currently, it is believed that there are enzyme systems for alkane degradation which have still not been characterized and that may include new proteins unrelated to those already known [41]. Moreover, in many alkane degraders more than one alkane oxidation system have been observed, which have been reported exhibiting overlapping substrate ranges [39, 40]. These observations point out that in order to characterize and explore metabolic diversity and functions involved in alkane degradation one should take into consideration the high diversity of enzymes capable of initiating such metabolism.

5.1.2. Aromatic hydrocarbons

The aerobic bacterial catabolism of aromatic compounds involves a wide variety of peripheral pathways that activate structurally diverse substrates into a limited number of common intermediates that are further cleaved and processed by a few central pathways to the central metabolism of the cell [55]. Metabolic pathways and encoding genes responsible for the degradation of specific members of a highly diverse range of aromatic compounds have been characterized for many isolated bacterial strains, predominantly from the Proteobacteria and Actinobacteria phyla [56]. Degradation by such isolates is typically initiated by members of one of the three superfamilies: the Rieske non-heme iron oxygenases (RNHO), the flavoprotein monooxygenases (FPM) and the soluble diiron multicomponent monooxygenases (SDM). Further metabolism is achieved through di- or trihydroxylated aromatic intermediates. Alternatively, activation is mediated by CoA ligases where the formed CoA derivates are subjected to selective hydroxylation [58, 53]. In the case of hydrophobic pollutants, such as benzene, toluene, naphthalene, biphenyl or polycyclic aromatics, aerobic degradation is usually initiated by activation of the aromatic ring through oxygenation reactions catalyzed by RNHO enzymes or, as intensively described for toluene degradation, through members of SDM enzymes [56].

Further intermediates can be catalyzed by two kinds of enzyme, intradiol and extradiol dioxygenases, which represent two classes of phylogenetically unrelated proteins [58]. These enzymes are key enzymes in the degradation of aromatic compounds, and many of such proteins and their encoding sequences have been described, purified and charac-terized in the last decades [56]. While all intradiol dioxygenases described so far belong to the same superfamily, the extradiol dioxygenases include at least three members of different families. Type I extradiol dioxygenases (e.g. catechol 2,3-dioxygenases and 1,2-dioxygenases) belong to the vicinal oxygen chelate superfamily enzymes. Type II extra-diol dioxygenases are related to LigB superfamily (e.g. protocatechuate 4,5-dioxygenases)

and the type III enzymes belongs to the cupin superfamily (e.g. gentisate dioxygenases) [53]. However, members of novel superfamilies performing crucial steps in aromatic metabolic pathways are still being discovered [56, 53].

The knowledge of metabolic properties of isolates has allowed the monitoring of the ability of microorganisms to mineralize aromatic hydrocarbons in soils. Typically, these studies have used primers designed based on conserved gene regions and focused on RNHO or SDM as targets for initiating degradation, or on Extradiol dioxygenases (EXDO) cleaving the aromatic ring [59]. These studies range from those searching for a narrow range of genes similar or identical to those observed in type strains using non-degenerated primers to those searching for subfamilies of homologous genes using degenerated primers [59]. However, due to the immense heterogeneity of such enzymes [57], there will never be a pair of primers that will reliably cover the huge diversity of a catabolic gene family in nature [53].

5.2. Anaerobic degradation

5.2.1. Aromatic hydrocarbons

We have already described the main mechanism for degradation of aromatic compounds in aerobic conditions, where oxygen is not only the final electron acceptor but also co-substrate of two key processes: hydroxylation and cleavage of the aromatic ring by oxygenases. In contrast, in the absence of oxygen, microorganisms use a complete different pathway, based in reductive reactions to attack the aromatic ring [61].

The biochemistry of some anaerobic degradation pathways of aromatic compounds has been studied to some extent; however, the genetic determinants of all these processes and the mechanisms involved in their regulation are much less studied [55]. Recent advances in genome sequencing have led to the complete genetic information for six bacterial strains that are able to anaerobically degrade aromatic compounds using different electron acceptors and that belong to different taxonomic groups of bacteria: denitrifying betaproteobacteria, *Thauera aromatica* and *Azoarcus* sp. EbN1, two alphaproteobacteria, the phototroph *Rhodopseudomonas palustris* strain CGA009 and the denitrifying *Magnetospirillum magneticum* strain AMB-1, and two obligate anaerobic deltaproteobacteria, the iron reducer *Geobacillus metallireducens* GS-15 and the fermenter *Syntrophus aciditrophicus* strain SB [55]. It is worth remembering that, in recent years, important inferences and generalizations have been made about the genetics involved in hydrocarbon metabolism based on these isolated bacteria under conventional laboratory conditions. However, potential novel genes, enzymes and metabolic pathways responsible for degradation processes are probably harbored by yet uncultivated bacteria.

The best understood and apparently the most widespread of these anaerobic mechanisms is the radical-catalyzed addition of fumarate to hydrocarbons, yielding substituted succinate derivatives. This reaction has been recognized for the activation of several alkyl-substituted benzenes as well for n-alkanes [62]. However, understanding of this fumarate-dependent hydrocarbon activation is most advanced in the case of toluene. The key enzyme in this process is the enzyme benzylsuccinate synthase. All enzymes required for β-oxidation of benzylsuccinate are encoded by the *bbs* operon. Subsequent degradation of benzoyl-CoA proceeds via

reductive dearomatization, hydrolytic ring cleavage, β-oxidation to acetyl-CoA units and terminal oxidation to Co_2 [63]. In contrast to the anaerobic metabolism of toluene, degradation of ethylbenzene (and probably other alkylbenzenes with carbon chain of at least 2) is entirely different, despite the chemical and structural similarities between the two compounds, and involves a direct oxidation of the methylene carbon via (S)-1-phenylethanol to acetophenone [55]. Ethylbenzene is anaerobically hydroxylated and dehydrogenated to acetophone, which is then carboxyled and converted to benzoylCoA as the first common intermediate of the two pathways [62].

Genetics of the enzymatic system have been only characterized for these two mechanisms for anaerobic hydrocarbon activation. Genes encoding pathways that involve fumarate addition are typically organized in two operons. One operon includes the three structural genes of the protein catalyzing fumarate addition and the other includes genes required for converting succinate derivates to benzoyl-CoA [64]. Gene sequences and organization are relatively conserved among nitrate-reducing bacteria but differ somewhat from those of the iron reducer *G. metallireducens* [64] and substantially from those of the hexane-degrading nitrate reducer strain HxN1 [65]. Hydrocarbon dehydrogenation pathway is also organized in two operons. One operon contains the structural genes for the first two reactions (ethylbenzene dehydrogenase and 1-phenylethanol dehydrogenase) and the other contains the structural genes for acetophone carboxylase [64].

Kane et al. [66] developed the first real-time polymerase chain reaction (PCR) method to quantify hydrocarbon utilizers based on *bss*A genes of nitrate-reducing Betaproteobacteria. Since then, there have been several additional studies investigating the presence and/or distribution of anaerobic hydrocarbon utilizers in anaerobic environments via functional gene surveys of *bss*A, extending the range of detectable hydrocarbon-degrading microbes to iron and sulfate-reducing Deltaproteobacteria and revealing partially novel, site specific degrader populations [67, 68]. Other *bss*A-based detection studies in impacted environments, as well as studies that combine field metabolomics and molecular tools, are described by other authors [69, 70, 71]. Despite of the role of benzylsuccinate synthase in aromatic hydrocarbon degradation and its use as a biomarker are well documented, there is no study on the presence of this gene in oil reservoirs.

5.2.2. Aliphatic hydrocarbons

Anaerobic degradation of alkanes has not been extensively studied as for some aromatic compounds. The presumable reasons include the greater attention given to BTEX compounds (benzene, toluene, ethylbenzene and xylenes) because of their classification as priority pollutants [71], also the fact that anaerobic growth with n-alkanes is even slower than that with the alkylbenzenes, and finally the fact that long chain alkanes are poorly soluble and often prevents the cultivation of cells homogeneously in the medium [72]. However, anaerobic degradation of alkanes is equally relevant, since alkanes are quantitatively the most important hydrocarbon components of petroleum, and some are acutely toxic and difficult to remediate [71]. Several anaerobic bacteria capable of degrading n-alkanes with 6 or more carbons in

length, particularly hexadecane (C16), using sulfate or nitrate as electron acceptors have been isolated [72, 73].

The two main mechanisms of anaerobic degradation of n-alkanes described involve unprecedented biochemical reactions that differ completely from those employed in aerobic hydrocarbon metabolism [73]. The first involves activation at the subterminal carbon of the alkane by the addition of fumarate, analogously to the formation of benzyl succinate during anaerobic degradation of toluene, however further reactions are completely different involving dehydrogenation and hydration [72]. Studies conducted with established axenic cultures have indicated that anaerobic metabolism of oil allkanes predominantly proceeds via addition of fumarate to the double bound [72]. Although alkylsuccinate metabolites have rarely been detected in oil reservoir fluids [74, 75], they have been reported in oil-contaminated environments as well as in oilfield facilities, where their detection is indicative of *in situ* microbial degradation of oil alkanes [71, 75]. Alkylsuccinic acids as intermediates of anaerobic alkane oxidation were first studied by Gieg and Suflita [76] when surveying these metabolites in aquifers contaminated with condensate gas, natural gas liquids, gasoline, diesel, alkanes and BTEX. They found alkylsuccinates originating from C3 to C11 alkanes, as well as putative metabolites originating from compounds with one degree of unsaturation, such as alkenes or alicyclic alkanes. Since this report, other studies have detected alkylsuccinate derivates in petroleum contaminated groundwater systems [76], coal beds [70] and oil fields [74, 77]. The formation of alkylsuccinates is catalyzed by a strictly anaerobic glycyl radical enzyme which has been termed as alkylsuccinate synthase or (1-methyl-alkyl)succinate synthase (Ass or Mas). The genes encoding Ass have recently been identified in the alkane degrading sulfidogenic bacteria *D. alkenivoras* AK-01 [78] and *Desulfoglaeba alkanedexens* ALDC[T] [71], as well as in nitrate reducing strains HxN1 [65] and OcN1 [79], all affiliated to the Proteobacteria phylum [80]. Recently, Callaghan et al. [71] detected *ass*A genes in a propane-utilizing mixed culture and in a paraffin-degrading enrichment culture maintained under sulfate-reducing conditions. Despite of no genes for benzyl-and alkylsuccinate synthase were found when environmental metagenome datasets of uncontaminated sites were analyzed in Callaghan et al [71], the authors consider that *ass*A gene could be a useful biomarker for anaerobic alkane metabolism.

The second mechanism for alkane anaerobic degradation is the carboxylation, mainly developed from the growth pattern of the sulfate-reducing strain Hxd3 [81], tentatively named as *Desulfococcus oleovorans*. This strain differs from other alkane degraders for converting C-even alkanes into C-odd cellular fatty acids whereas growth on C-odd alkanes resulted in C-even cellular fatty acids [81, 72]. More recently, Callaghan et al. [82] suggested that a carboxylation-like mechanism analogous to the activation strategy previously proposed by So et al. [81] was the probable route for the anaerobic biodegradation of hexadecane in an alkane-degrading, nitrate-reducing consortium. However, in both cases, the hypothetical fatty acid intermediate (2-ethylalkanoate) that should result from the incorporation of inorganic carbon at C-3 of the alkane has never been detected. There is an on-going debate about this initial activation mechanism. From an energetic point of view, the carboxylation of alkanes is not feasible under physiological conditions, unless the concentration of the fatty acid (2-ethylalkanoate) is in the micromolar order of magnitude or less [80].

Other alternative activation mechanisms are proposed for the anaerobic degradation of alkanes. For instance, the mechanism referred as "unusual oxygenation" is used by the strain *Pseudomonas chloritidismutans* AW-1^T, that is assumed to produce its own oxygen via chlorate respiration used for subsequent metabolism of alkanes [60]. Other alternative mechanism considers that activation in the anaerobic methanogenic system may be initiated by an anaerobic hydroxylation reaction [83].

6. Mechanisms involved in oil biodegradation in petroleum reservoirs

From those microorganisms studied in oilfields, methanogens have received particular attention since they have been isolated and molecularly detected in both low- and high-temperature reservoirs [88, 89]. Their physiological characteristics and potential activity possibly involved in methanogenesis occurring in oil reservoirs have been demonstrated [90]. Furthermore, recently, Jones et al. [20] provided evidence that the patterns of hydrocarbon degradation observed in biodegraded petroleum reservoirs were the result of methanogenic processes. Therefore, microbiological and biogeochemical investigations have indicated that methanogenesis is a widely distributed process in petroleum reservoirs, although still poorly understood [90]. Methanogenesis is the terminal process of biomass degradation. Acetate and hydrogen are the most important immediate precursors for methanogenesis, and are converted into methane by acetoclastic and hydrogenotrophic methanogens, respectively [91]. Acetate can also be a precursor for methanogenesis through syntrophic acetate oxidation coupled to hydrogenotrophic methanogenesis, which is mediated by syntrophic bacteria and methanogenic archaea [92, 93, 94, 95]. Interestingly, acetate is generally abundant in many petroleum reservoirs, at concentrations ranging between 0.3 and 20 mM [96] hence, acetate metabolism is considered an important methane production process in those environments [90].

Cultivation-dependent and -independent approaches have shown the presence of acetoclastic and hydrogenotrophic methanogens and putative syntrophic acetate-oxidizing bacteria in reservoirs [88, 89, 102], indicating that there should be two different pathways of acetate metabolism in the environment, namely acetoclastic methanogenesis and syntrophic acetate oxidation coupled with hydrogenotrophic methanogenesis. Some previous studies suggested that syntrophic acetate oxidation was most likely to occur in petroleum reservoirs, based on molecular biological analysis [89] and thermodynamic calculations [98]. In Jones et al. [20], the composition of oil in microcosms exhibiting methanogenic oil degradation is compared to patterns observed in biodegraded oils from the Gullfaks field in the North Sea. Analysis of the methanogenic communities from oil-degrading microcosms revealed a strong selection for CO_2-reducing methanogens against acetoclastic methanogens, and gas isotope modeling also revealed that to match the d13C of methane and carbon dioxide from biodegraded petroleum reservoirs 75–92% of methanogenesis should be via the CO_2 reduction pathway [20, 11].

The reason why syntrophic acetate oxidation predominates over acetoclastic methanogenesis in oil reservoirs remains unclear. There is evidence from studies of oil contaminated aquifers that crude oil can have a detrimental effect on acetoclastic methanogenesis and, in situations
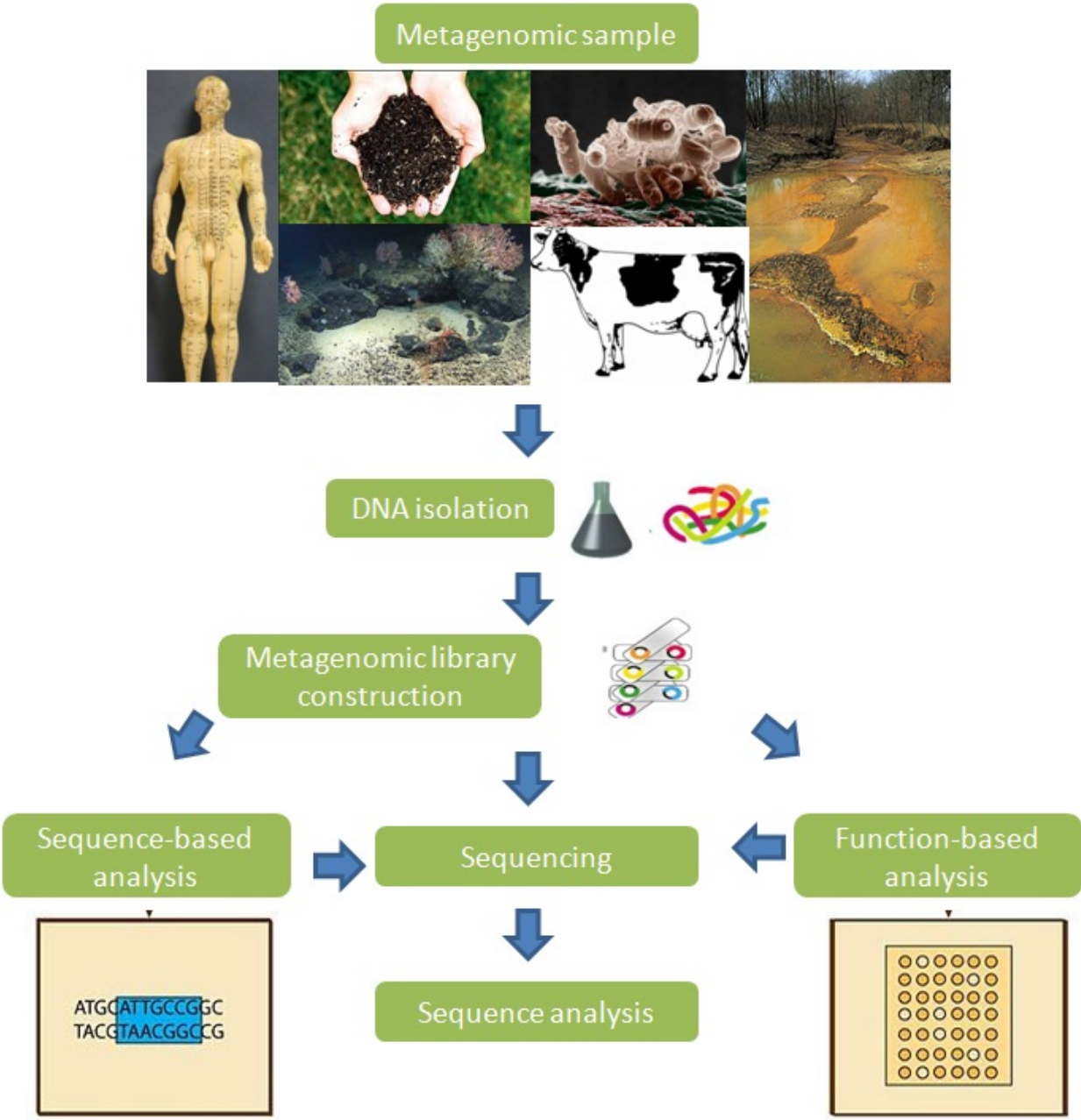
where acetoclastic methanogenesis is inhibited, methanogenic alkane degradation via syntrophic acetate oxidation may be thermodynamically the most favorable alternative pathway [11]. Nonetheless, one recent report suggests that acetoclastic methanogenesis may predominate in some methanogenic oil-degrading systems [19]. Although there is currently great interest in how much each of the two pathways contributes to methane production in petroleum reservoirs, no studies are being conducted to address this question [90].

7. Metagenomics as a tool for a better comprehension of biodegradation

As stated previously, cultivation-based methods have traditionally been utilized for studying the microbiology in oil fields and have yielded valuable information about microbial interactions and their relations with hydrocarbons [42]. However, nowadays, it is known that only a small fraction of the microbial diversity in nature (1-10%) can be grown in the laboratory [84, 85, 86]. Therefore, it is assumed that the ecological functions of the majority of microorganisms in nature and their potential applications in biotechnology remain obscure [87].

In metagenomics, total DNA is extracted from appropriately chosen environmental samples, propagated in the laboratory by cloning techniques, submitted to sequence or function-based screenings and/or subjected to large-scale sequence analysis (Fig. 2). Functional screening of metagenomic libraries offer the advantage that it does not rely on sequence homology to known genes, and for this reason, has allowed the isolation of different enzyme classes from several environments. The probability (hit rate) of identifying a certain gene depends on multiple factors that are intrinsically linked to each other: the host–vector system, size of the target gene, its abundance in the source metagenome, the assay method, and the efficiency of heterologous gene expression in a surrogate host [99].

One of the first studies using metagenomics to study microbial degradation of aromatic compounds was performed by Suenaga and colleagues [100], who constructed a metagenomic library from activated sludge for industrial wastewater. The library was functionally screened for extradiol dioxygenase activities (enzymes for aromatic degradation) and 38 clones were subjected to sequencing analysis [101]. As a result, various types of gene subsets were identified that were not similar to the previously reported pathways performing complete degradation. Moreover, the authors discussed the fact that aromatic compounds in the environment may be degraded through the concerted action of various fragmented pathways. Sierra-Garcia [101] reported the organization of hydrocarbon degradation genes of selected metagenomic fosmid clones derived from a metagenomic library from Brazilian petroleum reservoir and functional screening for hydrocarbon degradation activities. The author found many putative proteins of different aerobic and anaerobic well described catabolic pathways, however the complete catabolic pathways described for hydrocarbon degradation in previous studies were absent in the fosmid clones. Instead, the metagenomic fragments comprised genes belonging to different pathways, showing novel gene arrangements where hydrocarbon compounds were degraded through the concerted actions of these fragmented pathways. These results suggest that there are marked differences between the degradation genes found

Figure 2. Schematic representation of the different steps for metagenomic analysis.

in microbial communities derived from enrichments of oil reservoir sample and those that have been previously identified in bacteria isolated from contaminated or pristine environments.

However, function-based screening of metagenomic libraries for xenobiotic degradation genes is often considered problematic because of insufficient and biased expression of the heterologous genes in the host *Escherichia coli* [99]. Only a few efforts have been made to solve these problems. In Uchiyama et al. [103], a novel method for function-driven screening is described, which was termed substrate-induced gene expression screening (SIGEX). This high-through-

put screening approach employs an operon trap gfp expression vector in combination with fluorescence-activated cell sorting. The screening is based on the fact that catabolic-gene expression is induced mainly by specific substrates and is often controlled by regulatory elements located close to catabolic genes [103]. Using this approach, Uchiyama et al. [103] isolated aromatic-hydrocarbon-induced genes from a metagenomic library derived from groundwater. In Ono et al. [104] another screening strategy was based on functional comple‐mentation of a *Pseudomonas putida* host strain containing a naphthalene degrading pathway devoid of the naphthalene dioxygenase (NDO) encoding gene. Two clones were able to restore the ability of the host strain to use naphthalene as a sole carbon source and their genes were similar but no identical to already known operons. The authors refer to the use of other host strains for the construction of metagenomic libraries instead of the well-established *E. coli* as a simpler and economical way to perform function-driven screening in comparison to other reported systems such as SIGEX [103].

In the context of this chapter, several aspects of the hydrocarbon degradation need to be studied to obtain a comprehensive overview of the biodegradation processes that take place in oil reservoirs or petroleum impacted environments. These studies should take into consid‐eration the high diversity of enzymes capable of initiating such metabolism as well as the implementation of integrated studies combining culture and molecular techniques, linking with metabolomics or compound-specific isotope analysis and microcosm studies for a better resolution of in situ microbial activity in petroleum reservoirs.

8. Conclusions and research needs

The understanding about biodegraded petroleum reservoirs have advanced considerably in recent years, but the organisms responsible for the *in situ* activity and a quantitative under‐standing of the factors which control in-reservoir oil biodegradation remain far from complete. The inaccessibility of petroleum reservoirs and inherent difficulties of microbiological sampling from commercially operating oil wells have required a multidisciplinary approach to delineating the study of subsurface petroleum biodegradation, and to date there are still prevailing paradigms relating to hydrocarbon biodegradation processes. This multidiscipli‐nary approach to study *in situ* petroleum degradation should consider molecular biology, microbiology, and geological and geochemical parameters in order to establish the key organisms, biochemical reactions and mechanisms involved in such complex associations. Indeed, the isolation of anaerobic microorganisms capable of utilizing hydrocarbons is essential for a comprehensive understanding of their role and behavior in anoxic habitats and their complex interactions within methanogenic hydrocarbon-degrading communities. In addition, novel approaches, combining functional metagenomics, transcriptomics, metabolo‐mics and other molecular surveys in microcosms are urgently required to better allow access to a more realistic phylogenetic and metabolic diversity governing oil biodegradation in petroleum reservoirs.

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