# Anaerobic biodegradation of saturated and aromatic hydrocarbons

Friedrich Widdel* and Ralf Rabus†

Saturated and aromatic hydrocarbons are wide-spread in our environment. These compounds exhibit low chemical reactivity and for many decades were thought to undergo biodegradation only in the presence of free oxygen. During the past decade, however, an increasing number of microorganisms have been detected that degrade hydrocarbons under strictly anoxic conditions.

**Addresses**
Max-Planck-Institut für Marine Mikrobiologie, Celsiusstrasse 1, D-28359 Bremen, Germany
*e-mail: fwiddel@mpi-bremen.de
†e-mail: rrabus@mpi-bremen.de

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**Abbreviations**
**EPR** electron-paramagnetic resonance
**PFL** pyruvate formate lyase
**RNR** ribonucleotide reductase
**SRB** sulphate-reducing bacteria

## Introduction

Compounds that consist exclusively of carbon and hydrogen are termed hydrocarbons. 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 (π-bonds). It is, therefore, common to classify hydrocarbons according to their bonding features into four groups: the alkanes (saturated hydrocarbons), alkenes, alkynes, and aromatic hydrocarbons. Within each of the groups of non-aromatic (aliphatic) hydrocarbons we can further distinguish between straight-chain (e.g., *n*-alkanes), branched-chain and cyclic (alicyclic) compounds. Aromatic hydrocarbons may be mono- or polycyclic, and many important compounds in this class also contain aliphatic hydrocarbon chains (e.g., alkylbenzenes).

The availability of hydrocarbons as fuels and starting compounds for a vast range of chemical syntheses is of fundamental importance for our industrialised civilisation. Nearly all of these hydrocarbons are of natural origin or chemically synthesised directly from natural hydrocarbons. Hydrocarbons are naturally formed by long-term geochemical reactions of buried biomass or as metabolites in living organisms. Hence, anthropogenic activity has not introduced hydrocarbons as a novel class of compounds into the environment, but rather led to an increase in their accumulation; this can be deleterious as in the case of oil spills [1,2].

The presence of hydrocarbons in the biosphere throughout the history of life may explain why many microorganisms have acquired pathways to make use of these compounds as growth substrates (Figure 1). The study of such microorganisms is of basic scientific (e.g., biochemical), environmental and biotechnological interest.

Bacteria and fungi that utilise hydrocarbons in the presence of oxygen have been known since the beginning of the 20th century. The fact that oxygen is not available in all environments where hydrocarbons occur (e.g., in deep sediments and in oil reservoirs) has repeatedly evoked the question as to whether or not the biodegradation of hydrocarbons is possible under anoxic conditions, and if so to what extent. It was not until the late 1980s that novel types of microorganisms were definitively shown to degrade hydrocarbons under strictly anoxic conditions (Figure 1). So far, studies have shown that these microorganisms activate hydrocarbons by unprecedented biochemical mechanisms that differ completely from those employed in aerobic hydrocarbon metabolism. The present article focuses on saturated and aromatic hydrocarbons, which are the main constituents of gas and oil [3]. For information about anaerobic degradation of alkenes and alkynes, the reader is referred to other overview articles [4,5].

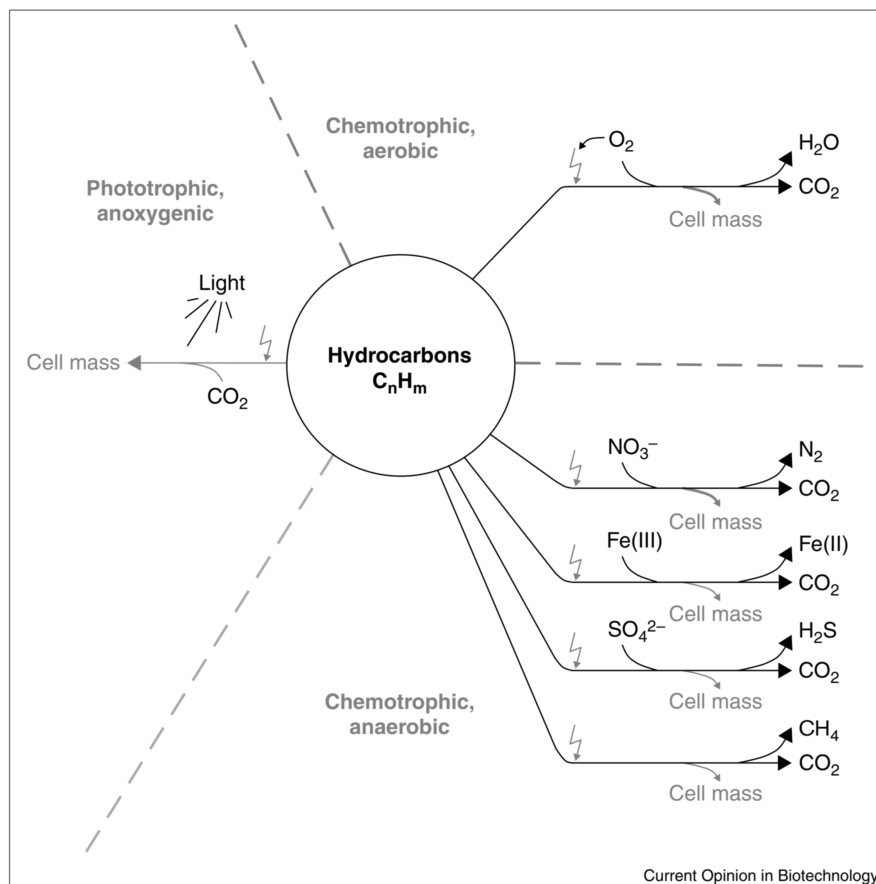
## Formation of aliphatic and aromatic hydrocarbons

### Biological formation

Among hydrocarbons formed by living organisms, one may distinguish between compounds with (assumed) protective or other advantageous functions and degradation products mostly from energy metabolism (i.e., 'exhaust' products).

The majority of biogenic hydrocarbons of the first category are alkenes and include the enormous variety of monoterpenes found in higher plants; monoterpenes may function as deterrents, inhibitors of fungal or bacterial growth or attractants, but often their role is unknown [4,6]. Saturated and aromatic hydrocarbons may also have an advantageous function in living organisms. Various *n*-alkanes or simple methyl-branched alkanes have been detected in bacteria [6,7], plants [6], and animals [6,8]. Highly methyl-branched alkanes derived from isoprene units are common in archaea [9]. One may speculate that certain alkanes stabilise lipid membranes or increase the water-repelling effect of protective waxes. The biosynthesis of alkanes is poorly understood. The predominance of C-odd chains among *n*-alkanes suggests synthesis from common C-even fatty acids by loss of the carboxyl group. However, simple decarboxylation of a saturated fatty acid is very unlikely from a mechanistic point of view. 1-Alkene biosynthesis from saturated fatty acids by hydrogen and carboxyl group elimination in an aerobic radical mechanism has been demonstrated [10]. Alkane formation by subsequent saturation of the non-activated double bond would again be difficult to explain on the basis of known enzyme mechanisms. On the other hand, it has been suggested that alkanes (R–H) in plants are produced

**Figure 1**




Experimentally verified possibilities for the microbial utilisation of hydrocarbons. In all chemotrophic reactions, a part of the hydrocarbon is oxidized for energy conservation (catabolism) and another part is assimilated into cell mass. In the long-established aerobic oxidation of hydrocarbons (upper right), oxygen is not only the terminal electron acceptor, but is also needed for substrate activation (oxygenase reactions). The anaerobic pathways involve novel hydrocarbon activation mechanisms that differ completely from the aerobic mechanisms. Jagged arrows indicate hydrocarbon activation. (For the various stoichiometric equations and free-energy changes of hydrocarbon oxidation see [5].)

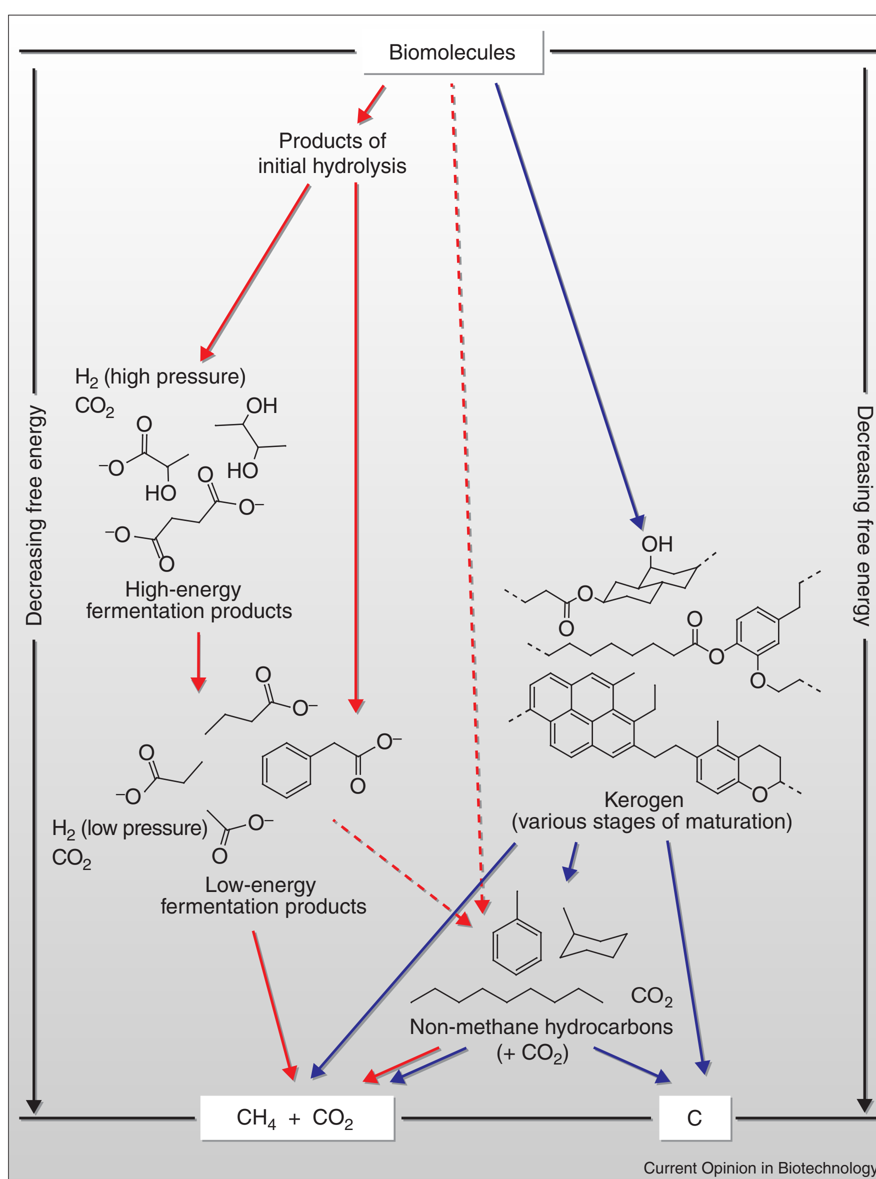
from aldehydes (R–CH=O, from fatty acid reduction) via hydrogen-atom abstraction, decarbonylation of the acyl radicals (R–C•=O) and the readdition of hydrogen by involvement of a cobalt tetrapyrrole [11]. The occurrence of such a cofactor in plants would be unique; the only known cobalt tetrapyrrole, coenzyme $B_{12}$, has otherwise never been detected in plants (J Rétey, personal communication). Aromatic hydrocarbons with an assumed protective function are *p*-isopropyltoluene (also called *p*-cymene) in plants [12] and naphthalene produced by a termite [13].

By far the most important hydrocarbon among biological degradation products is methane. There is much debate about a biological versus geochemical origin of methane in various reservoirs [3,9,14]. Biological origin is evident from a low $^{13}C/^{12}C$ isotope ratio [9,14]. The globally most important methane reservoirs are presumably gas hydrates; from their estimated volumes [15•], a content of around $10 \times 10^{12}$ tonnes carbon can be calculated. Biological methane is produced by diverse, strictly anaerobic archaea. They utilise acetate and biogenic $C_1$ compounds (methanol, methylamines and methylsulphides) that undergo net-dismutation reactions, or $CO_2$ that is reduced with $H_2$ or other hydrogen donors from fermentation processes [9,16]. Via fermentative, syntrophic and methanogenic microorganisms, biomass can in principle be completely converted to methane and $CO_2$ [9]. The process as a whole can be viewed as a gradual defunctionalisation and dismutation of the original biomolecules. Reactions continue until the final, thermodynamically stable states of carbon are reached in the form of methane and $CO_2$ (Figures 2,3). Methanogenic communities thus obtain the maximum free energy available from carbon compounds in the absence of external electron acceptors. Even aromatic [5,17,18] or saturated [19••,20•] hydrocarbons can be converted by microbial associations to methane.

The formation of ethane and higher gaseous alkanes from natural or added ethyl compounds or higher alkyl compounds, respectively, has been observed in natural microbial communities and in cultures of methanogens [9,21–23]; however, these hydrocarbons were only found in trace concentrations.

Aromatic hydrocarbons can also be formed as biological degradation products. *Tolumonas auensis* converts phenylacetate (produced from the fermentative breakdown of phenylalanine) during mixotrophic growth to toluene [24]. This reaction is formally a decarboxylation, but the mechanism is not understood. The anaerobic production of

**Figure 2**




The biological (red) and geochemical (blue) degradation of buried biomass in the absence of electron acceptors from an energetic point of view. Carbon reaches its stable state in the form of $CH_4$, $CO_2$ (also $HCO_3^-$ or $CO_3^{2-}$), and native carbon (see also Figure 3). For kerogen, only a few hypothetical and arbitrary structural possibilities are depicted. It is not known to what extent certain non-methane hydrocarbons in sediments are formed by biological catalysis (dotted red arrows). In order to understand the energetic state, the $CO_2$ formed (and $H_2O$; not shown) must also be taken into consideration; for instance, toluene alone is an energy-rich, endergonic compound ($G_f^0 = +110$ kJ mol$^{-1}$) in comparison to phenylacetate ($G_f^0 = -205$ kJ mol$^{-1}$), but the reaction phenylacetate + $H^+ \rightarrow$ toluene + $CO_2$ (see also text) is exergonic ($\Delta G^{0\prime} = -40$ kJ mol$^{-1}$) and both products together have 'less energy' than the reactants. The formation of inorganic carbon ($G_f^0 = -394.4$ kJ mol$^{-1}$ gaseous $CO_2$) may be generally regarded as a driving force.

*p*-cymene from alkenoic and oxygen-containing monoterpenes under conditions of methanogenesis provides another example of the biological formation of an aromatic hydrocarbon from degradation reactions [25].

## Geochemical formation

Saturated and aromatic hydrocarbons are formed over geological periods by the reactions of buried biomass, especially in marine sediments. Locally intense hydrocarbon production has led to the formation of oil and gas accumulations [3]. Saturated and aromatic hydrocarbons comprise on average ~80% (by mass) of oil constituents. The organic carbon in estimated oil reserves [3] amounts to ~$0.23 \times 10^{12}$ tonnes of carbon.

The geochemical transformation processes leading to the formation of hydrocarbons are extremely complex, and the literature in the field of organic geochemistry should be consulted for details [3,26]. Early defunctionalisation and condensation reactions of sedimented and buried biomass at moderate temperature (≤50°C), termed diagenesis (sometimes including biodegradative reactions), lead to the formation of structurally highly complex polymers designated kerogen. Kerogen in sediments of various ages is by far the most abundant form of organic carbon on our planet. The total mass of kerogen corresponds to greater than $10^{15}$ tonnes of carbon [3]; this is much more than the amount that could reduce all of the free oxygen on our planet. Reactions at higher temperature (and pressure), termed katagenesis, increase the hydrophobic character of kerogen by further defunctionalisation, and release parts of the organic carbon as aliphatic and aromatic hydrocarbons. High-temperature and high-pressure reactions at deep sites,

termed metagenesis, finally lead to the production of methane and $CO_2$, and/or to native carbon. In conclusion, organic carbon from geochemical transformation also approaches its energetically final state, but this happens via reactions that are far less specific, less complete and much slower than those of anaerobic microbial processes. An important difference between microbial and geochemical carbon transformation is the pronounced production in the latter process of chemically sluggish, numerous non-methane hydrocarbons as a 'pre-final', metastable state (Figure 2). Still, there is the open question as to whether or not microorganisms are involved in the production of some non-methane hydrocarbons.

## Hydrocarbon-degrading anaerobic bacteria and their activation mechanisms

In aerobic bacteria growing on hydrocarbons, $O_2$ is not only the terminal electron acceptor for respiratory energy conservation, but also an indispensable reactant in the activation mechanism (Figure 1) [2,27]. By the action of monooxygenases (on aliphatic and certain aromatic hydrocarbons) or dioxygenases (on aromatic hydrocarbons), one or two oxygen atoms, respectively, are directly incorporated from $O_2$ leading to hydroxylated products. The realisation that enzymatically activated oxygen as a strong oxidant is used to overcome the chemical sluggishness of hydrocarbons has for some decades favoured the view that hydrocarbons are not biodegradable under anoxic conditions. However, since the late 1980s, an increasing number of novel microorganisms have been shown to utilise saturated and aromatic hydrocarbons as growth substrates under strictly anoxic conditions. These microorganisms use nitrate, ferric iron or sulphate as electron acceptors for anaerobic respiration, grow in syntrophic cocultures with other anaerobes or grow by anoxygenic photosynthesis (Figures 1,4). Methane, the smallest and most stable hydrocarbon, is oxidized by archaea in a syntrophic association with sulphate-reducing bacteria (SRB).

There is no biochemical agent under anoxic conditions that exhibits the properties of the oxygen species involved in aerobic hydrocarbon activation; hence, the mechanisms of anaerobic hydrocarbon activation have to be completely different from oxygenase reactions. Indeed, all of the anaerobic activation reactions of hydrocarbons are mechanistically unprecedented in biochemistry.

### Methane

In marine sediments, methane diffusing upwards from deep zones often disappears long before any contact with oxygen is possible [28–32]. This anaerobic methane consumption is accompanied by sulphate reduction to sulphide at distinct rates [30,31,33••]. Residual methane is $^{13}C$-enriched, again indicating biological consumption [31]. In addition, inorganic carbon ($CO_2$, $HCO_3^-$, $CO_3^{2-}$) in the zone of methane depletion is relatively depleted in $^{13}C$ [31]; this suggests that $CO_2$ from the oxidation of isotopically light methane dilutes the isotopically heavier background of inorganic carbon. Upon addition of $^{14}C$-labelled methane to anoxic marine sediment, the formation of radioactive $CO_2$ could be measured [30,31,33••].

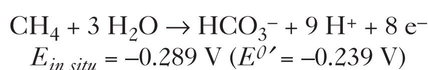
To date, no microorganism has been isolated that carries out a net oxidation of methane with electron acceptors other than $O_2$. A partial conversion of $^{14}CH_4$ to $^{14}CO_2$ during the net production of methane has been measured in cultures of methanogenic archaea [34,35], suggesting a certain reversibility of methanogenesis. Studies with marine sediment led to the conclusion that anaerobic oxidation of methane is mediated in a consortium of archaea and SRB, with the former converting methane to $CO_2$ and $H_2$ and the latter scavenging $H_2$ by oxidation with sulphate [32]. The assumption that such consortia exist was further supported by the discovery of specific, strongly $^{13}C$-depleted lipids and lipid-associated compounds, such as crocetane (2,6,11,15-tetramethylhexadecane), in the zone of anaerobic methane oxidation [33••,36•,37••,38]. In addition, 16S rRNA gene sequences retrieved from this zone indicated the presence of a distinct group of the *Methanosarcinales* [37••]. Furthermore, whole-cell hybridization assays with 16S rRNA-targeted fluorescent probes in anoxic sediment samples from a marine gas hydrate area revealed cell aggregates of archaea surrounded by bacteria; they exhibited close relationships to *Methanosarcinales* and sulphate-reducers of the *Desulfosarcina* branch (δ-*Proteobacteria*), respectively [33••]. The volume-related anaerobic methane oxidation rate in the studied gas hydrate area (up to $5 \times 10^{-3}$ mol dm$^{-3}$ day$^{-1}$ [33••]) was high in comparison to methane oxidation rates in many other sediments (between $1 \times 10^{-6}$ and $67 \times 10^{-6}$ mol dm$^{-3}$ day$^{-1}$ [5,30–32]). Specific rates calculated per biomass (dry mass) of consortia in the sediment were also strikingly high ($42 \times 10^{-3}$ mol g$^{-1}$ day$^{-1}$). This is within the range of specific rates of various SRB growing with various organic substrates under optimum conditions in the laboratory [5,33••].

The free-energy change ($\Delta G$) of anaerobic methane oxidation according to:

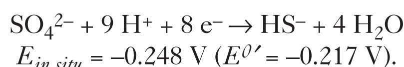
$$CH_4 + SO_4^{2-} \rightarrow HCO_3^- + HS^- + H_2O$$

is usually less negative (less exergonic) than about –40 kJ mol$^{-1}$ $CH_4$ or $SO_4^{2-}$ [5,33••]. If one assumes an approximately equal energy share between the partners, the partial pressure of hydrogen as an intermediate would correspond to a dissolved concentration in the range of $10^{-9}$ M $H_2$ (calculated for 25°C; at an *in situ* temperature of ~5°C the concentration would be even lower). On the basis of calculated diffusive fluxes [39] and roughly estimated substrate utilization kinetics [5], such low hydrogen concentrations do not easily offer an explanation for the high methane oxidation rates [33••]. Experimental evidence for hydrogen production from methane could not be provided [40]. Other organic compounds utilized by *Methanosarcinales*, and thus presenting hypothetical products of reverse methanogenesis (e.g., acetate and methanol), would be at least as problematic to account for the observed anaerobic

methane oxidation rates. Formate as an intermediate could be present at kinetically more favourable concentrations [39]. It is true that formate as electron donor is regarded as energetically nearly equivalent to $H_2$. However, their standard states underlying such energetic calculations are different. The standard state of $H_2$ is 101 Pa (1 atm), which is in equilibrium with dissolved concentrations of ~1 mM. The standard state of formate is the dissolved state with an activity of 1, corresponding to approximately 1 M. Hence, formate concentrations can be significantly higher (depending on the $CO_2/HCO_3^-$ concentration) than energetically equivalent dissolved hydrogen concentrations and thus an attractive explanation is that formate acts as a shuttle for carrying reducing equivalents between syntrophic partners. In the case of the consortia, one would have to explain how formate can be formed by *Methanosarcinales* despite the fact that none of the members of this group is able to utilize this compound. The use of a redox-active biomolecule other than a typical growth substrate as a shuttle for reducing equivalents between syntrophic partners has been suggested for an anaerobic coculture that oxidises acetate [41]. For any interspecies transfer of reducing equivalents, a shuttle with a midpoint potential close to that of the partial reactions of the partners would be kinetically most advantageous. If one assumes that all eight reducing equivalents (electrons) formed from one molecule of methane are transported singly or in pairs via the same shuttle mechanism, the redox potential ($E_{in\ situ}$) of the partial reaction (if viewed as being in equilibrium) of each partner under the actual conditions can be calculated:



$$CH_4 + 3\ H_2O \rightarrow HCO_3^- + 9\ H^+ + 8\ e^-$$
$$E_{in\ situ} = -0.289\ V\ (E^{0\prime} = -0.239\ V)$$

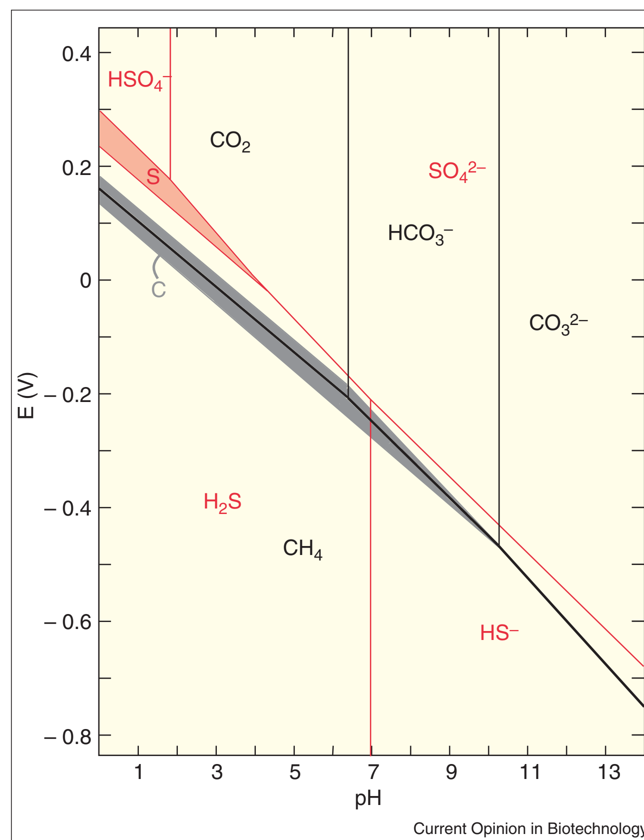


$$SO_4^{2-} + 9\ H^+ + 8\ e^- \rightarrow HS^- + 4\ H_2O$$
$$E_{in\ situ} = -0.248\ V\ (E^{0\prime} = -0.217\ V).$$

(The *in situ* conditions underlying the calculated values are indicated in Figure 3, which also depicts these redox potentials at pH 7.5 as assumed here. $E^{0\prime}$ refers to standard conditions except for $H^+$ activity [= $10^{-7}$].)

An electron shuttle (X) with a midpoint potential near to or in the above range (i.e., not far from –0.270 V) could be operative without an extremely high or an extremely low ratio between the concentrations (activities) of the oxidized and reduced form (viz. $[X_{oxidized}]/[X_{reduced}]$, according to the Nernst equation). On the other hand, such a shuttle would have to be kept in tight association with the cell surfaces to avoid diffusive loss. The hydrophobic cofactor methanophenazine (estimated midpoint potential close to –0.255 V), which has been detected as an electron transport component (in addition to a cytochrome) in a pure culture of *Methanosarcina* [42], appears as one candidate that could, in principle, be part of an interspecies electron-transfer system. Different working hypotheses for the interaction between methane-utilising and sulphate-reducing microorganisms are summarized in Figure 5.

**Figure 3**




Stability diagrams (redox potential *E* versus pH) of carbon (thick black line) and sulphur (thin red line). The redox potential is relative to the standard hydrogen electrode. The stability range of native carbon (graphite) is only approximately indicated in grey. At moderate temperature and pressure, the formation of native carbon is kinetically impeded; biological reactions leading to native carbon are unknown. Which stable forms dominate in a system depends not only on pH and the redox potential, but also on the molar ratio between carbon and total reducing equivalents (C/[H]). The diagram shows that redox transitions of carbon and sulphur are energetically 'close' to each other. Accordingly, the redox span for energy conservation by anaerobic methane oxidation with sulphate as electron acceptor is very small, and thus presents a reaction that is bioenergetically intriguing (in addition to interesting mechanistic and kinetic aspects). Borderlines were calculated from free-energy data and estimated activity coefficients in seawater [5,33••] for the following conditions: T = 298K; partial pressure of $CH_4 = 10^5$ Pa; activity of dissolved $CO_2$, $HCO_3^-$ and $CO_3^{2-} = 5 \times 10^{-3}$; activity of dissolved $H_2S$ and $HS^- = 10^{-3}$; activity of dissolved $HSO_4^-$ and $SO_4^2 = 2 \times 10^{-3}$ (activities of $HCO_3^-$, $HS^-$ and $SO_4^{2-}$ correspond to realistic concentrations of $10^{-2}$, $2 \times 10^{-3}$ and $2 \times 10^{-2}$ M, respectively, in marine sediments).

In addition to the low net free-energy change and kinetic restrictions in the electron shuttle, the high activation energy of methane presents another obstacle that has to be overcome during anaerobic oxidation (see Conclusions).

## Non-methane alkanes

Since the 1940s, the possibility of an anaerobic oxidation of alkanes of various chain lengths has been repeatedly examined to understand the geochemically important and

**Figure 4**

| Natural relationships | Organisms | Hydrocarbons utilised | Type of metabolism | References |
|---|---|---|---|---|
| α | *Rhodospirillum* | | | |
| | *Blastochloris sulfoviridis strain* ToP1 | To | Ph | [69] |
| | *Roseobacter* | | | |
| | *Strain* BS-TN | To | Dn | (unpublished) |
| β | *Ralstonia* | | | |
| | *Thauera aromatica* strains T1,K172 | To | Dn | [59,61] |
| | *Thauera selenatis* | | | |
| | *Azoarcus* spp., various strains | To | Dn | [5,60,64] |
| | *Azoarcus* spp. strains T, mXyN1, M3, Td3, Td15 | *mXy, To* | Dn | [58,60,62,63] |
| | *Azoarcus* sp. strain EbN1 | Eb, To | Dn | [62] |
| | *Azoarcus* sp. strain EB1 | Eb | Dn | [87] |
| | *Azoarcus* sp. strain PbN1 | Eb, Pb | Dn | [62] |
| | *Azoarcus* sp. strain pCyN1 | *p*Cy, *p*Et, To | Dn | [12] |
| | *Azoarcus* sp. strain HxN1 | Alk ($C_6$–$C_8$) | Dn | [45] |
| | *Azoarcus indigens* | | | |
| | *Rhodocyclus* | | | |
| | Strain OcN1 | Alk ($C_8$–$C_{12}$) | Dn | [45] |
| γ | *Escherichia coli* | | | |
| | *Vibrio* sp. NAP-4 | Nap | Dn | [95] |
| | *Halomonas* sp.NS-TN | To | Dn | (unpublished) |
| | *Pseudomonas* sp. NAP-3 | Nap | Dn | [95] |
| γ* | *Ectothiorhodospira* | | | |
| | Strain HdN1 | Alk ($C_{14}$–$C_{20}$) | Dn | [45] |
| δ | *Desulfovibrio* | | | |
| | Strain TD3 | Alk ($C_6$–$C_{16}$) | SR | [46] |
| | Strain NaphS2 | Nap | SR | [94] |
| | Clone 30 | Be | SR | [54] |
| | Strain mXyS1 | *m*Xy, *m*Et, To | SR | [67] |
| | Strain EbS7 | Eb | SR | (unpublished) |
| | *Desulfobacterium* | | | |
| | Strain oXyS1 | *o*Xy, *o*Et, To | SR | [67] |
| | *Desulfobacula toluolica* | To | SR | [65] |
| | Clone SB29 | Be | SR | [54] |
| | Strain Hxd3 | Alk ($C_{12}$–$C_{20}$) | SR | [43] |
| | Strain Pnd3 | Alk ($C_{14}$–$C_{17}$) | SR | [44] |
| | Strain AK01 | Alk ($C_{13}$–$C_{18}$) | SR | [47] |
| | *Desulfuromonas* | | | |
| | *Geobacter metallireducens* | To | FR | [57] |
| | *Syntrophus* | | | |
| | Clones B1–B3 | Alk ($C_{16}$) | Sy | [19] |
| ε | *Helicobacter* | | | |
| *Proteobacteria* | | | | |
| | Other bacterial phyla | Unknown | | |
| Bacteria | | | | |
| Archaea | Members of *Methanosarcinales* | Me | Sy | [33,37] |
| | Other archaeal lineages | Unknown | | |
| Eukarya | | Unknown | | |

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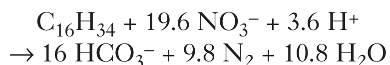
**Figure 4 legend**

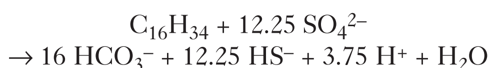
Diversity and affiliation of bacteria with the capacity to degrade saturated (blue) and aromatic (red) hydrocarbons in the absence of oxygen. Branch lengths are not indicative of quantitative phylogenetic distances. For orientation, some generally known relatives that do not degrade hydrocarbons anaerobically are included in black. The designation 'Clones' refers to enriched bacteria that were phylogenetically classified, but so far have not been isolated. Abbreviations for hydrocarbons: Alk, alkanes (range of carbon chain lengths in parentheses); Be, benzene; pCy, *p*-cymene; mEt, *m*-ethyltoluene; oEt, *o*-ethyltoluene; Eb, ethylbenzene; Nap, naphthalene; Pb, *n*-propylbenzene; To, toluene; *m*Xy, *m*-xylene; *o*Xy, *o*-xylene. Abbreviations for the mode of anaerobic metabolism: Dn, denitrification ($NO_3^-$ reduced to $N_2$); FR, Fe(III) reduction (inorganic or chelated ferric iron reduced to ferrous iron); Ph, photosynthesis (anoxygenic; photoheterotropic growth); SR, sulfate reduction ($SO_4^{2-}$ reduced to $HS^-$). Hydrocarbon-degrading bacteria without a reference present unpublished findings (K Zengler, O Kniemeyer, F Widdel).

economically undesirable process of bacterial sulphate reduction in oil reservoirs and during oil production. Alkanes, as major oil constituents, were regarded as potential electron donors and carbon sources for SRB. In addition, possible anaerobic reactions of alkanes were studied in connection with biochemical investigations of aerobic alkane-utilizing microorganisms. Before the monooxygenase reaction was generally accepted as the initial step of aerobic alkane degradation, an oxygen-independent dehydrogenation to 1-alkenes with subsequent epoxidation or hydration of the double bond was suggested as an alternative mechanism; the latter reaction sequence would, theoretically, offer the possibility for anaerobic alkane utilization. Later, however, anaerobic growth of the formerly investigated species and anaerobic alkane dehydrogenation could not be repeated or was viewed critically [27,43,44,45•]. The anaerobic degradation of alkanes with sulphate or nitrate was first demonstrated by the quantitative measurement of substrate consumption with novel isolates that differed from any known species [43,45•,46,47]. Furthermore, alkane degradation by methanogenic communities was also shown [19••,20•]. Overall equations for the anaerobic degradation of *n*-hexadecane — a frequently studied alkane — are shown below (free-energy changes given for liquid *n*-hexadecane, pH 7, anion activities of $10^{-2}$ and standard pressure of gases).



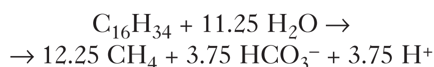
$$C_{16}H_{34} + 19.6\ NO_3^- + 3.6\ H^+ \rightarrow 16\ HCO_3^- + 9.8\ N_2 + 10.8\ H_2O$$

$\Delta G' = -983$ kJ per mole $N_2$ formed



$$C_{16}H_{34} + 12.25\ SO_4^{2-} \rightarrow 16\ HCO_3^- + 12.25\ HS^- + 3.75\ H^+ + H_2O$$

$\Delta G' = -61$ kJ per mole $HS^-$ formed



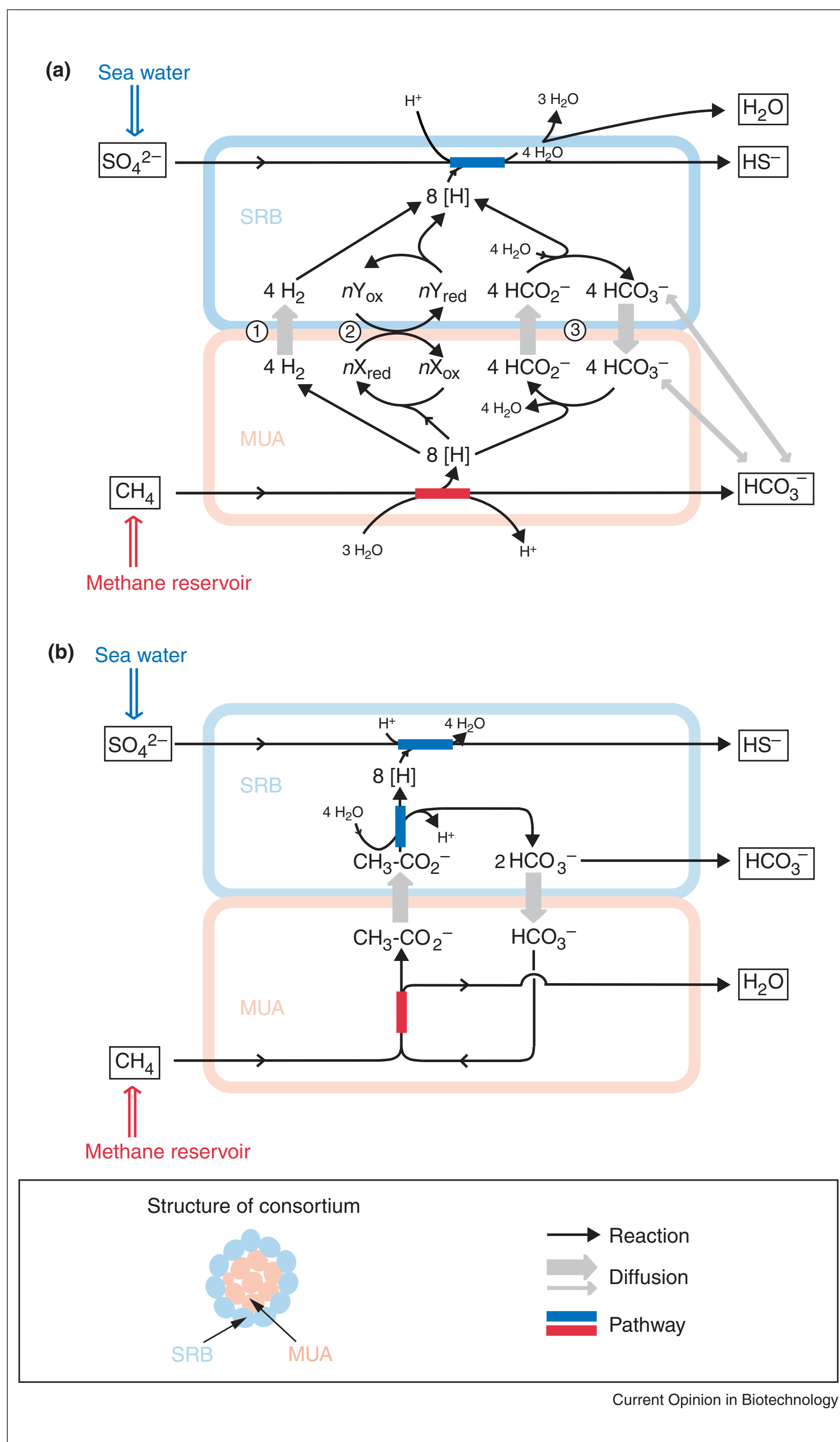
$$C_{16}H_{34} + 11.25\ H_2O \rightarrow 12.25\ CH_4 + 3.75\ HCO_3^- + 3.75\ H^+$$

$\Delta G' = -33$ kJ per mole $CH_4$ formed

($\Delta G'$ generally refers to non-standard conditions and pH 7.)

Fatty acid analysis in two SRB led to the assumption that anaerobic alkane activation occurs by the addition of a carbon compound (a $C_1$ compound in one initial hypothesis), and that the site in the alkane chain for such addition may not be the same in different species [44]. The reaction of C addition was further supported by labelling studies showing that the methyl group in branched fatty acids formed upon growth with *n*-alkanes originated from the terminal carbon of the substrate [48•]. In a denitrifying bacterium with the capacity for alkane degradation, one of the proteins that was specifically formed during growth on *n*-hexane exhibited N-terminal sequence similarity with the small subunit (BssC) of the anaerobic toluene-activating enzyme (see below) found in denitrifying bacteria (A Behrends, P Ehrenreich, J Heider, T Hurek, S Ratering, F Widdel unpublished results). These results suggested that alkane activation might have a similar mechanism to that of toluene activation, which involves fumarate and yields the substituted succinate, benzylsuccinate. Indeed, substituted succinates with alkane-derived alkyl chains were detected in a sulphate-reducing enrichment culture [49••] and a denitrifying strain [50••] growing with *n*-dodecane or *n*-hexane, respectively. The inclusion of authentic standards and labelling studies clearly indicated that *n*-hexane was activated at carbon-*2* in connection with an addition to fumarate, yielding (1-methylpentyl)succinate (Figure 6a); the additional formation of some (1-ethylbutyl)succinate indicated a by-reaction of the alkane at carbon-3. Interestingly, the alkylsuccinates occurred as two diastereomers. The formation of stereoisomers is exceptional among enzymatic reactions. Assuming that the reaction with fumarate is stereoselective, as in toluene activation (see below), the formation of the stereoisomers may be due to relaxed stereospecificity at the alkane carbon; this suggests that the diastereomers are nonracemic. Furthermore electron-paramagnetic resonance (EPR) spectroscopy showed the presence of an organic radical, possibly a glycyl radical, in *n*-hexane-grown cells but not in *n*-hexanoate-grown cells [50••]. This further supports the idea that alkane activation resembles, in principle, anaerobic toluene activation which most likely involves a glycyl radical (see below). A unifying mechanistic scheme is depicted in Figure 7. The energy to be overcome during activation of an alkane at the secondary carbon atom is 33 kJ $mol^{-1}$ higher than in the case of toluene (Figure 8); for a hypothetical alkane activation at the primary carbon atom the difference is even higher, 49 kJ $mol^{-1}$. The overall reaction of hydrocarbon addition to fumarate is exergonic [50••] (see also Conclusions).

**Figure 5**




Hypothesised alternative modes of interaction in consortia that perform anaerobic methane oxidation with sulphate as terminal electron acceptor (see text for details). **(a)** Methane oxidation involving interspecies transfer of reducing equivalents (1) via $H_2$, (2) via assumed redox shuttles or (3) via formate. A redox shuttle involving two components, as shown here, is one possibility; a shuttle of one component shared by both cells is also theoretically possible. **(b)** Methane oxidation involving interspecies transfer of acetate. Possible membrane-association of the reaction pathways is not depicted.

The biochemistry of the further degradation of alkylsuccinates has not been elucidated, but is expected to lead to fatty acid metabolism [44,48•]. If different alkylsuccinates are further degraded by analogous mechanisms, the fatty acids derived from alkanes activated at carbon-2 should differ by one carbon atom from fatty acids derived from alkanes activated at carbon-3 (the activation energy would be rather similar). Hence, different fatty acid profiles in some SRB grown on the same alkane may result from different sites of initial attack [44,48•].

The only other comparable anaerobic activation reaction is that used for the synthesis of diabolic acid

(15,16-dimethyltriacontanedioic acid), a component of the lipid fraction in *Butyrivibrio fibriosolvens*. The underlying mechanism has been suggested to comprise a simultaneous homolytic C–H cleavage at the subterminal carbon of two palmitate molecules by a $B_{12}$ enzyme, followed by condensation of the radical-carrying chains [51]. This reaction involves a net production of two hydrogen atoms (presumably in the form of free 5′-deoxyadenosine) per molecule of diabolic acid.

**Benzene**

Evidence for the anaerobic degradation of benzene, the most stable aromatic hydrocarbon, has been provided by studies with enriched populations in or from sediments containing different electron acceptors [5,17,52–54]. A sediment-free sulphate-reducing consortium was analyzed on the basis of 16S rRNA gene sequences (Figure 4) [54]. To date no pure cultures of bacteria have been described that degrade benzene anaerobically. The mechanism of the initial reaction of benzene degradation in the absence of molecular oxygen is unknown. Initiation by abstraction of a hydrogen atom to yield a phenyl radical would require an activation energy even higher than in the case of methane (Figure 8). Benzene activation may, therefore, involve another activation principle.

**Toluene**

The anaerobic biodegradation of hydrocarbons has been most intensely studied with toluene. In comparison with other aromatic (or saturated) hydrocarbons, toluene allows the relatively rapid growth of microorganisms (doubling time ≥6 h). The study of anaerobic toluene degradation has provided important clues as to our understanding of anaerobic hydrocarbon metabolism. Details have been reviewed recently [5,55] and within the scope of the present article discussion of this topic is restricted to essentials.

The first evidence for anaerobic toluene degradation came from studies with aquifer columns containing nitrate as electron acceptor [56]. Anaerobic toluene degradation was then repeatedly demonstrated in enriched microcosms with various electron acceptors, in pure cultures of newly isolated bacteria that reduce iron(III) [57], nitrate [58–64] or sulphate [65–67], in a binary (syntrophic) culture reducing external fumarate to succinate [68], and in an anoxygenic phototroph [69•].

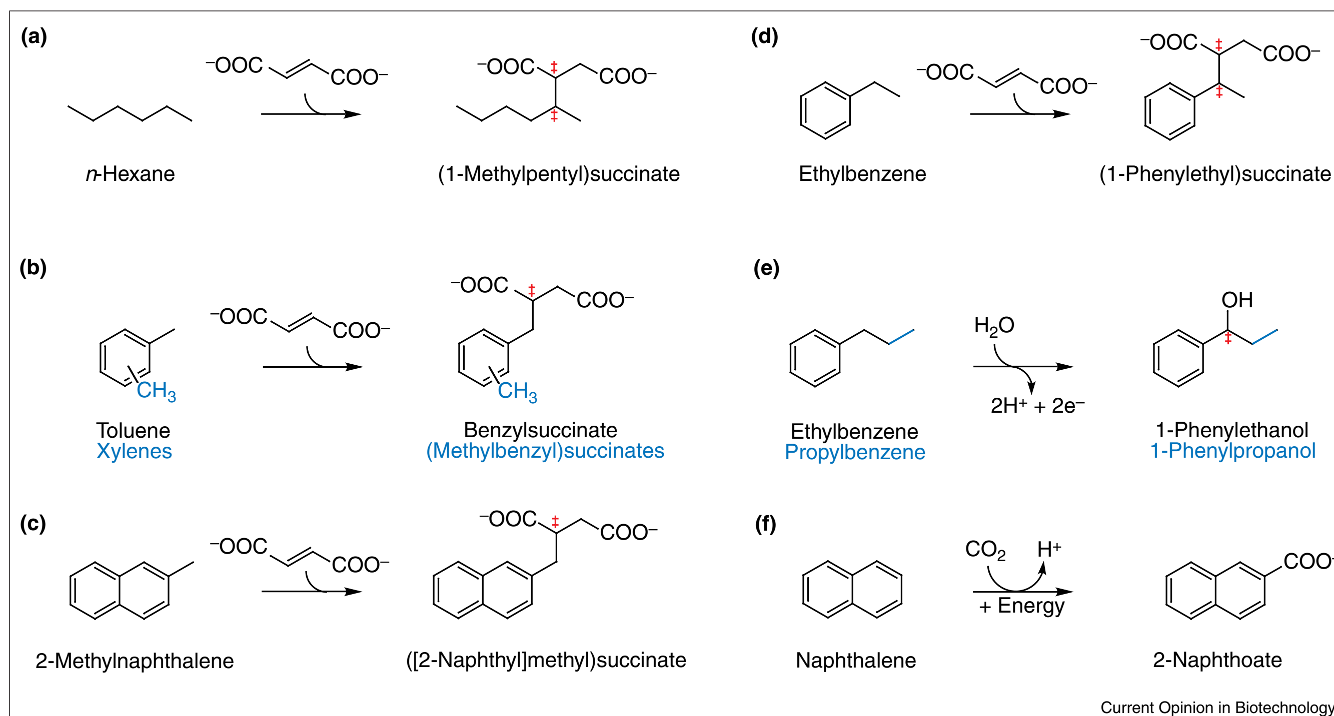
The identification of benzylsuccinate in a toluene-degrading sulphate-reducing enrichment culture [70] as well as in a denitrifying strain [71] was an important discovery and formed the basis for the elucidation of the anaerobic metabolism of toluene and other hydrocarbons. It could be shown that benzylsuccinate was not a by-product, but the initial intermediate formed from toluene and fumarate in a carbon–carbon addition reaction that did not require other cosubstrates (Figure 6b) [72]. Formation of the same product was also demonstrated in a phototrophic [69•] and an enriched methanogenic [18] culture. Stereochemical analysis in denitrifying bacteria showed the formation of *R*(+)-benzylsuccinate [73,74]. Experiments with deuterium-labelled toluene revealed that the hydrogen/deuterium atom that has to be removed before toluene can be added to fumarate is retained in the product [73]. Structural and mechanistic properties of the toluene-activating enzyme, benzylsuccinate synthase, were elucidated by genetic [75,76] and enzymatic [76] approaches. One of the structural genes (Figure 9) revealed a region with high similarity to genes encoding pyruvate formate lyase (PFL) and class III ribonucleotide reductase (RNR) [75,76]; the latter two are known to involve glycyl radicals in the polypeptide chain. Purified benzylsuccinate synthase from *Thauera aromatica* (strain K172) was recognized as a heterohexamer ($\alpha_2\beta_2\gamma_2$) with a native molecular mass of 200 kDa [76]. The presence of a glycyl radical was further confirmed by protein fragmentation upon exposure to oxygen [76], and by a characteristic EPR signal in *T. aromatica* strains [77•] (J Heider, personal communication). Furthermore, the gene encoding the putative, activating (radical-generating) enzyme was detected on the basis of sequence similarities with the activator genes of PFL and RNR [75,76].

The occurrence of a glycyl radical in biochemical reactions was originally detected in PFL, which catalyses the mechanistically unusual cleavage of pyruvate (+ coenzyme A) into acetyl-CoA and formate in a number of fermentative bacteria [78]. Somewhat later, involvement of a glycyl radical was also found in class III RNR [79]. Benzylsuccinate synthase presents a third class of glycyl radical enzymes. The activating enzymes of PFL and RNR generate the glycyl radical via cleavage of *S*-adenosylmethionine by one-electron reduction, yielding methionine and an adenosyl radical: the adenosyl radical abstracts a hydrogen atom from a glycine residue leading to the formation of a glycyl radical (i.e., -NH-•CH-CO- in the polypeptide chain). The glycyl radical is supposed to be a storage radical which then generates a thiyl radical (–S•) as the reactive form. Assuming that the organic radical in anaerobic toluene and alkane activation (see above) is also initiated with *S*-adenosylmethionine, the unifying mechanistic scheme depicted in Figure 7 can be postulated.

RNA analysis demonstrated that the expression of genes related to benzylsuccinate formation are induced by toluene and confirmed their previously observed arrangement in an operon [76,80•]. In both strains of *T. aromatica*, genes were identified that exhibited significant sequence homology to sensor/regulator proteins of two-component systems and were suggested to function in the regulation of toluene metabolism [81,82]. The promotor controlling transcription of the operon with the genes involved in benzylsuccinate metabolism in *T. aromatica* was recently identified [83•].

Benzylsuccinate is further metabolized, somewhat in analogy to the β-oxidation of fatty acids, to acetyl-CoA and benzoyl-CoA [74,83•]. Benzoyl-CoA undergoes reductive dearomatization and ring cleavage followed by reactions that again resemble those in the β-oxidation of fatty acids [84].

Figure 6




The initial reactions during the anaerobic degradation of saturated and aromatic hydrocarbons. Double daggers mark chiral carbon atoms. **(a–d)** The most common activation mechanism that has been detected in several physiological types of anaerobes is a radical reaction of hydrocarbons with fumarate, yielding substituted succinates. **(e)** Ethylbenzene (or *n*-propylbenzene) in denitrifiers is dehydrogenated to yield a secondary alcohol. **(f)** In the anaerobic degradation of naphthalene, carboxylation is the initial or an early step.

The substrate range of the benzylsuccinate-forming activity was tested with partially purified benzylsuccinate synthase from *Azoarcus* strain T and found to include xylenes, fluorotoluenes and the alkene, 1-methyl-1-cyclohexene [5].

### Xylenes and *p*-cymene

Among the alkylbenzenes with two or more alkyl substituents, xylenes (dimethylbenzenes) are the most relevant ones found in oil and as chemicals. Several strains that can degrade toluene can also grow with *m*-xylene [5,58,60,62,63,67]. Furthermore, the degradation of higher homologues has also been observed, among which *p*-isopropyltoluene is a wide-spread plant hydrocarbon [12].

There is evidence that anaerobic degradation of *m*-xylene proceeds, in analogy to that of toluene, via *m*-methylbenzylsuccinate (Figure 6b) to *m*-methylbenzoyl-CoA [5,85]. Further degradation of *m*-methylbenzoyl-CoA would be possible by reactions analogous to those of benzoyl-CoA, because the methyl group does not interfere with the reactions of a regular β-oxidation.

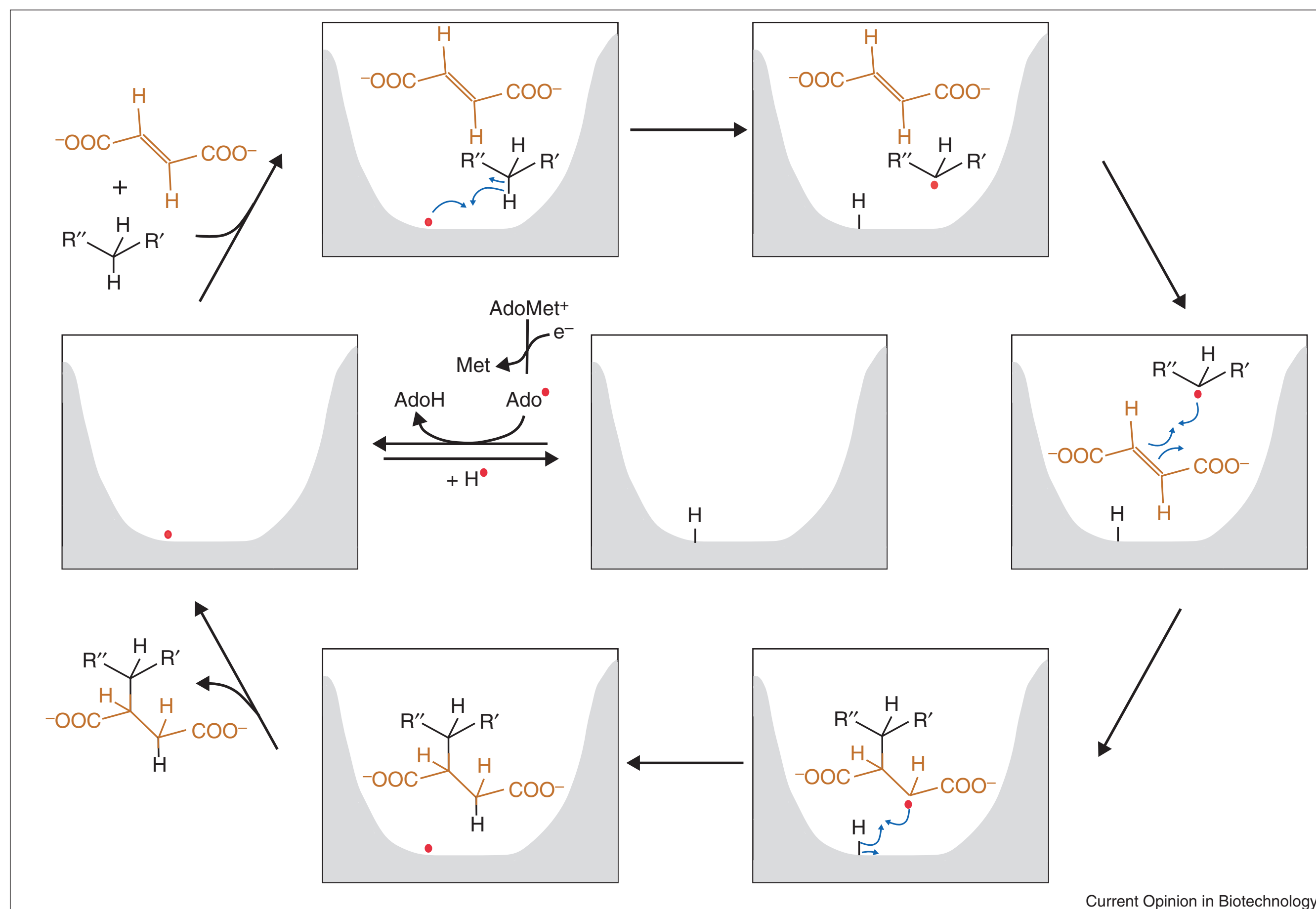
Degradation of *o*-xylene and *p*-xylene, which appear to be poorly and rarely utilized by anaerobic bacteria [67,86], may be also initiated by reaction with fumarate (Figure 6b), the products being *o*-methylbenzoyl-CoA and *p*-methylbenzoyl-CoA, respectively. However, upon ring cleavage the methyl groups would prevent one round of regular β-oxidation and thus require additional mechanisms for complete substrate oxidation. Interestingly, *p*-isopropyltoluene is rapidly utilized by denitrifying strains [12], suggesting an effective mechanism to by-pass the blockage of β-oxidation after cleavage of the assumed intermediate *p*-isopropylbenzoyl-CoA. The capacities for *p*-cymene and toluene degradation in strain pCyN1 are specifically induced by the substrates [12].

### Ethylbenzene and propylbenzene

Denitrifying *Azoarcus* strains [62,87] and a novel gas-vesicle-containing sulphate-reducing bacterium (Figure 4; O Kniemeyer and F Widdel, unpublished results) have been shown to degrade ethybenzene anaerobically in pure cultures.

Ethylbenzene oxidation by the denitrifiers is thought to proceed via dehydrogenation to 1-phenylethanol (Figure 6e) and acetophenone, carboxylation and activation to yield 3-oxo-3-phenylpropionyl-CoA, and thiolytic cleavage to acetyl-CoA and benzoyl-CoA [5,62,87,88,89••]. Ethylbenzene dehydrogenase, which produces (*S*)-1-phenylethanol in *Azoarcus* strains [89••], was shown to be a new molybdenum/iron–sulphur/haem protein localised in the periplasm (O Kniemeyer

**Figure 7**




Generalised reaction scheme of anaerobic hydrocarbon activation via a radical mechanism with fumarate as cosubstrate. R′ represents $CH_3$ (but in some cases could be $C_2H_5$); R″ represents $C_nH_{2n+1}$ (alkyl), $C_6H_5CH_2$ (benzyl) or other aromatic groups. The scheme is a simplification. For instance, it cannot explain the loss (exchange) of the hydrogen atom at the fumarate carbon where the hydrocarbon is being attached, as observed during *n*-hexane degradation [50••]. Also, the stereochemistry [73,74] and the supposed radical transition within the polypeptide from a glycyl (-NH-•CH-CO-) to a thiol (-SH) residue yielding a thiyl (-S•) radical (see text) are not included in the scheme. The assumption that the radical for anaerobic hydrocarbon activation is generated from *S*-adenosylmethionine (AdoMet) still needs experimental verification; this assumption is based on biochemical analogies with the known radical generation by AdoMet in the reactions of PFL [78] and class III RNR [79].

and J Heider, personal communication). The natural electron acceptor is unknown. The capacity for ethylbenzene degradation was shown to be induced [88,89••,90].

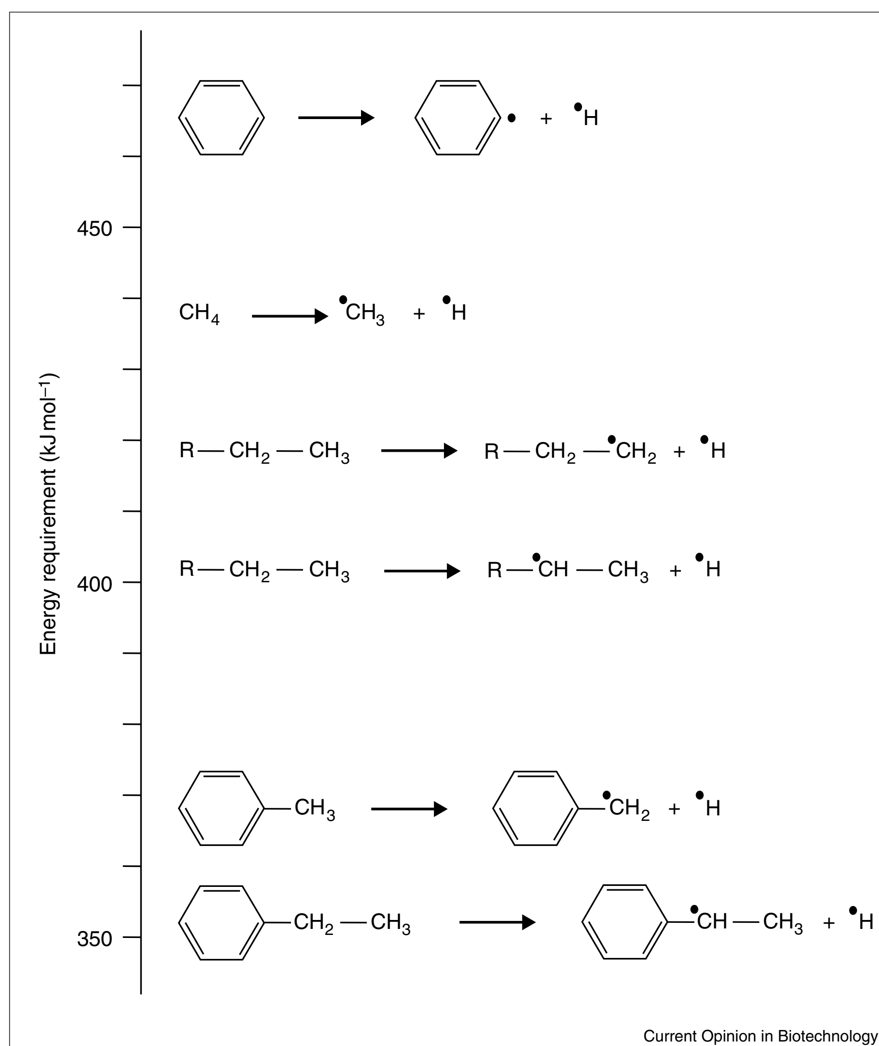
There is evidence that the initial anaerobic reaction of ethylbenzene in SRB differs completely from the reaction in denitrifiers and is analogous to toluene activation. In a sulphate-reducing enrichment culture utilising ethylbenzene, (1-phenylethyl)succinate was detected indicating ethylbenzene addition to fumarate [91•] (Figure 6d). Furthermore, the sulphate-reducing strain isolated with ethylbenzene is unable to oxidize 1-phenylethanol and acetophenone (O Kniemeyer, F Widdel, unpublished results), which are intermediates and growth substrates in ethylbenzene-degrading denitrifiers. In the ‘low-potential’ metabolism of SRB, activation via a reductively generated radical (see above) is apparently ‘easier’ to achieve than dehydrogenation, which has a relatively high redox potential (1-phenylethanol/ethylbenzene, $E^{0\prime} \approx +0.03$ V; estimated by analogy with thermodynamic data of other alcohols/hydrocarbons).

*Azoarcus* strain PbN1 utilizes *n*-propylbenzene in addition to ethylbenzene. The pathway of propylbenzene oxidation is assumed to proceed in analogy to that of ethylbenzene (Figure 6e) [5,62] and might even involve the same enzymes for activation and subsequent steps (O Kniemeyer, J Heider, personal communication).

## Naphthalene, phenanthrene and 2-methylnaphthalene

Hints on anaerobic naphthalene degradation originally came from studies with sediment communities under conditions of sulphate reduction [5,92,93]. Complete degradation of naphthalene was demonstrated by quantitative growth experiments with a novel type of SRB [94•]. Experiments with radiolabelled substrate revealed naphthalene oxidation by pure cultures of denitrifying bacteria [95]. Identification of

**Figure 8**




Energy requirements for the homolytic C–H bond cleavage of some hydrocarbons. Activation of the upper three compounds in anaerobic biological systems may not occur as depicted here (see text); these hypothetical reactions are included merely for comparison. To consider the energetic feasibility of the activation mechanisms via the radicals shown, the differences between the energy values rather than the absolute values indicated on the scale are significant. The abstracted hydrogen atom can only exist bound to a (transient) acceptor with its own hydrogen-bond energy that has to be subtracted. (The figure was drawn according to values given in [108]. The energy requirement for ethylbenzene is an estimate.)

2-naphthoic acid as a metabolite in sulphate-reducing cultures enriched with naphthalene suggested an initial activation via carboxylation to 2-naphthoate [93,96] (Figure 6f). This finding is in agreement with the observation that the sulphate-reducing isolate can utilize 2-naphthoate but not 1-naphthoate [94•]. The identification of other metabolites in a sulphate-reducing enrichment culture indicated the further metabolism of 2-naphthoate (presumably as activated acid) via subsequent reduction of the two rings to yield decalin-2-carboxylate (or an activated form).

Degradation of the tricyclic aromatic hydrocarbon phenanthrene under anoxic conditions was demonstrated in sediment cultures with $^{14}C$-labelled substrate [92,93]. Mass spectrometric analysis indicated the formation of a phenanthrene carboxylate isomer as the initial product, again suggesting substrate carboxylation [93].

Alkylnaphthalenes (and higher polycyclic aromatic hydrocarbons with alkyl groups) occur in great structural variety in crude oil [3], but little is known about their degradation. In comparative enrichment studies with sulphate and 1- and 2-methylnaphthalene, only the latter compound led to bacterial growth and sulphide production (F Widdel, unpublished results). Alkylnaphthalenes are expected to undergo activation more easily than naphthalene (as alkylbenzenes are easier to activate than benzene) and to follow initial reactions comparable to those of alkylbenzenes. Indeed, the identification of naphthyl-2-methylsuccinic acid as a metabolite in a sulphate-reducing enrichment culture grown on 2-methylnaphthalene (Figure 6c) supports an activation mechanism analogous to that of toluene [97•].

## Environmental and other aspects of anaerobic hydrocarbon degradation

### Anaerobic bioremediation

Many studies of the anaerobic biodegradation of the hydrocarbons in natural habitats, microcosms and enrichment cultures (e.g., [52–57,60,63,86,91•,92,98•,99•]) were initiated to determine whether or not bioremediation

processes are possible in deep, anoxic petroleum-contaminated or fuel-contaminated sediments and aquifers. A basic idea for augmented bioremediation is to make electron acceptors in injected water available at concentrations higher than that of dissolved oxygen from air. The concentration of $O_2$ in air-saturated water (8.6 mg/L at 25°C) has the capacity to oxidize, for instance, no more than 2.8 mg toluene/L. Nitrate and sulphate are much more soluble. For example, even gypsum, a form of sulphate with low solubility, with a saturation concentration corresponding to around 2 g $CaSO_4$/L would allow the anaerobic oxidation of 300 mg toluene/L. Aromatic hydrocarbons are of particular concern because of their toxic effects, including carcinogenic properties in the case of benzene [100]. There is no doubt that the degradation of petroleum and refined products is much faster under oxic than anoxic conditions, as can be easily demonstrated in comparative enrichment cultures with and without air. Furthermore, aerobic microorganisms seem to degrade a wider range of hydrocarbon compounds than anaerobic microorganisms. Still, an argument in support of the development of anaerobic bioremediation procedures is the observation that benzene, toluene, xylenes and ethylbenzene are degradable without oxygen; these are the most water-soluble aromatic hydrocarbons (saturation concentrations at 25°C are 1800, 580, around 200 and 125 mg/L, respectively) and spread most easily. Alkanes are usually regarded to have no or little toxicity, apart from volatile alkanes at high concentrations which have mainly a narcotic effect. Nevertheless, the degradation of alkanes as a highly hydrophobic background that adsorbs aromatic hydrocarbons may increase bioavailability of the latter for microbial degradation. The extent of the degradation of hydrocarbons from oil and the groups of microorganisms involved were studied in enrichment cultures with sulphate [46,101] or nitrate [99•] as electron acceptors. If crude oil is present in growth-limiting amounts, the portion that can be oxidized under anoxic conditions can be estimated from the amount of reduced electron acceptor (e.g., sulphate or nitrate). For example, in sulphate-reducing and denitrifying cultures, the consumption of *n*-alkanes and alkylbenzenes together amounted to ~12 or 3%, respectively, of the crude oil [46,99•]. Further hydrocarbons not degraded in these enrichment cultures, but shown to be degradable in other cultures or habitats, are trimethylbenzene isomers [102], naphthalene, 2-methylnaphthalene (see above) and pristane (2,6,10,14-tetramethylpentadecane) [103]. With these capabilities, and possibly further as yet unknown capacities, a higher percentage of crude oil and gasoline may be degraded without oxygen, in particular if incubation times are longer than a couple of months as used for enrichment cultures. For monitoring the extent of anaerobic biodegradation, not only chemical hydrocarbon analysis, but also the identification of polar metabolites [91•,99•,104,105•] and isotope fractionation studies [98•,105•,106•] may be valuable tools. Anaerobic bacterial hydrocarbon oxidation causes significant $^{13}C$-enrichment in the residual part of the organic substrate.

## Aspects from biogeochemistry and oil field microbiology

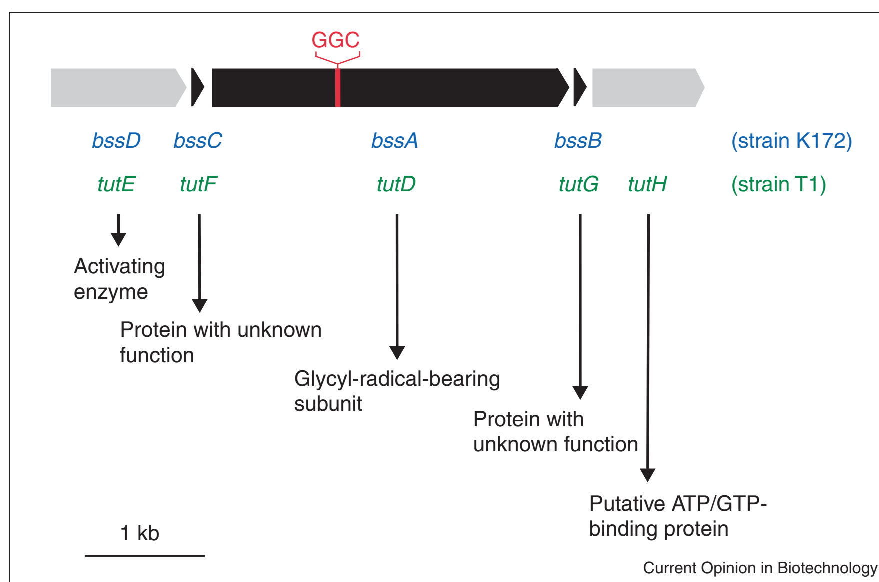
The study of anaerobic hydrocarbon degradation also has implications for our understanding of biogeochemical processes in marine sediments and petroleum reservoirs.

Methane is an important compound in the global carbon cycle and a trace gas that contributes to atmospheric photochemical reactions and influences the heat budget of our biosphere [15•]. Anaerobic oxidation of methane in marine sediments is a globally relevant sink without which the methane input into our atmosphere would be 5–20% higher [31]. Knowledge of the growth properties of anaerobic methane-oxidizing communities and their control by biotic and abiotic factors (e.g., bioturbation, temperature, redox conditions, concentrations of reactants and products) are essential to predict how the consumption of methane is affected by natural or anthropogenic environmental changes.

The utilization of non-methane hydrocarbons by SRB has been regarded as a source of sulphide and sulphur (formed by incomplete oxidation with oxygen) during maturation of petroleum reservoirs [107]. This assumption, as well as that of an anaerobic alteration of the petroleum composition [106•], has been supported by the enrichment and isolation of SRB able to grow by utilising hydrocarbons directly from crude oil [46,101]. The utilisation of oil hydrocarbons for bacterial sulphate reduction may also significantly contribute to the undesirable production of hydrogen sulphide in oil production [101]. Hydrogen sulphide is toxic, stimulates corrosion of steel, diminishes the value of oil and gas by increasing the sulphur content, and forms FeS precipitates that impede oil-water separation and in addition reduce the permeability of reservoir rocks if water is injected for oil extraction. Knowledge of the bacteria involved is a prerequisite for the development of countermeasures.

The anaerobic degradability of several hydrocarbons from crude oil does not necessarily contradict their obvious preservation in reservoirs. First, oil in reservoirs is trapped in the pores of rocks, such that molecular diffusion into aqueous surroundings where bacteria can, in principle, develop is very limited. Second, many anaerobic bacteria formerly buried with sediments may have died due to substrate limitations or high temperature during catagenesis; also extremely high salt concentrations may limit the diversity of bacteria that can develop in stratal waters. On the other hand, there are assumptions that anaerobic bacteria deposited with the original sediments have survived millions of years; such survival appears unlikely without a slow, more or less constant supply of substrates by migration processes over geological periods [101]. In any case, suitable growth conditions for hydrocarbon-degrading anaerobes are likely to be established by the mobilization of oil upon water injection, the introduction of sulphate (especially if seawater is injected), and the decrease of temperature and salinity. The repeatedly observed increase in sulphate reduction after the onset of oil production may reflect the gradual spreading and growth of SRB.

**Figure 9**




The organisation of the genes involved in anaerobic toluene metabolism in denitrifying *T. aromatica* strains K172 [76,83•] and T1 [75,80•]. The bases encoding the radical-carrying glycine are indicated in red.

## Conclusions

The study of the anaerobic degradation of hydrocarbons has brought to light a variety of novel anaerobic bacteria and degradative capacities (Figures 4,6). Such investigations elucidate the possibilities and limits of anaerobic bioremediation and deepen our understanding of microbial processes in sediments and oil fields. Another important outcome is the recognition of novel reaction mechanisms that are unprecedented in biochemistry and that might be of heuristic value for biomimetic approaches to the development of catalysts. It is true that a variety of catalysts and chemical reactions are successfully applied in petrochemistry. However, chemically catalysed reactions of hydrocarbons are usually of low specificity and not suited to the efficient synthesis of pure substances, whereas biological hydrocarbon activation and subsequent reactions are specific. On the other hand, biological alkane activation especially under anoxic conditions is slow: the rates observed in anaerobes are in the range 1 mmol hydrocarbon/h/g total cell protein [5] (the rate for pure active enzyme may be a factor of >10 higher).

The unique mechanistic aspects of the anaerobic biological activation of hydrocarbons, especially of alkanes, become evident if compared with known chemical or aerobic biological reactions. The formation of functionalized products from alkanes, as the least reactive hydrocarbons, requires very harsh conditions or special catalysts for activation [108–113], as a few examples will demonstrate. Alkanes can be chemically activated by highly reactive free radicals, such as Cl• (e.g., from photolysis of $Cl_2$) or •OH (e.g., from $H_2O_2$ and $Fe^{2+}$); the formed alkyl radicals undergo further reactions (often chain reactions) leading to chlorinated or functionalized products, respectively [108]. Among ionic mechanisms, the attack of alkanes with superacids has gained much attention [109,110]. Carbonium ions (>$C^+[H_2]$–) formed by protic superacids decay to carbenium ions (>$C^+$–) and $H_2$; carbenium ions, which tend to undergo rearrangements, can add to non-hydrocarbon molecules and thus lead to the formation of functionalized products [109–112]. Since the 1970s, an increasing number of transition metal complexes have been shown to react with the C–H bonds of alkanes. In comprehensive presentations of the subject, high-valent and low-valent metal complexes have been distinguished [110,111]. Reactions with metal complexes can also lead to functionalized products. Functionalization usually involves a strong oxidant, such as a high-valent metal complex with an oxygen atom as ligand, or requires another 'driving force' such as light in the carbonylation of alkanes to aldehydes with a low-valent metal (e.g., rhodium) complex. Monooxygenases, the biological catalysts in aerobic alkane functionalization, present high-valent metal complexes of iron that reach formal oxidation states of Fe(IV) or Fe(V) [110–112]. The active oxygen atom is formed by partial reduction and cleavage of $O_2$ bound to an Fe centre, which may be mononuclear (P450 and other monooxygenases) or dinuclear (methane monooxygenase). The oxygen atom either abstracts a hydrogen atom from the alkane, with subsequent reaction of the •OH radical with the alkyl radical to form the alcohol (rebound mechanism) or inserts directly into the C–H bond to yield the alcohol; the assumption of the latter mechanism is favoured in the case of methane.

Bacteria that oxidize alkanes anaerobically live at normal temperatures often in reducing (e.g., sulphidic) environments where no agents with oxidizing properties similar to those of oxygen species or high oxidation states of iron (>III) can be generated. These bacteria must nonetheless harbor enzymes

with 'harsh' and/or catalytically unique reaction centres to overcome the sluggishness of the substrate. Despite this, the initial activation step must not be energetically expensive. In particular, methane-oxidizing consortia and sulphate-reducers or syntrophs (in association with methanogens) gain little energy from the overall reactions and therefore cannot dissipate much energy for substrate activation. From this point of view, the radical mechanism involving fumarate appears an elegant solution for several types of hydrocarbons (Figure 6), and even for polar aromatic compounds with methyl groups [114•]. The overall reaction of hydrocarbon addition to fumarate is clearly in favour of the product ($\Delta G^{0} \sim -30$ kJ mol$^{-1}$) [50••], but is by no means extremely exergonic ('dissipative') like alkane activation with $O_2$ (e.g., $C_3H_8 + O_2 + NADH + H^+ \rightarrow C_3H_7OH + H_2O + NAD^+$; $\Delta G = -371$ kJ mol$^{-1}$). The anaerobic oxidation of methane with sulphate with its very low net energy gain may require an initial reaction that is even less exergonic than that of higher alkanes (or might need coupling to an energy-conserving mechanism), and thus may have to operate close to equilibrium. This postulate is in favour of an activation that is, or resembles, a reversal of methanogenesis. The final step in methanogenesis is thought to be the liberation of $CH_4$ from a Ni(II)–$CH_3$ centre by protonation [16]. Even if there are arguments against an exact reversal of this mechanism — of methane deprotonation (hypothetical p$K_a$ value is as high as 48 [108]) — a nickel centre (or the same nickel centre) as in the formation of methane may be involved. This type of methane activation would be the biological equivalent of the chemical alkane activation with a low-valent metal complex (see above). In analogy with the activation of $H_2$ by hydrogenase, which has almost the same bond energy as the C–H bond in methane (but can be readily activated due to the non-directed H–H bond [112]), such a methane-activating enzyme would deserve the designation methanase. It will be interesting to compare the subsequent reactions of the activated methyl group with those in methanogenesis [16].

## Acknowledgements

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- • of special interest
- •• of outstanding interest


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