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Metabolism of alkylbenzenes, alkanes, and other hydrocarbons in anaerobic bacteria

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Abstract

Aromatic and aliphatic hydrocarbons are the main constituents of petroleum and its refined products. Whereas degradation of hydrocarbons by oxygen-respiring microorganisms has been known for about a century, utilization of hydrocarbons under anoxic conditions has been investigated only during the past decade. Diverse strains of anaerobic bacteria have been isolated that degrade toluene anaerobically, using nitrate, iron(III), or sulfate as electron acceptors. Also, other alkylbenzenes such as *m*-xylene or ethylbenzene are utilized by a number of strains. The capacity for anaerobic utilization of alkylbenzenes has been observed in members of the α-, β-, γ- and δ-subclasses of the Proteobacteria. Furthermore, denitrifying bacteria and sulfate-reducing bacteria with the capacity for anaerobic alkane degradation have been isolated, which are members of the β- and δ-subclass, respectively. The mechanism of the activation of hydrocarbons as apolar molecules in the absence of oxygen is of particular interest. The biochemistry of anaerobic toluene degradation has been studied in detail. Toluene is activated by addition to fumarate to yield benzylsuccinate, which is then further metabolized via benzoyl-CoA. The toluene-activating enzyme presents a novel type of glycine radical protein. Another principle of anaerobic alkylbenzene activation has been observed in the anaerobic degradation of ethylbenzene. Ethylbenzene in denitrifying bacteria is dehydrogenated to 1-phenylethanol and further to acetophenone; the latter is also metabolized to benzoyl-CoA. Naphthalene is presumably activated under anoxic conditions by a carboxylation reaction. Investigations into the pathway of anaerobic alkane degradation are only at the beginning. The saturated hydrocarbons are most likely activated by addition of a carbon compound rather than by desaturation and hydration, as speculated about in some early studies. An anaerobic oxidation of methane with sulfate as electron acceptor has been documented in aquatic sediments. The process is assumed to involve a reversal of methanogenesis catalyzed by Archaea, and scavenge of an electron-carrying metabolite by sulfate-reducing bacteria. Among unsaturated non-aromatic hydrocarbons, anaerobic bacterial degradation has been demonstrated and investigated with *n*-alkenes, alkenoic terpenes and the alkyne, acetylene.

A) Introduction

Hydrocarbons are wide-spread compounds in the environment. Several hydrocarbons are natural metabolites and part of the lipophilic fraction in microorganisms, plants and animals (Birch & Bachofen 1988). A major reservoir and source of hydrocarbons is petroleum (Tissot & Welte 1984). Petroleum hydrocarbons are introduced into the environment due to their extensive use as fuels and chemicals. But already before their industrial use, locally significant accumulations of these hydrocarbons must have occurred in the biosphere via natural seeps. Hence, it is very likely that various hydrocarbons have been ubiquitous through life's history; this may explain why many types of microorganisms have evolved metabolic capacities to utilize these compounds as electron donors for aerobic

or anaerobic respiration, and as carbon sources for cell synthesis.

Mineralization of aromatic and aliphatic hydrocarbons has long been considered to be feasible only under oxic conditions. The biochemical rational behind this dogma was based mainly on the notion that in aerobic hydrocarbon-degrading bacteria molecular oxygen is obligatorily required as a cosubstrate in the initial enzymatic hydrocarbon activation. These transformation reactions are catalyzed by the well-known mono- or dioxygenases (Britton 1984; Bühler & Schindler 1984; Gibson & Subramanian 1984). Hence, the biochemical strategy for hydrocarbon activation under oxic conditions is to introduce a hydroxyl group (or hydroxyl groups) into the apolar molecule by means of activated oxygen as a powerful oxidizing agent. The lack of molecular oxygen in anoxic environments precludes such mode of activation. Since mineralization of hydrocarbons is nevertheless observed in anoxic environments, anaerobic hydrocarbon-degrading microorganisms must have different biochemical means to convert the apolar hydrocarbon substrate to a compound that carries a functional group. The presence of functional groups such as hydroxyl, carbonyl or carboxyl groups in organic compounds is a prerequisite for subsequent reactions to channel the substrate into central oxidative pathways (e.g., β-oxidation, TCA cycle). As very recent research on anaerobic alkylbenzene degradation indicates, it appears that at least two fundamentally different strategies exist for initiating anaerobic alkylbenzene degradation: (i) Transformation of methyl-substituted benzenes involves an enzymatic addition to a carbon-compound as co-substrate, most likely via a radical mechanism, whereas (ii) alkyl-substituted benzenes with alkyl side chains with two and more carbon atoms in denitrifiers are activated by dehydrogenation of the benzylic carbon.

The present review focuses on the anaerobic degradation of aromatic and saturated hydrocarbons. These are the main products of transformation processes (microbial processes, diagenesis and catagenesis; Tissot & Welte, 1984) of organic material buried in sediments and, therefore, represent the major fractions of petroleum and gas in natural reservoirs, and of refined products derived therefrom. In addition, novel findings from the study of the anaerobic degradation of alkenes as a wide-spread class of plant metabolites, and of acetylene as a representative of alkynes are included. Of the various studies dealing with anaerobic degradation of hydrocarbons, mainly those that have been carried out with pure cultures under the aspects of physiology and biochemistry are presented. For *in situ* and microcosm studies the reader is referred to the other articles of this issue. However, some *in situ* and microcosm studies are included, especially in cases where anaerobic degradation of the hydrocarbon has not been documented so far in pure cultures.

B) Overall energetics, diversity and physiology of anaerobic bacteria that degrade hydrocarbons

Microbial mineralization of hydrocarbons under conditions of nitrate reduction, Fe(III) reduction, sulfate reduction and methanogenesis is an exergonic process, and, thus, is theoretically suitable for microbial energy conservation (Table 1). At standard activities of reactants and products (and pH = 7), the free energy available per mol of electron acceptor consumed for the mineralization of a hydrocarbon is largely invariant of the type (aliphatic vs. aromatic) and size of the molecule (e.g., C_6H_6 vs. $C_{18}H_{34}$) of the substrate. However, the real activities of reactants and products in natural environments may lead to an *in situ* ΔG that differs significantly from the $\Delta G^{0\prime}$ value, especially in the case of large numerical differences in the stoichiometry of soluble reactants or products. Several factors may limit the rate of hydrocarbon mineralization and, thus, are relevant for estimating and predicting rates of *in situ* degradation of fuel components. These factors include the abundance of the relevant microbes, the availability of the hydrocarbon via diffusion, the kinetics and energetics of the initial hydrocarbon-activating reaction, and the efficiency of the subsequent reactions leading to CO_2. Of the aromatic and aliphatic hydrocarbons, the BTEX compounds (benzene, toluene, ethylbenzene, xylenes), naphthalene, as well as hexane, octane and hexadecane have been serving as representative compounds in laboratory studies to unravel the metabolic principles and mechanisms involved in anaerobic degradation of hydrocarbons.

Alkylbenzenes and other aromatic hydrocarbons

Alkylbenzenes

In studies of anaerobic degradation of aromatic hydrocarbons, degradation of toluene has been most

Table 1. Stoichiometry and energetics of mineralization of aromatic and aliphatic hydrocarbons under conditions of denitrification, nitrate ammonification, iron(III) reduction, and methanogenesis. The table presents theoretical equations, irrespective of whether the reaction has been observed or not

Chemical equation	Change in free energy[1] $\Delta G^{0\prime}$
Denitrification	
$C_6H_6 + 6NO_3^- \rightarrow 6HCO_3^- + 3N_2$	-496.2 kJ/mol NO_3^-
$C_6H_5(CH_3) + 7.2NO_3^- + 0.2H^+ \rightarrow 7HCO_3^- + 3.6N_2 + 0.6H_2O$	-493.6 kJ/mol NO_3^-
$C_6H_4(CH_3)_2 + 8.4NO_3^- + 0.4H^+ \rightarrow 8HCO_3^- + 4.2N_2 + 1.2H_2O$	-492.4 kJ/mol NO_3^-
$C_6H_5(C_2H_5) + 8.4NO_3^- + 0.4H^+ \rightarrow 8HCO_3^- + 4.2N_2 + 1.2H_2O$	-495.0 kJ/mol NO_3^-
$C_{10}H_8 + 9.6NO_3^- + 1.2H_2O \rightarrow 10HCO_3^- + 4.8N_2 + 0.4H^+$	-492.9 kJ/mol NO_3^-
$C_{14}H_{10} + 13.2NO_3^- + 2.4H_2O \rightarrow 14HCO_3^- + 6.6N_2 + 0.8H^+$	-490.6 kJ/mol NO_3^-
$CH_4 + 1.6NO_3^- + 0.6H^+ + 3H_2O \rightarrow 1HCO_3^- + 0.8N_2 + 4.8H_2O$	-475.9 kJ/mol NO_3^-
$C_6H_{14} + 7.6NO_3^- + 1.6H^+ \rightarrow 6HCO_3^- + 3.8N_2 + 4.8H_2O$	-492.8 kJ/mol NO_3^-
$C_8H_{18} + 10NO_3^- + 2H^+ \rightarrow 8HCO_3^- + 5N_2 + 6H_2O$	-493.1 kJ/mol NO_3^-
$C_{16}H_{34} + 19.6NO_3^- + 3.6H^+ \rightarrow 16HCO_3^- + 9.8N_2 + 10.8H_2O$	-493.7 kJ/mol NO_3^-
Nitrate ammonification (example of toluene)	
$C_6H_5(CH_3) + 4.5NO_3^- + 2H^+ + 7.5H_2O \rightarrow 7HCO_3^- + 4.5NH_4^+$	-493.1 kJ/mol NO_3^-
Iron(III) reduction (selected examples)[2]	
$C_6H_6 + 30Fe(OH)_3 + 24HCO_3^- + 24H^+ \rightarrow 30FeCO_3 + 72H_2O$	-39.6 kJ/mol Fe
$C_6H_5(CH_3) + 36Fe(OH)_3 + 29HCO_3^- + 29H^+ \rightarrow 36FeCO_3 + 87H_2O$	-39.1 kJ/mol Fe
$CH_4 + 8Fe(OH)_3 + 7HCO_3^- + 7H^+ \rightarrow 8FeCO_3 + 21H_2O$	-35.4 kJ/mol Fe
$C_{16}H_{34} + 98Fe(OH)_3 + 82HCO_3^- + 82H^+ \rightarrow 98FeCO_3 + 246H_2O$	-39.1 kJ/mol Fe
$C_6H_5(CH_3) + 36\alpha$-FeO(OH) $+ 29HCO_3^- + 29H^+ \rightarrow 36FeCO_3 + 51H_2O$	-12.3 kJ/mol Fe
Sulfate reduction	
$C_6H_6 + 3.75SO_4^{2-} + 1.5H^+ + 3H_2O \rightarrow 6HCO_3^- + 3.75H_2S$	-49.6 kJ/mol SO_4^{2-}
$C_6H_5(CH_3) + 4.5SO_4^{2-} + 2H^+ + 3H_2O \rightarrow 7HCO_3^- + 4.5H_2S$	-45.6 kJ/mol SO_4^{2-}
$C_6H_4(CH_3)_2 + 5.25SO_4^{2-} + 2.5H^+ + 3H_2O \rightarrow 8HCO_3^- + 5.25H_2S$	-43.5 kJ/mol SO_4^{2-}
$C_6H_5(C_2H_5) + 5.25SO_4^{2-} + 2.5H^+ + 3H_2O \rightarrow 8HCO_3^- + 5.25H_2S$	-47.6 kJ/mol SO_4^{2-}
$C_{10}H_8 + 6SO_4^{2-} + 6H_2O + 2H^+ \rightarrow 10HCO_3^- + 6H_2S$	-44.3 kJ/mol SO_4^{2-}
$C_{14}H_{10} + 8.25SO_4^{2-} + 2.5H^+ + 9H_2O \rightarrow 14HCO_3^- + 8.25H_2S$	-40.7 kJ/mol SO_4^{2-}
$CH_4 + SO_4^{2-} + H^+ \rightarrow HCO_3^- + H_2S + H_2O$	-17.0 kJ/mol SO_4^{2-}
$C_6H_{14} + 4.75SO_4^{2-} + 3.5H^+ \rightarrow 6HCO_3^- + 4.75H_2S + H_2O$	-44.2 kJmol /SO_4^{2-}
$C_8H_{18} + 6.25SO_4^{2-} + 4.5H^+ \rightarrow 8HCO_3^- + 6.25H_2S + H_2O$	-44.6 kJ/mol SO_4^{2-}
$C_{16}H_{34} + 12.25SO_4^{2-} + 8.5H^+ \rightarrow 16HCO_3^- + 12.25H_2S + H_2O$	-45.6 kJ/mol SO_4^{2-}
Methanogenesis (selected examples)	
$C_6H_6 + 6.75H_2O \rightarrow 2.25HCO_3^- + 3.75CH_4 + 2.25H^+$	-32.5 kJ/mol CH_4
$C_6H_5(CH_3) + 7.5H_2O \rightarrow 2.5HCO_3^- + 4.5CH_4 + 2.5H^+$	-28.5 kJ/mol CH_4
$C_{16}H_{34} + 11.25H_2O \rightarrow 3.75HCO_3^- + 12.25CH_4 + 3.75H^+$	-28.6 kJ/mol CH_4

[1]Free energy of formation (ΔG_f^0, in kJ/mol) of hydrocarbons used in the presented equations: C_6H_6, benzene (lq): +124.4; $C_6H_5(CH_3)$, toluene (lq): +114.2; $C_6H_4(CH_3)_2$, *m*-xylene (lq): +108; $C_6H_5(C_2H_5)$, ethylbenzene (lq): +130; $C_{10}H_8$, naphthalene (c): +201.0; $C_{14}H_{10}$, phenanthrene (c): +268.3; CH_4, methane (g): -50.3; C_6H_{14}, hexane (lq): -3.8; C_8H_{18}, octane (lq): +6.41; $C_{16}H_{34}$, hexadecane (lq): +52.
Free energy of formation (ΔG_f^0, in kJ/mol) of iron oxides used in the presented equation: $Fe(OH)_3$, ferric hydroxide (amorphous): -699; α-FeO(OH), goethite (c): -488.6; $FeCO_3$, siderite (c), -666.7.
ΔG_f^0 values were taken from Dean 1992, D'Ans Lax 1983, Ehrenreich et al. 2000, Stumm & Morgan 1981, Thauer et al. 1977.
For adequate comparison of the energetics of the indicated reactions, $\Delta G^{0\prime}$-values have to be related to the same number of electrons transferred. Hence, the values for denitrification and iron (III) reduction must be multiplied by 1.6 and 8, respectively, resulting in free energy changes per 8 mol electrons (viz. per 1.6 mol NO_3^- and 8 mol Fe, respectively). Alternatively, comparison of $\Delta G^{0\prime}$-values may be achieved by relating values to one mol electrons transferred. Furthermore, the actual concentrations of reactants and products in a habitat or a cultivation system have to be considered, which may result in free energy changes different from the indicated $\Delta G^{0\prime}$ values.
[2]Most equations and energy values are given for amorphous ferric hydroxide (ferrihydrite); for comparison, one equation (example of toluene) is also given with geothite as a crystalline and energetically less favourable electron acceptor.

frequently observed. After the demonstration of toluene degradation in anoxic microcosms (e.g., Grbić-Galić & Vogel 1987; Kuhn et al. 1988), pure cultures of iron(III)-reducing (Lovley et al. 1989; Lovley & Lonergan 1990), denitrifying (Dolfing et al. 1990; Evans et al. 1991; Schocher et al. 1991, Fries et al. 1994), and sulfate-reducing bacteria (Rabus et al. 1993; Beller et al. 1996) were readily shown to grow with toluene as the only organic substrate; most of these cultures were directly isolated with toluene. In a syntrophic co-culture of a sulfate-reducing bacterium with *Wolinella succinogenes* in sulfate-free medium, oxidation of toluene was achieved by coupling to the reduction of fumarate, iron(III) or nitrate yielding succinate, iron(II) or ammonia, respectively; interspecies electron transfer was assumed to occur via hydrogen or an organic redox component (Meckenstock 1999). A phototrophic bacterium, *Blastochloris sulfoviridis* strain ToP1, assimilates toluene (and CO_2) anaerobically with light as the energy source (Zengler et al. 1999a). Some of the denitrifiers enriched and isolated with toluene (strains T and Td15) were shown to grow also with *m*-xylene. Other denitrifying strains (mXyN1, M3) and a sulfate-reducing strain (mXyS1) were directly isolated from enrichment cultures with *m*-xylene. Sulfate-reducing strain oXyS1 was isolated with *o*-xylene (for references see Table 2). Other alkylbenzenes utilized by pure cultures of denitrifying bacteria are ethylbenzene, propylbenzene and the aromatic monoterpene, *p*-cymene. An alkylbenzene that is obviously very slowly degraded is *p*-xylene; degradation has been observed in enriched bacterial communities (Edwards et al. 1992; Häner et al. 1995; Chen and Taylor 1997), but not so far in pure cultures. Anaerobic degradation of 1,3,5-trimethylbenzene and 1,2,4-trimethylbenzene was shown in enrichment cultures utilizing N_2O as electron acceptor (Häner et al. 1997).

The majority of isolated strains of denitrifying bacteria that degrade alkylbenzenes is of freshwater origin and belongs to the *Azoarcus-Thauera* branch of the β-subclass of Proteobacteria. However, toluene-degrading denitrifiers were also isolated from marine habitats; these strains affiliated with the α- or γ- subclass (Zengler, Llobet-Brossa, Nadalig, Dinh, Widdel, unpublished). The toluene-utilizing phototroph, *B. sulfoviridis* is a member of the α-subclass.

Benzene

Under anoxic conditions benzene is usually less readily degraded than toluene and some other alkylbenzenes. Benzene oxidation has been documented in natural or enriched bacterial communities with nitrate (Burland and Edwards 1999), ferric iron in the presence of a chelator (Rooney-Varga et al. 1999), or sulfate (Lovley et al. 1995; Phelps et al. 1998) as electron acceptors, or under conditions of methanogenesis (Grbić-Galić & Vogel 1987; Kazumi et al. 1997). Degradation in a pure culture could not be demonstrated so far, even though benzene is usually included in substrate tests of anaerobic bacteria that degrade other aromatic hydrocarbons such as toluene.

Polycyclic aromatic hydrocarbons

Anaerobic degradation of naphthalene and in some instances of phenanthrene has been measured in microbial communities in soil or sediment under conditions of denitrification (Mihelcic & Luthy 1988; Al-Bashir et al. 1990) or sulfate reduction (Coates et al. 1996; Langenhoff et al. 1996; Coates et al. 1997; Bedessem et al. 1997; Zhang & Young 1997). Cell suspensions of several aerobically isolated *Pseudomonas* species were reported to degrade naphthalene and other polycyclic aromatic hydrocarbons provided as a mixture, with concomitant reduction of nitrate (McNally & Lueking 1998). From a sulfate-reducing enrichment culture growing with naphthalene as the only organic substrate, a pure culture was isolated and shown to degrade the aromatic hydrocarbon completely to carbon dioxide (Galushko et al. 1999). 16S rRNA gene analysis revealed close relationship to a sulfate-reducing bacterium that utilizes *m*-xylene (Harms et al. 1999a) and a molecular clone retrieved from a sulfate-reducing enrichment culture with benzene (Phelps et al. 1998).

In comparative degradation studies with sediment and methylnaphthalenes under conditions of sulfate reduction, only 2-methylnapththalene was degradable within an incubation time of 2 months and yielded an enrichment culture (Widdel, unpublished).

Table 2. Pure cultures of anaerobic bacteria that degrade aromatic or aliphatic hydrocarbons

Species and/or strain	Hydrocarbon metabolized	Doubling time[a] [h]	Dissimilatory growth yield on hydrocarbon [g dry mass per mol hydrocarbon dissimilated]	Specific rate of hydrocarbon degradation [nmol min^{-1} mg^{-1}][p]	Reference for strain isolation
Denitrifying bacteria					
Thauera aromatica K172	Toluene	Toluene, 6	Toluene, 49[b]	Toluene, 20–50[c,e], 12[d]	Anders et al. (1995)
Thauera aromatica T1	Toluene		Toluene, 103	Toluene, 56[d,f]	Evans et al. (1991)
Azoarcus sp. strain T	Toluene, *m*-xylene		Toluene, 60[b]	Toluene, 5–12[c], 15[d,g]	Dolfing et al. (1990)
Azoarcus tolulyticus Tol4	Toluene	Toluene, 4.3	Toluene, 49.6[o]	Toluene, 50[c,h]	Zhou et al. (1995)
Azoarcus tolulyticus Td15	Toluene, *m*-xylene				Fries et al. (1994)
Strain ToN1	Toluene				Rabus & Widdel (1995)
Strain EbN1	Ethylbenzene, toluene	Ethylbenzene, 11	Ethylbenzene, 114	Ethylbenzene, 18[c,i]	Rabus & Widdel (1995)
Azoarcus sp. strain EB1	Ethylbenzene	Ethylbenzene, 14		Ethylbenzene, 60[d,j]	Ball et al. (1996)
Strain PbN1	Ethylbenzene, propylbenzene				Rabus & Widdel (1995)
Strain mXyN1	Toluene, *m*-xylene				Rabus & Widdel (1995)
Strain T3	Toluene				Hess et al. (1997)
Strain M3	Toluene, *m*-xylene				Hess et al. (1997)
Strain pCyN1	*p*-Cymene, toluene, *p*-ethyltoluene	*p*-Cymene, 12	*p*-Cymene, 71	*p*-Cymene, ~30[c,k]	Harms et al. (1999b)
Strain pCyN2	*p*-Cymene	*p*-Cymene, 16	*p*-Cymene, 65	*p*-Cymene, 30–35[c,k]	Harms et al. (1999b)
Strain HxN1	Alkanes (C_6–C_8)	Hexane, 11	Hexane, 65		Ehrenreich et al. (2000)
Strain OcN1	Alkanes (C_8–C_{12})				Ehrenreich et al. (2000)
Strain HdN1	Alkanes (C_{14}–C_{20})		Hexadecane, 161		Ehrenreich et al. (2000)
Ferric iron reducing bacterium					
Geobacter metallireducens GS15	Toluene				Lovley & Lonergan (1990)
Sulfate reducing bacteria					
Desulfobacula toluolica Tol2	Toluene	Toluene, 27	Toluene, 29	Toluene, 6[c,l]	Rabus et al. (1993)
Strain PRTOL1	Toluene	Toluene, 36		Toluene, 35–40[c,m]	Beller et al. (1996)
Desulfobacterium cetonicum	Toluene				Harms et al. (1999b)
Strain oXyS1	Toluene, *o*-xylene, *o*-ethyltoluene	*o*-Xylene, 75			Harms et al. (1999b)
Strain mXyS1	Toluene, *m*-xylene, *m*-ethyltoluene, *m*-cymene	*m*-Xylene, 55			Harms et al. (1999b)
Strain NaphS2	Naphthalene	Naphthalene, ~170	Naphthalene, ~39	Naphthalene, 3–4	Galushko et al. (1999)
Strain Hxd3	Alkanes (C_{12}–C_{20}), 1-hexadecene	(Stearate, 26)			Ackersberg et al. (1991)
Strain Pnd3	Alkanes (C_{14}–C_{17}), 1-hexadecene				Ackersberg et al. (1998)
Strain TD3	Alkanes (C_6–C_{16})				Rueter et al. (1994)
Strain AK-01	Alkanes (C_{13}–C_{18})	Hexadecane, 72	Hexadecane, 14.8[m]	Hexadecane, ~20[c,n]	So and Young (1999a)

If available from literature, doubling times, growth yields and calculated specific rates of hydrocarbon consumption are included. The table does not include anaerobic bacteria that grow on alkenes and acetylene (see text).

[a] Doubling times are usually estimated from the early growth phase; with increasing cell density, hydrocarbon utilizing bacteria tend to exhibit linear rather than exponential growth (viz. decreasing growth rate).

[b] Using N_2O as electron acceptor Schocher et al. (1991).

[c] Calculated from dissimilatory molar growth yield and doubling time, assuming that 1 g of cells contains 0.5 g protein.

[d] Determined in cell suspensions.

[e] Calculated from Biegert et al. (1996).

[f] Calculated from Evans et al. (1991).

[g] Calculated from Beller & Spormann (1997b).

[h] Calculated from Chee-Sanford et al. (1996).

[i] Calculated from Rabus & Widdel (1995).

[j] Calculated from Ball et al. (1996).

[k] Calculated from Harms et al. (1999b).

[l] Calculated from Rabus et al. (1993).

[m] Calculated from Beller & Spormann (1997).

[n] Calculated from So & Young (1999b).

[o] Total growth yield [g dry mass/total hydrocarbon totally consumed].

[p] Related to mg of cell protein.

Alkanes

Long chain alkanes

The capacity for biological degradation of alkanes was known for several decades only from aerobic microorganisms (McKenna & Kallio 1965; Britton 1984; Bühler & Schindler 1984). There were some early reports on anaerobic oxidation of *n*-alkanes by strains of sulfate-reducing (Novelli & ZoBell 1944; Davis and Yarbrough 1966) or denitrifying (Traxler & Bernard 1969) bacteria, but strains were not preserved and results were later viewed critically or not confirmed (Swain et al. 1978; Griffin & Traxler 1981; Aeckersberg et al. 1991; Monpert 1996). During the past decade, however, anaerobic oxidation of *n*-alkanes, which served as the only organic growth substrates, was repeatedly demonstrated in strictly anoxic, quantitative growth experiments with pure cultures of novel types of sulfate-reducing (Aeckersberg et al. 1991; Rueter et al. 1994; Aeckersberg et al. 1998; So & Young 1999a) and denitrifying (Ehrenreich et al. 2000) bacteria. In addition, *n*-alkane degradation in anoxic bacterial communities enriched under conditions of denitrification (Bregnard et al. 1996; Bregnard et al. 1997), sulfate reduction (Coates et al. 1997; Caldwell et al. 1998) or methanogenesis (Zengler et al. 1999b; Anderson and Lovley 2000) has been reported. Degradation of the isoprenoid alkane, pristane (2,6,10,14-tetramethylpentadecane), was observed in denitrifying bacterial populations (Bregnard et al. 1996; Bregnard et al. 1997). A striking nutritional feature is the distinct, narrow range of *n*-alkanes that is utilized by each individual strain (Table 2); for instance, denitrifying strain HxN1 utilized only hexane, heptane and octane. Similar specialized capacities with respect to alkane utilization have been observed among aerobic alkane-degrading microorganisms (Britton 1984).

All sulfate-reducing bacteria with the capacity to degrade alkanes are members of the δ-subclass of Proteobacteria. Two denitrifying strains (HxN1 and OcN1) belong to the β-subclass, while one denitrifying strain (HdN1) belongs to the γ-subclass. Strain HxN1 is closely related to *Azoarcus* species that utilize toluene or other alkylbenzenes. However, strain HxN1 is unable to grow with aromatic hydrocarbons.

Methane

Methane, which can be regarded as the first member of the homologous series of alkanes, is formed by microbial (Ferry 1993) and biogeochemical (Tissot & Welte 1984) processes. Globally important reservoirs are gas accumulations in geological strata (Tissot & Welte 1984) and gas hydrates, in particular those in deep sea sediments (Dillon & Paull 1983; Zatsepina & Buffett 1997). Often biologically formed methane dominates. Because of the important role of methane in the biological carbon cycle and as an atmospheric trace gas (e.g., Conrad 1995; Petit et al. 1999), its microbial oxidation has been frequently investigated. Aerobic methane-oxidizing bacteria are established genera of prokaryotes. The pathway of methane oxidation and assimilation, that is initiated by methane monooxygenase, has been studied in detail (Dalton 1992). Because of the abundance of methane in almost every anoxic aquatic habitat, the possibility of an anaerobic methane oxidation has been also under investigation.

First hints on anaerobic methane oxidation came from biogeochemical investigations in marine sediment, where sulfate acts as the terminal electron acceptor. Evidence is based on three independent approaches. First, methane in marine habitats has been often shown to disappear definitely below the oxic zone, and the depth profile of the methane concentration exhibits a concave-up curvature indicating an anaerobic sink (Reeburgh 1976; Barnes & Goldberg 1976; Martens & Berner 1977; Devohl & Ahmed 1981; Alperin & Reeburgh 1984; Hoehler et al. 1994). A maximum of the sulfate reduction rate in the depth profile was observed to coincide with the zone of anaerobic methane depletion (Alperin & Reeburgh 1985; Iversen & Jørgensen 1985; Reeburgh & Alperin 1988; Hansen et al. 1998). Second, $^{13}C/^{12}C$ isotope analyses are in favour of an anaerobic methane oxidation. Residual methane in the zone of its anaerobic depletion is ^{13}C-enriched, indicating biological consumption (Alperin et al. 1988). In addition, inorganic carbon (CO_2, HCO_3^-, CO_3^{2-}) in the zone of methane depletion was shown to be relatively depleted in ^{13}C (Reeburgh 1980, Reeburgh & Alperin 1988; Blair & Aller 1995); this suggested that carbon dioxide from the oxidation of isotopically light methane added to the signature of the isotopically heavier background of inorganic carbon. Third, after addition of ^{14}C-labeled methane to anoxic marine sediment cores or slurries, formation of radioactive carbon dioxide could be measured (Reeburgh 1980; Iversen & Blackburn 1981; Alperin & Reeburgh 1984, 1985; Iversen & Jørgensen 1985; Hoehler et al. 1994; Hansen et al. 1998). The rates of anaerobic methane oxidation calculated from the biogeochemical data were usually

relatively low in comparison to carbon oxidation and sulfate reduction rates in upper sediment zones with fresh organic input. Often, anaerobic methane oxidation rates were between 1×10^{-6} and 67×10^{-6} mol dm^{-3} day^{-1} (Reeburgh 1980; Devohl & Ahmed 1981; Iversen & Jørgensen 1985; Alperin & Reeburgh 1985, 1988; Reeburgh & Alperin 1988; Alperin et al. 1988; Hoehler et al. 1994; Blair & Aller 1995). However, at a gas seep (Aharon & Fu 2000) or in a gas hydrate area (Boetius et al. 2000), sulfate reduction rates as high as 2.5×10^{-3} (calculated from profile) and 5×10^{-3} mol dm^{-3} day^{-1} (directly measured), respectively, were attributed to methane as the main or only electron donor; this implies that methane oxidation rates at these sites have very similar or the same values (see equation below). The high values of the latter rates are particularly striking if compared to rates of sulfate-reducing bacteria in laboratory cultures. The volumetric sulfate reduction rate in the gas hydrate area is three times higher than that in a well-growing culture of a toluene-degrading sulfate-reducing bacterium (Rabus et al. 1993).

So far, no microorganism has been enriched and isolated that can oxidize methane anaerobically with an external electron acceptor such as sulfate or nitrate. None of the anaerobic bacteria that degrade higher alkanes (Table 1) could be grown with methane as organic substrate. A partial conversion of $^{14}CH_4$ to $^{14}CO_2$ during methanogenesis but no net oxidation of methane has been measured in cultures of methanogenic archaea (Zehnder & Brock 1979; Zehnder & Brock 1980); this finding suggested for the first time the occurrence of a "reverse methanogenesis". Since biologically produced methane, which is typically used for labeling experiments, may contain traces of carbon monoxide as a by-product, ^{14}C-methane was purified from this contaminant and applied to various anaerobic microorganisms (Harder 1997). Again, a partial conversion of methane to CO_2 without net consumption was demonstrated in methanogenic archaea. The reaction with the purified methane was not detectable in cultures of sulfate-reducing and homoacetogenic bacteria. Incubation experiments with marine sediment led to the conclusion that anaerobic oxidation of methane is mediated via metabolic interaction between archaea and sulfate-reducing bacteria in a 'consortium' (Hoehler et al. 1994; Hansen et al. 1998); archaea were suggested to convert methane to CO_2 and H_2, followed by scavenge of H_2 and sulfide production by sulfate-reducing bacteria.

The assumption that anaerobic oxidation of methane is catalyzed by methanogenic archaea or at least by a phylogenetically closely related group receives much support from microbiological *in situ* analysis on the basis of biomarkers and 16S rRNA sequences. Special isoprenoid lipids and hydrocarbons such as crocetane (2,6,11,15-tetramethylhexadecane) detected in the zone of methane depletion exhibited an unusually low $^{13}C/^{12}C$-ratio. These markers were assumed to belong to the methane-utilizing anaerobes (Elvert & Suess 1999; Hinrichs et al. 1999; Boetius et al. 2000); also, lipid fatty acids attributed to sulfate-reducing bacteria were isotopically light. 16S rRNA gene sequences retrieved from this zone represent a distinctive cluster within the *Methanosarcinales* and were tentatively assigned to the methane-oxidizing microorganisms (Hinrichs et al. 1999). Whole-cell hybridization assays with specific, 16S rRNA-targeted fluorescent probes in marine sediment samples taken above gashydrates revealed aggregated consortia of defined types of archaea and bacteria; they exhibited close relationships to *Methanosarcinales* and sulfate-reducers of the *Desulfosarcina* branch (*δ-Proteobacteria*), respectively (Boetius et al. 2000). The specific sulfate reduction rate (related to cell dry mass of countable sulfate-reducing bacteria) that was attributed to anaerobic oxidation of methane, was up to 42×10^{-3} mol g^{-1} day^{-1}. This is within the range of specific rates of various sulfate-reducing bacteria in cultures grown under optimum conditions in the laboratory ($15 - 430 \times 10^{-3}$ mol g^{-1} day^{-1}; Rabus et al. 2000).

The free energy yield from anaerobic methane oxidation with sulfate at realistic activities in marine sediments is relatively low, as shown in the following example (assumed partial reactions and net reaction):

$$CH_4 + 3H_2O \rightarrow HCO_3^- + 4H_2 + H^+$$

$$\Delta G = -15.7\text{kJ/mol} \qquad (1)$$

$$SO_4^{2-} + 4H_2O + H^+ \rightarrow HS^- + 4H_2O$$

$$\Delta G = -15.7\text{kJ/mol} \qquad (2)$$

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

$$\Delta G = -31.4\text{kJ/mol} \qquad (3)$$

(Values were calculated for conditions as follows: T = 298 K; pH = 7.5; CH_4 partial pressure = 10^5 Pa; H_2 partial pressure = 0.12 Pa; SO_4^{2-} concentration = 2×10^{-2} M; HCO_3^- concentration = 10^{-2} M; HS^- concentration = 2×10^{-3} M; activity coefficients of SO_4^{2-}, HCO_3^- and HS^- in seawater of 0.1, 0.5 and 0.5, respectively.)

The net free energy change (Eq. 3) has to be shared between two partners. For an assumed equal share of the free energy as above, the H_2 partial pressure would have to be as low as approximately 0.12 Pa (corresponding to 0.9×10^{-9} M dissolved H_2). Such partial pressures have been measured in marine habitats (Scranton et al. 1984; Hoehler et al. 1994). The redox potential (E of $2H^+/H_2$) at this partial pressure and a pH of 7.5 is -0.269 V (relative to standard hydrogen electrode).

However, it is presently difficult to reconcile such low hydrogen concentrations with the highest specific sulfate reduction rate that has been observed and attributed to methane oxidation (see above). If the methane concentration approaches zero as in many anoxic sediments, the concentration of hydrogen as an intermediate would even become lower. It is true that the close association between archaea and sulfate-reducing bacteria would enable an effective hydrogen transfer via molecular diffusion. Nevertheless, even with an "optimistic" combination of the most favorable kinetic parameters reported for cells of sulfate-reducing bacteria (for overview see Widdel 1988), viz. V_{max} = 1.9 mol H_2 g^{-1} day^{-1} and $K_M = 0.7 \times 10^{-6}$ mol H_2 l^{-1}, the high rates of methane oxidation cannot be explained on the basis of H_2 transfer. The V_{max}/K_M value of 2.7×10^6 l g^{-1} day^{-1} (as the first-order rate constant at substrate concentrations far below K_M) would yield an oxidation rate (at 0.9×10^{-9} mol H_2 l^{-1}) of 2.4×10^{-3} mol H_2 g^{-1} day^{-1} or 0.6×10^{-3} mol CH_4 g^{-1} day^{-1} (related to cell dry mass). For an explanation of the high rate of anaerobic oxidation of methane, such as attributed to the aggregates, one would have to assume even more favourable kinetic properties than used in the present calculation. Since members of the *Methanosarcinales* and *Desulfosarcina*-branch are metabolically versatile, also a transfer of metabolites other than H_2 may be assumed. However, organic compounds known as methanogenic substrates would require concentrations even lower than that of hydrogen to allow reverse methanogenesis and an approximately equal energy share of both partners (acetate, 3×10^{-11} M; concentrations of methanol and methylsulfide even lower). Hydrogen/electron carriers with midpoint potentials close to the redox potential calculated above would allow kinetically more favourable concentrations for a transfer of reducing equivalents to the sulfate-reducing partners.

In addition to the low net free energy change, the high activation energy presents another difficulty for an adequate mechanistic understanding of the anaerobic oxidation of methane. The energy barrier that has to be overcome during cleavage of a C–H bond in methane is by 70 kJ/mol higher than in the case of the methyl group of toluene. In comparison, the barrier for C–H cleavage at the terminal or subterminal carbon of a higher alkane is by 51 and 33 kJ/mol, respectively, higher than for toluene (March 1992). Further investigations into the anaerobic oxidation of methane are expected to reveal new insights into reaction mechanisms as well as into bioenergetic and kinetic principles of bacteria thriving with chemically sluggish "low-energy" substrates.

Unsaturated aliphatic hydrocarbons

Alkenes

Alkenes are more reactive than aromatic hydrocarbons and alkanes and usually not found as constituents of petroleum. It is assumed that the structurally diverse alkenes from living organisms (e.g., Birch & Bachofen 1988) are gradually saturated or (in the case of cyclic compounds) aromatized after burial in sediments. Such reactions are part of the geochemical transformations (diagenetic and catagenetic processes) that lead to petroleum (Tissot & Welte 1984). Aromatization of unsaturated cyclic hydrocarbons may be also biologically mediated, as shown in methanogenic enrichment cultures that utilized alkenoic monoterpenes and converted them partially to *p*-cymene (Harder & Foss 1999). An anaerobic degradation of alkenes containing one or more double bonds has been repeatedly documented. A methanogenic co-culture has been enriched on 1-*n*-hexadecene (Schink 1985a), and a denitrifying bacterium has been isolated with 1-*n*-heptadecene as organic substrate (Gilewicz et al. 1991). Also, some of the denitrifying and sulfate-reducing bacteria originally isolated with *n*-alkanes are able to grow with 1-alkenes (Table 2). Squalene, an isoprenoid alkene with six isolated double bonds, was slowly degraded in a methanogenic enrichment

culture (Schink 1985a). A wide-spread class of natural alkenes with documented biodegradability under anoxic conditions are monoterpenes (Hylemon & Harder 1999). Strains of a nutritionally versatile denitrifying bacterium, *Alcaligenes defragrans*, have been isolated with the monounsaturated hydrocarbons *p*-menth-1-ene, α-pinene, 2-carene, and the diunsaturated hydrocarbon α-phellandrene (Foss et al. 1998). The strains also used several other alkenoic monoterpenes including the open-chain compound myrcene that contains three double bonds. *A. defragrans* did not utilize aromatic hydrocarbons. Two denitrifiers, strains pCyN1 and pCyN2, that were isolated with the aromatic monoterpene hydrocarbon *p*-cymene also utilized a number of alkenoic monoterpenes (Harms et al. 1999b). Another class of natural alkenes are carotenoids without functional groups (e.g., β-carotene); in addition to the conjugated π-electron system, some of these carotenoids contain isolated double bonds or substituted benzene rings. Under anoxic conditions in the dark, carotenoids appear to be highly recalcitrant. β-Carotene did not support growth of methanogenic enrichment cultures (Schink 1985a). It has been repeatedly shown that carotenoids with or without functional groups are preserved in anoxic sediments over thousands of years (Overmann et al. 1993, and references cited therein). Strong adsorption to a matrix may contribute to the long-term stability of carotenoids in anoxic sediments.

Alkynes

Natural carbon compounds with chains containing C–C triple bonds (e.g., mycomycin) are rare, reactive secondary metabolites that have been detected in some fungi and a few other organisms in which these substances may function as deterrents; these compounds are non-hydrocarbons, viz. contain functional groups. Alkynes, viz. hydrocarbons with triple bonds, have not been detected among natural hydrocarbons in soils and sediments. It is, therefore, remarkable that anaerobic bacteria can be readily enriched with acetylene. A strict anaerobe, *Pelobacter acetylenicus*, has been isolated that fermented 2 mol acetylene to 1 mol ethanol and 1 mol acetate (Schink 1985b, Rosner & Schink 1995). Growth on acetylene was relatively fast, with a doubling of 5 to 5.5 h. The natural significance of the capacity for acetylene degradation is unknown. It has been assumed that the involved enzyme (see below) detoxifies natural alkyne derivatives or nitriles (Schink 1985b).

C) Biochemistry of anaerobic degradiation of hydrocarbons

The biochemistry of anaerobic degradation of hydrocarbons has been most intensely studied in the case of toluene. Toluene is a relatively water-soluble hydrocarbon (saturated aqueous solution at 24 °C contains 0.078% [v/v] or 7.3 mM) that can be easily applied in batch cultures (for instance from an inert carrier phase) and enzymatic assays. Furthermore, toluene-degrading anaerobes grow relatively fast such that active cell material can be grown sometimes within a few or a couple of days; denitrifiers may reach doubling times of around 6 h.

Aromatic hydrocarbons

Initial reactions and overall metabolic pathway of toluene

The biochemical pathway of anaerobic toluene mineralization has been studied in the denitrifying bacteria, *Azoarcus* strain T, *Thauera aromatica*, and *Thauera* sp. Strain T1 (Biegert et al. 1996; Beller & Spormann 1997a; Beller & Spormann 1998; Heider et al. 1998; Leuthner & Heider 1998; Leuthner et al. 1998; Coschigano et al. 1998; Beller & Spormann 1999; Krieger et al. 1999; Coschigano 1999). The initial reaction sequence leads from toluene to benzoyl-CoA (Figure 1). Benzoyl-CoA has been recognized previously as central intermediate in the anaerobic degradation of many aromatic compounds (Heider & Fuchs 1997; Harwood et al. 1999). Further metabolism of benzoyl-CoA proceeds via reduction of the aromatic ring, β-oxidation steps, ring cleavage, and subsequent mineralization via 3-oxopimelyl-CoA and glutaryl-CoA.

The first step in the anaerobic metabolism of toluene is mediated by an unusual enzymatic reaction, which is the addition of toluene at its methyl group to fumarate to form benzylsuccinate (Figure 1). Free benzylsuccinate was reported as a transient intermediate in anaerobic toluene oxidation by *in vitro* experiments and *in vivo* isotope trapping experiments in *T. aromatica* strain K172 (Biegert et al. 1996). Studies on the kinetics of benzylsuccinate formation from toluene and fumarate in *Azoarcus* sp. strain T showed that the *in vitro* rate of benzylsuccinate formation was about 30% of the *in vivo* rate of toluene consumption (Beller & Spormann 1997a). This

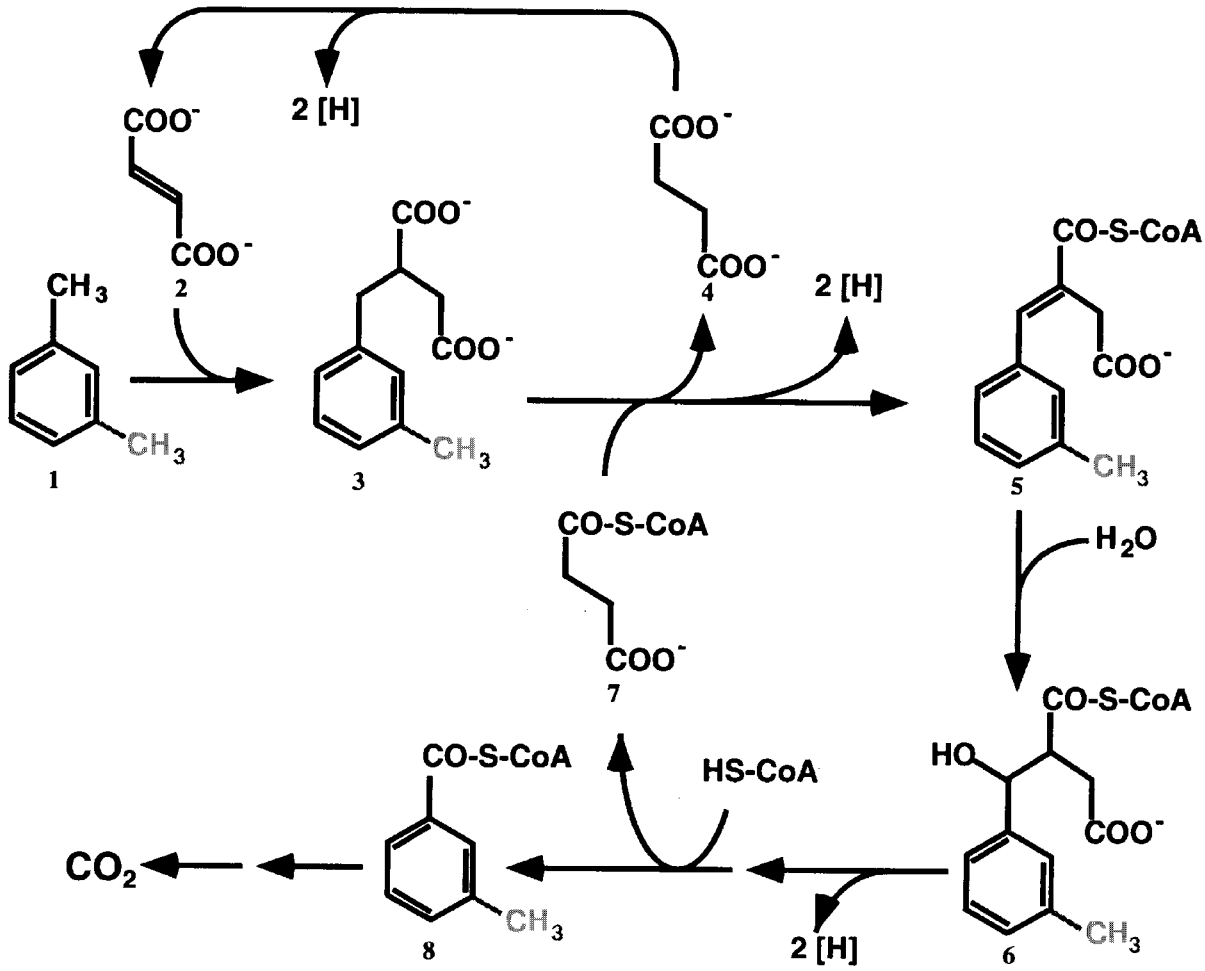

Figure 1. Pathways of anaerobic toluene and *m*-xylene degradation in denitrifying bacteria. Only the reactions leading from the hydrocarbons to benzoyl-CoA (or 3-methylbenzoyl-CoA) are shown in detail. For subsequent reactions of benzoyl-CoA, see Harwood et al. (1999). The pathways for oxidation of toluene (black structures) and *m*-xylene (black structures plus methyl group drawn in gray) involve identical reactions. Chemical designations (given only for toluene pathway): 1, Toluene; 2, fumarate; 3, benzylsuccinate; 4, succinate; 5, phenylitaconate; 6, 2-carboxymethyl-3-hydroxyphenylpropionyl-CoA; 7, succinyl-CoA; 8, benzoyl-CoA.

observation suggests that this reaction actually represents the first step in anaerobic toluene mineralization. Furthermore, *E*-phenylitaconate (possibly originating from the CoA thioester) was identified as an oxidation product of benzylsuccinate (Beller & Spormann 1997a). The enzymatic oxidation of benzylsuccinate to *E*-phenylitaconyl-CoA and benzoyl-CoA was dependent on the presence of coenzyme A (CoA), did not require ATP (Biegert et al. 1996) and was significantly increased when succinyl-CoA served as the source of CoA (Beller & Spormann 1998). Recently, a benzylsuccinate:succinyl-CoA CoA-transferase activity was detected in toluene-grown cells of *T. aromatica* (Leutwein & Heider 1999). It was concluded from these observations that benzylsuccinyl-CoA is oxidized to *E*-phenylitaconyl-CoA. A benzylsuccinyl-CoA dehydrogenase activity was detected when *bbsG* was overexpressed in *E. coli.* The BbsG protein was identified by two-dimensional PAGE as a peptide that was specifically present in cells of *T. aromatica* grown with toluene as the inducing substrate (Leutwein & Heider 1999). *E*-phenylitaconyl-CoA is proposed to be hydrated to 2-carboxymethyl-3-hydroxy-phenylpropionyl-CoA, which undergoes oxidation to benzoylsuccinyl-CoA. Subsequent thiolytic cleavage of benzoylsuccinyl-CoA yields benzoyl-CoA and succinyl-CoA (Figure 1). As will be shown below, an analogous pathway is involved in mineralization

of the dimethylbenzene *m*-xylene, which is, therefore, included in Figure 1.

The benzylsuccinate synthase reaction is the key reaction of anaerobic toluene metabolism and of considerable biochemical interest as a means for activation of aromatic hydrocarbons. Converting the unreactive methyl carbon of toluene to a methylene carbon, which is in β-position to a thioester (as in benzylsuccinyl-CoA), allows the subsequent oxidation by conventional β-oxidation reactions and cleavage to benzoyl-CoA and acetyl-CoA (Figure 1). The enzymatic addition reaction has several novel features that are unique in comparison to previously known, aerobic biochemical activation reactions of aromatic hydrocarbons. Firstly, enzymatic toluene addition to fumarate does not involve a net redox reaction. This is in contrast to all known toluene-transforming oxygenases, which oxidize the hydrocarbon substrate (Gibson & Subramanian 1984). Secondly, benzylsuccinate formation represents a unique biochemical reaction to form a new carbon-carbon bond and, thus, differs from carboxylations, aldolase-type and oxo-acid lyase-type reactions (Metzler 1977).

First insights into benzylsuccinate synthase were gained from a genetic approach using *Thauera* sp. strain T1 (Coschigano et al. 1998). Later on, Leuthner et al. reported the first biochemical isolation of benzylsuccinate synthase (BSS) from *T. aromatica* (Leuthner et al. 1998). The native BSS enzyme is an $\alpha_2\beta_2\gamma_2$ heterohexamer and contains a flavin cofactor (Leuthner et al. 1998; Beller & Spormann 1999). The three subunits are encoded by the genes *bssA*, *bssB* and *bssC*, and have a predicted size of 860, 73, and 57 amino acids, respectively (Leuthner et al. 1998).

Genes encoding benzylsuccinate synthase and other enzymes involved in toluene degradation

The genes encoding benzylsuccinate synthase as well as a putative BSS activase were cloned and sequenced from *T. aromatica* and *Thauera* sp. strain T1 (Coschigano et al. 1998; Leuthner et al. 1998; Coschigano 1999). The organization of the *bss* and *tut* gene cluster is shown in Figure 2. In recent studies of *T. aromatica*, polypeptides induced under growth conditions with toluene were identified by one- and two-dimensional PAGE (Leutwein & Heider 1999). Isolation and analysis of the genes coding for these polypeptides indicated that many genes are organized in an operon designated *bbs* (for beta-oxidation of benzylsuccinate). Based on similarities of predicted amino acid sequences, the proteins are postulated to have the following enzymatic activity: benzylsuccinate:succinyl-CoA CoA transferase (BbsEF), benzylsuccinyl-CoA dehydrogenase (BbsG), phenylitaconyl-CoA hydratase (BbsH), 3-hydroxyacyl-CoA dehydrogenase (BbsCD), and benzoylsuccinyl-CoA thiolase (BbsB) (Leuthner & Heider 2000).

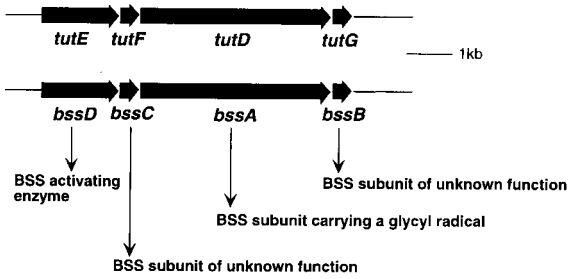

Figure 2. Organization of the genes encoding benzylsuccinate synthase (BSS) and the BSS-activating enzyme in the *bss* and *tut* gene cluster. The shown arrangement has been found in *Azoarcus* and *Thauera* strains. The functions of the gene products are indicated.

The predicted amino acid sequences of the large α-subunit of BSS, BssA in *T. aromatica* and *Azoarcus* sp. strain T, and TutD in *Thauera* strain T1, show 80% identity to each other. The C-terminal part of BssA shows strong homology to pyruvate formate-lyase (PFL) and anaerobic ribonucleotide reductase (NRD). The homology includes a conserved glycine (at position 828 in BssA) and cysteine (position 492). Both conserved amino acids have been shown to be essential in PFL and NRD. As indicated by genetic complementation studies in *Thauera* strain T1, these two amino acids are also essential for toluene utilization (Coschigano et al. 1998). The predicted amino acid sequences of each the β and γ subunit of BSS show a similar degree of homology to the corresponding sequence in the other microorganisms but not to any other protein in public databases. A gene, designated *bssD* and *tutE*, respectively, is located immediately upstream of the *bssCAB* and *tutFDGH* gene cluster, and its predicted amino acid sequence shows homology to a PFL-activating enzyme (Coschigano et al. 1998; Leuthner et al. 1998). The putative activating enzymes, BssD and TutE, are also very similar to each other (62%).

Pyruvate formate-lyase catalyzes the homolytic cleavage of the C_1–C_2 carbon bond of pyruvate to formate and acetyl-CoA (Knappe & Wagner 1995). PFL is found in many anaerobic or facultative microbes, and is a key enzyme in mixed acid fermentation.

Anaerobic NRD catalyzes the reduction of ribonucleotides to deoxyribonucleotides as a key reaction in DNA synthesis and is found in anaerobically grown *E. coli* and methanogenic archaea (for review, see Jordan & Reichard (1998)). PFL and anaerobic NRD are well-studied radical enzymes (Eklund & Fontecave 1999). The active enzyme carries an oxygen-sensitive radical at a specific glycine residue in the peptide chain backbone. Upon binding of the respective substrate, the glycyl radical abstracts a hydrogen atom from a cysteine, which is located at the active site. Transformation of either pyruvate or ribonucleotides (by PFL or NRD, respectively) proceeds via substrate radicals as reaction intermediates. The molecular similarities of the *bss* and *tut* gene cluster to *pfl* and *nrd* genes suggest that BSS may be a glycyl radical enzyme. Similar to observations with PFL, a proteolytic fragmentation pattern of BSS upon incubation with molecular oxygen is consistent with the presence of a glycyl radical in active BSS (Leuthner et al. 1998).

Catalytic properties of benzylsuccinate synthase

Purification of BSS from *Azoarcus* strain T provided a highly active enzyme fraction that was used to conduct studies with implications on the BSS reaction mechanism (Beller & Spormann 1999). During enzymatic addition of the methyl group of toluene to fumarate, a carbon-hydrogen bond of the methyl group is cleaved and the abstracted hydrogen atom is retained in the succinyl moiety of benzylsuccinate (Beller & Spormann 1997a). As indicated by stable isotope cross-over studies, the retained hydrogen atom and the entire benzyl group of the formed benzylsuccinate molecule are derived from the same toluene molecule (Beller & Spormann 1998). This observation indicates that BSS does not carry an abstracted H atom at the beginning and at the end of a reaction cycle (Beller & Spormann 1998). The BSS reaction is associated with a significant kinetic isotope effect of ($k_{obs,H}/k_{obs,D}$ = ca. 3), indicating that cleavage of a C–H bond or H atom abstraction from an enzyme intermediate may be the rate-determining step in the reaction (Krieger et al. 1999). The BSS reaction was also found to be highly stereospecific and yielded only *R*-(+)-benzylsuccinate (Beller & Spormann 1998). BSS from *T. aromatica* was also observed to form only *R*-(+)-benzylsuccinate (Leutwein & Heider 1999). These observations led to the scheme for the BSS reaction shown in Figure 3. In the absence of the substrates, BSS is in its active radical form. At the beginning of the reaction cycle,

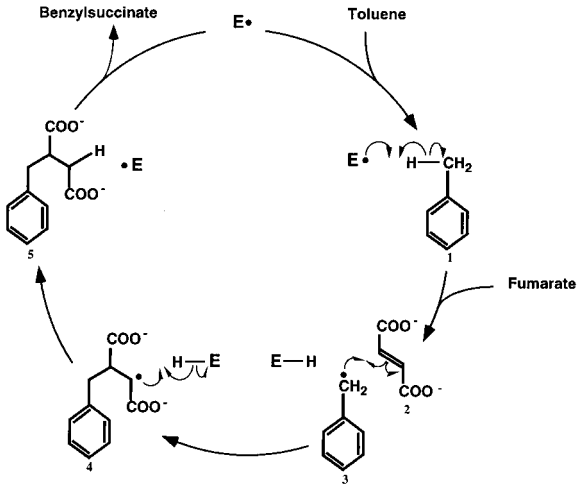

Figure 3. Proposed radical reaction cycle of benzylsuccinate synthase. The catalytically active form of BSS contains an enzyme-based radical (E•). At the beginning of the reaction cycle, toluene, an H atom is abstracted from the methyl group of toluene to form a benzyl radical. The abstracted H atom is transiently stored at benzylsuccinate synthase. The benzyl radical adds to the sp^2-hybridized C-2 carbon of fumarate to form an *R*-benzylsuccinyl radical. The abstracted H atom reacts with the benzylsuccinyl radical to yield *R*-benzylsuccinate and to regenerate the enzyme radical. Note, that the actual sequence of substrates binding to BSS and of products dissociating from BSS are currently unknown. Chemical designations: 1, Toluene, 2, fumarate; 3, benzyl radical; 4, benzylsuccinyl radical, 5, benzylsuccinate.

a C–H bond of the toluene methyl group is homolytically cleaved; the hydrogen atom is transferred to the enzyme and a benzyl radical is formed. This benzyl radical attacks the C–C double bond of fumarate and adds stereospecifically at the *re* face of the fumarate C-2 carbon. The benzylsuccinyl radical then abstracts the enzyme-bound hydrogen atom, which was previously abstracted from the methyl group of toluene. *R*-(+)-benzylsuccinate is released from the enzyme, such that BSS is returned to its active radical form after one reaction cycle.

Initial experiments with respect to the substrate specificity of BSS of *Azoarcus* strain T were conducted with the partially purified, highly active enzyme (Beller & Spormann 1999). In order to identify structural features of the substrates that are essential for or constraining BSS activity, transformation of various toluene and fumarate analogs or surrogates was investigated (Beller & Spormann 1999). The addition reaction of the following toluene surrogates to fumarate was observed: *o*-, *m*-, *p*-xylene, 2-fluorotoluene, 3-fluorotoluene, 4-fluorotoluene, benzaldehyde, and 1-methyl-1-cyclohexene. No reaction occurred with

styrene, 4-methyl-1-cyclohexene or methylcyclohexane. These observations suggest that enzymatic formation of the primary activation product of the enzyme-bound hydrocarbon substrate is favoured by electron delocalization in the benzyl radical, or in the allyl radical in the case of 1-methyl-1-cyclohexene. In contrast, a radical generated at the methyl group of 4-methyl-1-cyclohexene or methylcyclohexane would not be stabilized by delocalization, which is in agreement with the observation that these hydrocarbons were not transformed by BSS.

Substrate range studies with maleate, *trans*-glutaconate, and crotonate as possible fumarate surrogates showed that only maleate, which is the *cis* stereoisomer of fumarate, was transformed (Beller & Spormann 1999). Interestingly, toluene addition to maleate resulted in $>$98% formation of *R*-(+)-benzylsuccinate (Beller & Spormann 1999). This observation suggests that the binding of the C-1 carboxyl group and the adjacent CH=CH group of the dicarboxylic acid (as indicated in Figure 3) is much more important than binding of the second carboxyl group (C-4 region, see Figure 3). One may, therefore, assume that the relative spacial position of toluene and the C-1 and C-2 of the C4-aliphatic acid in the active site is the same regardless of whether the acid is fumarate or maleate (Beller & Spormann 1999).

Diversity of BSS-type reactions

Although benzylsuccinate synthase was studied initially in anaerobic toluene activation in denitrifying *Thauera* and *Azoarcus* species, there is increasing evidence that this type of enzymatic activation is wide spread in other metabolic and phylogenetic groups of bacteria. *In vitro* formation of benzylsuccinate from toluene and fumarate was demonstrated for sulfate-reducing bacteria (strain PRTOL1, *Desulfobacula toluolica*) of the δ-subclass of proteobacteria and for toluene-utilizing phototrophic *Blastochloris sulfoviridis* of the α-subclass (Beller & Spormann 1997b; Rabus & Heider 1998; Zengler et al. 1999a). Furthermore, anaerobic *m*-xylene mineralization is initiated by addition of *m*-xylene to fumarate to form (3-methylbenzyl)succinate (Krieger et al. 1999) (see below). *p*-Cymene (*p*-isopropyl-toluene) was converted to *p*-isopropylbenzoate by a newly isolated, *p*-cymene-degrading anaerobic bacterium, a finding which would be consistent with an activation of the methyl group, possibly also by a BSS-type activation (Harms et al. 1999b). Also recently, it was reported that in the anaerobic *m*-cresol-mineralizing *Desulfobacterium cetonicum*, 3-hydroxybenzylsuccinate accumulated during growth, suggesting that *m*-cresol degradation may be initiated also by a BSS-type reaction (Müller et al. 1999). However, anaerobic *p*-cresol mineralization is probably initiated by an anaerobic methylhydroxylase. Notably, the alkylbenzene ethylbenzene is not transformed by BSS (Rabus and Heider 1998; Beller and Spormann, unpublished, see below). In conclusion, observations suggest that BSS-type reactions are typically involved in the anaerobic degradation of methyl-substituted aromatic hydrocarbons and possibly other methyl-substituted aromatic compounds with low reactivity.

Benzylsuccinate, *E*-phenylitaconate and the methyl homologs are metabolites that are unique to anaerobic methylbenzene mineralization. Interestingly, benzylsuccinate and a dehydrogenation product, which was initially identified as benzylfumarate, were observed in culture supernatant of methylbenzene (toluene) mineralizing microcosms and cultures (Evans et al. 1992; Beller et al. 1992). Since utilization of these extracellular metabolites was not observed, these compounds were initially referred to as dead-end products. Because they accumulate in pure cultures, in laboratory microcosms, as well as in anoxic hydrocarbon-contaminated plumes undergoing biodegradation, benzylsuccinates and the corresponding *E*-phenylitaconates can serve as useful markers that indicate specifically anaerobic metabolism of methylbenzenes (Beller et al. 1995; see other chapter in this issue).

m-Xylene

Several bacteria capable of anaerobic toluene mineralization also catalyze the complete mineralization of the dimethylbenzene, *m*-xylene (see Table 2). *m*-Xylene mineralization was studied in detail in *Azoarcus* strain T, which is also capable of anaerobic toluene mineralization. Work by Krieger et al. (1999) revealed that *m*-xylene is converted to 3-methylbenzoate (or 3-methylbenzoyl-CoA) in a series of reactions that are analogous to those of anaerobic toluene conversion to benzoate (see above, Figure 1).

Addition of a methyl group of *m*-xylene to fumarate to form (3-methylbenzyl)succinate was catalyzed by permeabilized cells at a rate which is at least 15% of the *in vitro* activity of anaerobic *m*-xylene consumption. Further metabolism of (3-methylbenzyl)succinate to *E*-(3-

methylphenyl)itacontate (or a closely related isomer) and to 3-methylbenzoate (or the thioester) was also dependent on succinyl-CoA, suggesting the involvement of a succinyl-CoA transferase similar as in toluene degradation. Interestingly, cells of *Azoarcus* strain T that were grown on toluene catalyzed the transformation of *m*-xylene to (3-methylbenzyl)succinate, and cells grown on *m*-xylene catalyzed the transformation of toluene to benzylsuccinate *in vitro*. As was shown in substrate transformation studies with highly enriched BSS from *Azoarcus* strain T, benzylsuccinate synthase has a remarkably wide substrate range, which includes all three xylene isomers. It is therefore conceivable that one or more of the enzymes involved in toluene conversion to benzoyl-CoA function in the pathway of *m*-xylene oxidation to 3-methylbenzoate and transform the corresponding methylhomologs.

Ethylbenzene, propylbenzene

Only in the last few years have anaerobic bacteria been reported that are capable of mineralizing ethylbenzene and propylbenzene (Rabus & Widdel 1995; Ball et al., 1996). More detailed studies in two related *Azoarcus* strains provided insights into the pathway of ethylbenzene degradation (Rabus & Widdel 1995; Ball et al. 1996; Johnson & Spormann 1999). Similar as in anaerobic toluene mineralization, benzoyl-CoA appears to be a metabolic intermediate in anaerobic ethylbenzene mineralization. However, the initial step in the metabolism of ethylbenzene in denitrifiers is a dehydrogenation rather than an addition to another carbon compound as co-substrate. The proposed pathway of anaerobic ethylbenzene oxidation via 1-phenylethanol to benzoyl-CoA is shown in Figure 4.

Experimental evidence for the proposed pathway rests on the following major observations: First, stable isotope labeling studies of ethylbenzene-metabolizing cell suspensions of *Azoarcus* strain EB1 demonstrated that the hydroxyl and keto-group of 1-phenylethanol and acetophenone, respectively, are derived from ^{18}O-water (Ball et al. 1996). This indicates that ethylbenzene oxidation to 1-phenylethanol is truly an anaerobic dehydrogenation reaction. Second, activities of ethylbenzene dehydrogenase and 1-phenylethanol dehydrogenase were detected in cell-free extract of *Azoarcus* strain EB1 in amounts that are sufficient to explain the *in vivo* rate of ethylbenzene oxidation via these initial reactions (Johnson & Spormann 1999). Third, growth of cells metabolizing ethylbenzene or acetophenone was dependent on the presence of CO_2, in contrast to growth on benzoate, suggesting that a carboxylation is involved in both ethylbenzene and acetophenone mineralization in the pathway leading from acetophenone to benzoyl-CoA (Ball et al. 1996; Champion et al. 1999).

The physiological electron acceptor of the initial enzymatic reaction leading to 1-phenylethanol is most likely a quinone. Ethylbenzene dehydrogenase is a stereospecific enzyme yielding only *S*-1-phenylethanol. *S*-1-phenylethanol is subsequently oxidized in an NAD^+-dependent reaction to acetophenone by an *S*-1-phenylethanol dehydrogenase. Both enzyme activities have been measured in ethylbenzene-grown cells (Johnson & Spormann 1999). Conversion of acetophenone to benzoate (benzoyl-CoA) is proposed to proceed via carboxylation of acetophenone to benzoylacetate (Rabus & Widdel 1995; Ball et al. 1996; Johnson & Spormann 1999) in analogy to acetone degradation (Platen & Schink, 1989; Sluis et al. 1996; Sluis & Ensign 1997). Benzoylacetate is proposed to be activated to the CoA-thioester and then thiolytically cleaved into benzoyl-CoA and acetyl-CoA (Rabus & Widdel 1995; Ball et al. 1996; Johnson & Spormann 1999).

Denitrifying strain EbN1 can degrade both aromatic hydrocarbons, ethylbenzene and toluene, even though the latter is utilized less rapidly. Adaptation studies revealed that the pathways for the degradation of ethylbenzene and toluene are induced independently from each other by the respective hydrocarbon substrate (Champion et al. 1999).

Denitrifying strain PbN1 was isolated under anoxic conditions with *n*-propylbenzene as electron donor and carbon source (Rabus & Widdel 1995). Among other organic substrates, the strain utilizes also 1-phenyl-1-propanol and propiophenone as well as ethylbenzene, 1-phenylethanol and acetophenone. These properties and the close phylogenetic relationship to denitrifiers directly isolated on ethylbenzene (Rabus & Widdel 1995; Johnson & Spormann 1999) suggest that the metabolism of propylbenzene in strain PbN1 is a variant of the pathway of ethylbenzene (see above), viz. that it occurs via dehydrogenation. After carboxylation of the intermediate propiophenone (phenylethylketone) at the C–H-acidic methylene group next to the carbonyl function and subsequent ligation with coenzyme A, a 2-methyl-3-oxo acid thioester would result. As in the metabolism of common 2-methyl-branched fatty acid intermediates (e.g., as formed during isoleucine degradation), the methyl

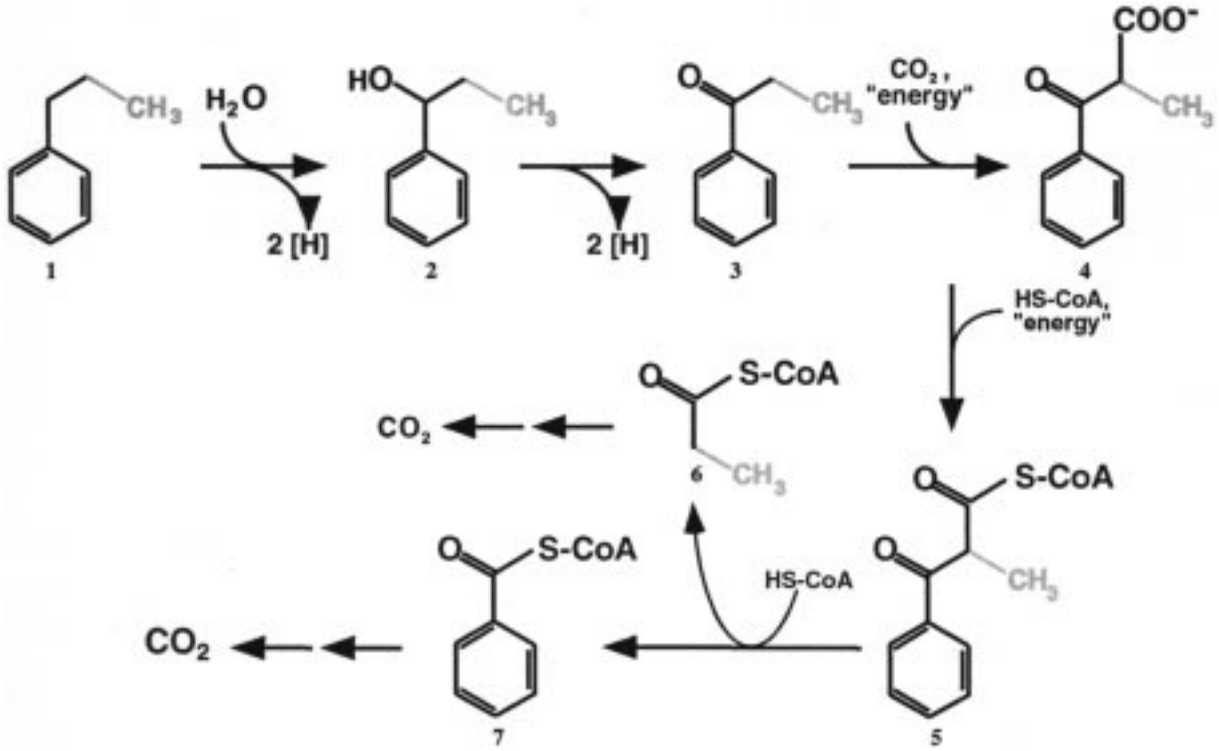

Figure 4. Pathway of anaerobic ethylbenzene (and propylbenzene) mineralization in denitrifying bacteria with emphasis on the reactions leading to benzoyl-CoA. Experimental support of this pathway was derived mainly from studies of ethylbenzene-mineralizing *Azoarcus* strain EB1. It is assumed that a similar pathway in operating in anaerobic propylbenzene mineralization (indicated by methyl group in gray). See text for evidence. The thiolytic cleavage product would be acetyl-CoA in the former and propionyl-CoA in the latter case. Chemical designation (given only for ethylbenzene pathway): 1, Ethylbenzene; 2, 1-phenylethanol; 3, acetophenone; 4, benzoylacetate; 5, benzoylacetyl-CoA; 6, acetyl-CoA; 7, benzoyl-CoA.

group in this position does in principle not interfere with a regular thiolytic cleavage; as a result, benzoyl-CoA and propionyl-CoA would be formed and metabolized further. An interesting question is whether and to which extent the same enzymes are involved in the degradation of propylbenzene and ethylbenzene, or whether more specific enzymes are synthesized for each hydrocarbon.

Naphthalene

In a marine enrichment culture of sulfate-reducing bacteria on napthalene, 2-naphthoate (naphthalene-2-carboxylate) was identified (Zhang & Young 1997). This finding suggested that anaerobic activation of naphthalene involves carboxylation. In agreement with this is the observation that a pure culture of a sulfate-reducing bacterium, strain NaphS2, that has been isolated with naphthalene as the only organic substrate, used in addition 2-naphthoate, but not 1-naphthoate (Galushko et al. 1999). A different initial mechanism of anaerobic degradation of naphthalene was suggested in a study of anaerobic freshwater microcosms in the presence of sulfate. In these, naphthalenol (naphthol; isomer not determined) was detected as a possible intermediate (Bedessem et al. 1997).

Aliphatic hydrocarbons

Alkanes

Containing exclusively apolar sigma bonds in the molecule, alkanes belong to the chemically least reactive organic compounds. Aerobic alkane-degrading microorganisms initiate the metabolism of their hydrocarbon substrates by monooxygenases (Britton 1984; Bühler & Schindler 1984). These enzymes generate a highly reactive oxygen species by partial reduction of O_2, resulting in the introduction of a hydroxyl group into the alkane molecule by a radical mech-

anism (White & Coon 1980; Groh & Nelson 1990). To our present knowledge, generation of oxidized forms of oxygen or compounds with comparable oxidizing properties and reactivity is not possible under anoxic conditions. In some early studies of alkane-degrading microorganisms, an oxygen-independent initial metabolism of alkanes via dehydrogenation to 1-alkenes and hydration to primary alcohols was suggested (Senez & Azoulay 1961; Chouteau et al. 1962; Azoulay et al. 1963; Davis & Yarbrough 1966; Wagner et al. 1967; Iizuka et al. 1969; Iida & Iizuka 1970). In later investigations, however, alkane dehydrogenation (desaturation) as an oxygen-independent activation mechanism was viewed critically (McKenna & Kallio 1965; Britton 1984; Bühler & Schindler 1984; Aeckersberg et al. 1991; Aeckersberg et al. 1998). Adaptation studies and analysis of cellular fatty acids with a sulfate-reducing bacterium, strain Hxd3 (originally isolated with *n*-hexadecane) indicated that 1-alkenes are not intermediates during anaerobic degradation of alkanes (Aeckersberg et al. 1998). Cellular fatty acids in strain Hxd3 were mainly C-odd during growth with n-hexadecane, and mainly C-even during growth with *n*-heptadecane. One explanation of these fatty acids patterns was the assumption of an alteration of the carbon chain length during the initial reactions by a C-odd carbon unit, e.g., by terminal addition of a one-carbon compound. However, in the closely related alkane-utilizing sulfate-reducing strain Pnd3, cellular fatty acids were mainly C-even upon growth with C-even alkanes and mainly C-odd upon growth with C-odd alkanes; in addition, unidentified fatty acids were formed. These findings suggested different modes of initial reactions in the two *n*-alkane-degrading sulfate-reducing strains. Still, a common principle in the mechanism of initial reactions of *n*-alkanes in both strains was considered by assuming that the site of carbon addition may be also the subterminal position in the chain (Aeckersberg et al. 1998). In a third isolate of an *n*-alkane-degrading sulfate-reducing bacterium (strain AK-01), the *n*-alkanes utilized as organic growth substrates influenced the pattern of cellular fatty acids in a similar manner as in strain Pnd3; in addition, 2-, 4- and 6-methyl-branched fatty acids were identified (So & Young 1999b). By labelling studies, the methyl branch of the fatty acids was shown to be the original terminal carbon of the *n*-alkane, suggesting addition of a carbon compound to the subterminal position (i.e., C-2 atom) of the *n*-alkanes; the carboxyl group was not derived from bicarbonate (So & Young 1999b). Recent two-dimensional gel electrophoresis of cell extracts of the alkane-degrading denitrifying strain HxN1 (Table 1) revealed specific formation of proteins during growth on *n*-hexane that were not formed on *n*-hexanoate. One of these proteins exhibited in its N-terminus a sequence similarity to the small subunit (BssC) of benzylsuccinate synthase in denitrifying bacteria, but not to any other known protein (A. Behrends, P. Ehrenreich, J. Heider, F.A. Rainey & F. Widdel, unpublished data). Results point at a mechanistic similarity between alkane activation and toluene activation in anaerobic bacteria. However, the energy barrier for C–H bond cleavage in an alkane as required for the reaction with a hypothetic organic co-substrate such as fumarate is higher than in the case of toluene. An alkyl radical cannot be stabilized by delocalization like the benzyl radical. A unique biosynthetic reaction that is, in principle, comparable to the activation of an alkane molecule is the anaerobic coupling of two palmitate molecules at their subterminal carbon atoms to form diabolic acid (15,16-dimethyltriacontanedioic acid). This long-chain dicarboxylic acid has been detected as a component in the lipid fraction of *Butyrivibrio fibriosolvens*. Investigations into the mechanism suggest 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 (Fitz & Arigoni 1992; Galliker et al. 1998). In this biosynthetic (anabolic) reaction, there is a net production of hydrogen atoms (2H per diabolic acid formed) that presumably appear at the free ligand formed upon cleavage of the cobalt-carbon bond; this would necessitate regeneration of the ligand of coenzyme B_{12} for the next catalytic round.

Alkenes

The initial reactions of alkene activation are still unknown. If alkane-utilizing bacteria use in addition alkenes, these could, in principle, be activated like alkanes at carbon positions remote from the double bond, even though such a reaction has not been demonstrated. However, most anaerobes degrading alkenes were directly enriched with these compounds and did not utilize saturated hydrocarbons (Schink 1985a; Gilewicz et al. 1991; Foss et al. 1998). This suggests that there are specific alkene-activating enzymes, and that the double bond is essential for their catalytic mechanism. Reactions appear in principle possible at the carbon atom next to the double bond, or at the double bond itself.

In the case of a reaction at the carbon adjacent to the double bond, hypothetical radical or cationic intermediates can be stabilized via delocalization. The promiscuous catalysis by benzylsuccinate synthase of 1-methyl-1-cyclohexene addition to fumarate (see section 'Catalytic properties of benzylsuccinate synthase') is such an example; the natural significance of this relaxed substrate specificity is unknown. Comparative substrate tests with various menthadienes (monoterpene alkenes with two double bonds) suggested that sp^2-hybridization of the ring carbon atom next to the methyl group is a prerequisite for degradability of the hydrocarbon by *Alcaligenes defragrans* (Hylemon and Harder 1999); however, there are no hints so far that the methyl group is the site of initial enzymatic attack as in the case of the model compound 1-methyl-1-cyclohexene in the toluene activating system.

If a reaction (e.g., addition of a proton) occurred at an isolated double bond of an alkene, the intermediate (e.g., carbenium ion) would not be stabilized by delocalization. It is true that reactions at double bonds such as hydrations or reductions are very common in the metabolism of all organisms; however, in these reactions there is at least one adjacent functional group in the substrate that favours formation of the ionic intermediate or concerted addition of an H^+ and HO^- ion, as for instance in the reaction of fumarase, aconitase or enoyl-CoA-hydratase reaction (for references see Buckel 1992). Nevertheless, there are examples of reactions at isolated double bonds without functional groups next to the double-bonded carbon atoms; this is always the case in hydrocarbon molecules. Carotenoids in anoxygenic phototrophic bacteria (e.g. neurosporene) can be hydrated at isolated double bonds to hydroxyl compounds (e.g. hydroxyneurosporene); the genes coding for the involved enzymes have been identified (Takaishi 1999). A bacterial enzyme has been purified and heterologously expressed that hydrates the isolated terminal double bond in the isopropyl side chain of limonene, an alkenoic monoterpene, yielding α-terpineol (Savithiry et al. 1997). In denitrifying *Alcaligenes defragrans*, a cometabolic conversion of the monoterpene isolimonene to isoterpinolene, viz. a shift of the terminal double bond in the side chain toward the ring (viz. to an energetically more stable position, has been observed (Heyen & Harder 1998)). In the carotene as well as in the monoterpenes, the double-bonds involve tertiary (branched) carbon atoms. Therefore, a catalysis of these reactions by protonation appears likely since the resulting carbenium ion at a tertiary carbon would be in an energetically favourable position. On the other hand, there are anaerobic reactions at isolated double bonds that are neither adjacent to a functional group nor involve a tertiary carbon atom. Long-chain unsaturated fatty acids can react at their double bond (or at one of their double bonds) with the methyl group (equivalent to a methyl cation) from S-adenosylmethionine to yield cyclopropane fatty acids or, if subsequent reduction takes place, methyl-branched fatty acids (Gurr & Harwood 1991; Grogan & Cronan 1997); these modified fatty acids are part of many lipids. Hydration of long-chain fatty acids at isolated double bond positions resulting in hydroxy acids has also been reported (Niehaus et al. 1970; Yang et al. 1993). In a 1-hexadecene-degrading methanogenic enrichment culture that also continued to grow on primary long-chain alcohols, hydration of the double bond to yield a primary alcohol (which would be an anti-Markownikoff orientation) has been speculated about, but so far only acetate and no early metabolite was detectable as an intermediate (Schink 1985a).

Alkyenes

Acetylene is a relatively reactive hydrocarbon, as obvious from its chemical properties. For instance, with Hg^{2+} and H^+ ions as catalysts, acetylene can be converted to acetaldehyde. Due to a certain C–H-acidity (pK_a = 25), acetylene can also combine with some metal ions such as monovalent copper from amine complexes. The fast metabolism of acetylene in *Pelobacter acetylenicus* indicates substantial reactivity of acetylene. Enzymatic measurements and analyses of intermediates suggested that degradation of acetylene in *P. acetylenicus* occurs via hydration to acetaldehyde (Rosner & Schink 1995), as observed in aerobic acetylene-degrading bacteria (DeBont & Peck 1980; Kanner & Bartha 1982). One part of the acetylene is then oxidized to acetate; the reducing equivalents are transferred to another part of the acetaldehyde; hence, the overall reaction is a dismutation to equimolar amounts of acetate and ethanol (Schink 1985b). Acetylene hydratase has been purified (Rosner & Schink 1995) and its catalytic properties as well as prosthetic groups have been studied. The enzyme, which is a monomer (molecular mass 83 kDa), contains an Fe_4S_4 cluster and tungsten as part of tungstopterine-guanine dinucleotide (Meckenstock et al. 1999). In a suggested model, acetylene binds to the tungsten(IV) site, fol-

lowed by the addition of water to the triple bond. The reaction of the enzyme appeared to be independent of the redox state of the iron-sulfur cluster.

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