Anaerobic degradation of oil hydrocarbons by sulfate-reducing and nitrate-reducing bacteria

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ABSTRACT

Crude oil is a complex mixture mainly composed of various saturated and aromatic hydrocarbons. Whereas degradation of hydrocarbons by oxygen-respiring microorganisms has been known for nearly one century, utilization of hydrocarbons under anoxic conditions has been investigated only during the past ten years. The present paper summarizes investigations into the anaerobic degradability of crude oil as a complex mixture of hydrocarbons. Anaerobic growth on crude oil was observed in enrichment cultures and pure cultures of sulfate-reducing bacteria. Several alkylbenzenes and *n*-alkanes were specifically consumed with concomitant reduction of sulfate to sulfide; consumed hydrocarbons together accounted for up to approximately 10% of the crude oil. Incompletely consumed alkylbenzenes exhibited an enrichment in ^{13}C versus ^{12}C. Sulfate-reduction with oil hydrocarbons may offer an explanation for ancient microbial processes in oil reservoirs where reduced sulfur species are present, and for the undesirable sulfide formation in oil production plants. Furthermore, an anaerobic consumption of alkylbenzenes and *n*-alkanes from crude oil in enrichment cultures and pure cultures of denitrifying bacteria could be demonstrated.

Introduction

Most crude oils are composed of more than 75% of aliphatic and aromatic hydrocarbons [54, 57]. Oil hydrocarbons belong to the large global fraction of organic carbon that has been preserved from an ancient biosphere due to burial followed by diagenetic and catagenetic transformation processes. The withdrawal of photosynthetically fixed carbon from reoxidation and recycling into the inorganic pool by bacteria and other organisms gave rise to our oxic atmosphere. The total mass of O_2 on our planet amounts to 1,200 Tt (1,185 Tt in the atmosphere and around 12 Tt dissolved in water reservoirs; values calculated from data summarized by Greenwood and Earnshaw [28]. One Tt = 10^{12} t). Assuming that for each molecule of O_2 generated by water cleavage one atom of carbon (C) is fixed from CO_2, according to the net equation of photosynthesis, 450 Tt organic C must have been

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deposited (C-loss due to diagenetic decarboxylation processes not considered). The real amount of biologically fixed organic C may be even higher since some O_2 has been consumed by reaction with originally reduced inorganic compounds such as ferrous minerals. Living organisms and dead biomass (in soil and water) on our planet contain 0.8 and 2.8 Tt C, respectively, which is 0.18% and 0.62%, respectively, of the total organic C (summarized from [13]). The estimated amounts in accessible fuel reservoirs are 0.23 Tt C in oil harboured in conventional reservoirs as well as in shales and sands, 0.044 Tt C in gas, 0.22 Tt C in lignite and 0.95 Tt C in coal (calculated from data summarized by [54, 57]). The sum is 1.4 Tt accessible fuel-C, which is 0.32% of the total organic C. Hence, one has to postulate that around 445 Tt, i.e. around 99% of photosynthetically produced and presently preserved organic C is distributed in sediments of various ages where its inaccessibility to biological reoxidation processes guarantees the maintenance of our oxic biosphere. The hydrocarbon content of this organic material is unknown. But even if one assumes that this enormous reservoir of organic carbon consists on the average of no more than 1% of hydrocarbons, as the less maturated kerogens (the diagenetically transformed biomass that gave rise to oil), it would add around 4 Tt hydrocarbon-C to the 1.4 Tt organic C in accessible fuel reservoirs. Hence, studying the biological degradation or degradability of hydrocarbons means directing our research interest not only to an environmentally and technologically important, but also to a globally rather dominant group of organic compounds.

Brief historical overview

Biological hydrocarbon oxidation was first demonstrated around the turn of the century in fungal and bacterial cultures (for summary see [15]). The biochemical mechanisms of hydrocarbon oxidation have been elucidated [14, 15]. The only aerobic activation mechanism of saturated hydrocarbons (open-chain and cyclic alkanes) that has been substantiated thus far is the monooxygenase reaction. Aromatic hydrocarbons are initially attacked by monooxygenases or dioxygenases, depending on the type of alkyl side chain or the type of microorganism [26].

During the 1940s, and again twenty years later, there were reports of an anaerobic oxidation of alkanes by sulfate-reducing bacteria of the genus *Desulfovibrio* [19, 42, 51]. However, cultures have not been preserved, or attempts to reproduce hydrocarbon oxidation by *Desulfovibrio* strains failed [1, 2]. Interest in possible hydrocarbon oxidation by sulfate-reducing bacteria arose from their frequent presence in oil production plants where these bacteria produce sulfide with its many undesirable effects (for overview see [17, 43, 45]. Even though sulfate-reducing bacteria were recognized as the source of sulfide produced in oil fields in the 1920s [7], their electron donor and carbon substrates in these habitats remained a matter of discussion for several decades. In his review written in 1958, ZoBell supposed that crude oil itself, provided it is dispersed in mineral solution, supports growth of certain sulfate-reducing bacteria; but at that time he expressed certain doubts about an anaerobic utilization of hydrocarbons as reported before [59]. Furthermore, geochemical studies invoked an interest in possible hydrocarbon oxidation by sulfate-reducing bacteria. Investigations into the genesis of sulfur deposits led to the assumption that hydrocarbons from oil formerly served as electron donors for sulfate-reducing bacteria and yielded sulfide that was subsequently oxidized with oxygen to sulfur [52].

The first hydrocarbons for which an anaerobic degradation could be unequivocally shown were alkylbenzenes; degradation of these, especially of toluene, was shown in enriched bacterial populations [27, 29, 33, 53] and in pure cultures of iron-reducing [37], denitrifying [4, 5, 20, 23, 24, 48, 55] and sulfate-reducing [10, 47] bacteria. In the meantime, several details of the biochemistry and underlying genes of anaerobic toluene activation have become known [9, 12, 18, 34, 46]. Toluene is activated by condensation with fumarate yielding benzylsuccinate. This is further oxidized by reactions somewhat resembling β-oxidation of fatty acids and yielding benzoyl-CoA and succinyl-CoA. Furthermore, anaerobic degradation of the unsubstituted aromatic hydrocarbons, benzene and naphthalene could be measured in enriched bacterial communities [21, 36, 58].

Consumption of an *n*-alkane as the only organic growth substrate under anoxic conditions was demonstrated in quantitative experiments with newly isolated, mesophilic types of sulfate-reducing bacteria under strict exclusion of air [2, 53]. Furthermore, three strains of denitrifying bacteria have been isolated and shown to grow anaerobically on defined *n*-alkanes [22; Behrends, Harder, Rainey, Widdel, unpublished]. The mechanism of anaerobic alkane oxidation is still insufficiently understood. Fatty acid analyses after anaerobic growth on *n*-alkanes suggested that in one type of sulfate-reducing bacterium the carbon chain of the alkane is altered at the end by one carbon atom during activation; one possibility would be the terminal addition of a C_1-unit. This mechanism may not occur in other species [3].

Growth of sulfate-reducing bacteria on crude oil

In addition to individual hydrocarbons, crude oil as a natural, complex mixture of hydrocarbons was also tested as growth substrate for sulfate-reducing bacteria. A mesophilic enrichment culture from an oil tank was shown to utilize alkylbenzenes from crude oil added as the only source of organic compounds to defined anoxic mineral medium [53]. Whole-cell hybridization with fluorescently labelled 16S rRNA-targeted oligonucleotide probes revealed that more than 95% of the enriched population were members of the suggested family of the *Desulfobacteriaceae* [45]. Members of this family of sulfate-reducing bacteria are distinctive from *Desulfovibrio* and *Desulfomicrobium* species, for which the family *Desulfovibrionaceae* has been suggested. This observation is in agreement with the finding that many sulfate-reducing bacteria that degrade aromatic compounds are members of the *Desulfobacteriaceae*. Subsequent attempts to isolate the microorganisms responsible for depletion of alkylbenzenes from crude oil in the enrichment culture yielded two types of novel sulfate-reducing bacteria. One strain oxidized *o*-xylene, *o*-ethyltoluene and toluene, the other strain oxidized *m*-xylene, *m*-ethyltoluene, *m*-isopropyltoluene and toluene [30]. The anaerobic consumption of alkylbenzenes by the enrichment culture caused an isotopic discrimination, as obvious from analysis of the remaining part of the respective hydrocarbons (Table 1).

Furthermore, utilization of *n*-alkanes from crude oil by sulfate-reducing bacteria has been demonstrated. A moderately thermophilic sulfate-reducing bacterium (optimum around 60 °C) isolated on *n*-decane consumed *n*-alkanes from crude oil especially in the range from C_8 to C_{11} [53]. Furthermore, an enrichment culture exhibited sulfate-dependent consumption of *n*-alkanes from oil [16].

Table 1. Fractionation of carbon isotopes of alkylbenzenes during their utilization from crude oil in a sulfate-reducing enrichment culture after 54 days of incubation. 1,2,4-Trimethylbenzene is included as a representative of alkylbenzenes that did not show noticable consumption in this enrichment culture.

Compound	Degradation (%)	^{13}C enrichment in remaining compound[a] (‰)
Toluene	100	ND[b]
o-Xylene	69	4.0
m/p-Xylene[c]	63	2.4
o-Ethyltoluene	77	6.0
m/p-Ethyltoluene[c]	58	2.6
1,2,4-Trimethylbenzene	0	0.3

[a] Reproducibility of the analysis was 0.3‰.

[b] ND, not determined because of complete consumption during the experiment.

[c] The *m*- and *p*-isomers could not be separated by the applied gas-chromotographic method. Additional analysis by another separation method revealed that the enrichment culture oxidized the *m*-isomers.

It can be calculated from the volume of oil added to the cultures of sulfate-reducing bacteria and from the amount of sulfide produced [53] that around 10% (vol/vol) of the crude oil was anaerobically consumed by alkylbenzene- and alkane-utilizing sulfate-reducing bacteria.

The enriched and isolated cultures of sulfate-reducing bacteria growing with crude oil may serve as model systems that help to understand microbiological processes in oil wells, tanks and pipelines. Even if sulfate-reducing bacteria under the presumably non-optimal growth conditions in oil reservoirs and production plants exhibit slower and poorer growth than in the enrichment culture, a utilization of alkylbenzenes and alkanes from crude oil as in the model culture should in principle be possible also under *in situ* conditions.

The assumption of an anaerobic utilization of oil hydrocarbons by sulfate-reducing bacteria has to be reconciled with the observation that alkylbenzenes are still present in oils. There might be several explanations: (a) Many unexploited oil reservoirs exhibit very low concentrations of sulfate [11, 40, 41]. Sulfate in these reservoirs has probably become the limiting factor for anaerobic oxidation processes, due to depletion by bacterial sulfate reduction during early diagenesis and eventually during oil maturation. (b) Oil in reservoirs is trapped in the pores of rocks [57] which may impede diffusion of substrates into aqueous niches where bacteria could develop. Only during the process of oil extraction by water injection may conditions for bacterial growth become favourable by mixing processes, formation of emulsions and new contact areas between oil and water. (c) Sulfate-reducing bacteria that were formerly buried with the ancient sediments may have died off in the reservoirs, due to unfavourable conditions (extreme temperature and salinity) at the deep sites of catagenesis, and due to sulfate depletion. During secondary oil recovery, new, viable bacteria are introduced with the injected water. Upon cooling and dilution, the temperature

and salinity, respectively, may become favourable for growth of a wide variety of sulfate-reducing bacteria. Also, high concentrations of sulfate are introduced if seawater is injected.

In addition to alkylbenzenes and *n*-alkanes in crude oil, there are other sources of reducing equivalents for sulfate-reduction, and of organic carbon for cell synthesis. Further potential substrates for sulfate-reducing bacteria in oil fields are (a) anaerobic biodegradation products from substances such as xanthan that are added during secondary oil recovery [17], (b) fatty acids from ancient maturation processes [6, 38], and (c) polar organic products formed by aerobic bacteria during growth on hydrocarbons [39].

Also extremely thermophilic sulfate-reducing archaea have been detected in oil reservoirs and oil fields [8, 35, 56; Stetter, this volume]. Their organic electron donors under *in situ* conditions are unknown. So far, archaea have not been shown to utilize hydrocarbons.

At temperatures higher than approximately 150 ˚C, sulfide may be formed by a chemical process [44]. Sulfide which is present initially reacts with sulfate to yield elemental sulfur. The latter attacks hydrocarbons and is reduced to sulfide. Sulfide then reacts with further sulfate. The net reaction is the reduction of sulfate to sulfide with hydrocarbons providing the reducing equivalents.

Growth of denitrifying bacteria on crude oil

Research on anaerobic degradation of oil hydrocarbons especially by denitrifying bacteria has been stimulated by the fact that dispersal of oil and fuel in the environment may lead to the contamination of deep aquifers. Since groundwater aquifers are frequently anoxic, knowledge of bacterial capacities for degradation of oil hydrocarbons in the absence of molecular oxygen is important to predict the fate of hydrocarbons and the effectiveness of bioremediation efforts under such conditions. Addition of nitrate to oil-contaminated sites, e.g. groundwater aquifers [25, 32], has been regarded as a potential means of enhancing bioremediation efforts on site. Furthermore, the study of anaerobic hydrocarbon oxidation by nitrate reducers may help to understand future side effects of the suggested control of sulfide production during oil production by the addition of nitrate [Jenneman, this volume].

Strains of denitrifying bacteria which had been originally enriched and isolated on toluene, ethylbenzene, propylbenzene, and *m*-xylene as defined substrates were shown to grow also on crude oil by utilization of alkylbenzenes [49]. In a subsequent study with denitrifying bacteria directly enriched from freshwater mud samples in the presence of crude oil as a source of organic substrates, we investigated which oil constituents are preferentially degraded by denitrifying bacteria [50]. The enrichment culture exhibited biphasic growth. Analyses of oil samples taken during incubation revealed that alkylbenzenes were only utilized during the first growth phase and *n*-alkanes only during the second growth phase. Toluene, ethylbenzene and *m*-xylene disappeared completely during the first growth phase within 10 days, while *o*-xylene, *o*-ethyltoluene and 1,2,4-trimethylbenzene were partially consumed. During the second growth phase, partial consumption of *n*-alkanes in the range of C_5-C_{12}, in particular of hexane, was observed. It could be calculated that 3.1% (vol/vol) of the oil was anaerobically oxidized by denitrification. Application of a newly designed, fluorescently labeled 16S rRNA-targeted oligonucleotide probe specific for the *Azoarcus/Thauera* group within the β-subclass of *Proteobacteria* allowed an affiliation of the vast majority of the enriched denitrifiers with this phylogenetic group. This result is in agreement with the observation that all denitrifiers

known so far to degrade alkylbenzenes belong to the *Azoarcus/Thauera* group. Recently, other oligonucleotide probes constructed according to sequences from isolated alkylbenzene-degrading denitrifiers related to *Azoarcus* species allowed the *in situ* detection of such bacteria in subsurface soil exposed to fuel hydrocarbons [31].

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