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Minireview

Anaerobic benzene degradation by bacteria

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Summary

Benzene is a widespread and toxic contaminant. The fate of benzene in contaminated aquifers seems to be primarily controlled by the abundance of oxygen: benzene is aerobically degraded at high rates by ubiquitous microorganisms, and the oxygen-dependent pathways for its breakdown were elucidated more than 50 years ago. In contrast, benzene was thought to be persistent under anoxic conditions until 25 years ago. Nevertheless, within the last 15 years, several benzene-degrading cultures have been enriched under varying electron acceptor conditions in laboratories around the world, and organisms involved in anaerobic benzene degradation have been identified, indicating that anaerobic benzene degradation is a relevant environmental process. However, only a few benzene degraders have been isolated in pure culture so far, and they all use nitrate as an electron acceptor. In some highly enriched strictly anaerobic cultures, benzene has been described to be mineralized cooperatively by two or more different organisms. Despite great efforts, the biochemical mechanism by which the aromatic ring of benzene is activated in the absence of oxygen is still not fully elucidated; methylation, hydroxylation and carboxylation are discussed as likely reactions. This review summarizes the current knowledge about the 'key players' of anaerobic benzene degradation under different electron acceptor conditions and the possible pathway(s) of anaerobic benzene degradation.

Introduction

Benzene is a common component of fuels, particularly gasoline, and an important raw chemical used as solvent or chemical intermediate. Due to its extensive use, benzene is a widespread anthropogenic contaminant in aqueous environments. Compared with other hydrocarbons, benzene is highly water-soluble (saturation: 24 mM at 25°C) and toxic; the US Environmental Protection Agency has classified benzene as a Group A human carcinogen. Chemically, benzene is stable under typical environmental conditions, as the compound is stabilized by the aromatic ring system (π-electron system) without any potentially reactive substituent.

The persistence of benzene in aqueous environments seems to be primarily controlled by the abundance of oxygen as benzene is often persistent under anoxic conditions. Aerobic benzene-degrading microorganisms are ubiquitous and have been known for a long time – the first report in regard to aerobic benzene-degrading microorganisms dates almost 100 years back (Söhngen, 1913). Benzene-degrading organisms contain mono- or dioxygenases which activate the aromatic nucleus by introducing molecular oxygen to yield phenol or *cis*-benzene dihydrodiol, compounds that are further oxidized to catechol (Gibson and Parales, 2000; Tao *et al*., 2004). The aromatic ring of catechol is finally cleaved by further dioxygenases in *ortho*- or *meta*-position (Vaillancourt *et al*., 2006).

Contaminant plumes in aquifers usually become anoxic due to the low solubility and rapid microbial consumption of oxygen. Therefore, knowledge about anaerobic benzene degradation is highly relevant to understand the fate of benzene in the environment. Until the beginning of 1980, aromatic hydrocarbons were thought to be generally recalcitrant under anoxic conditions (Atlas, 1981). In the last 25 years, it was realized that many hydrocarbons including aromatics are biodegradable under several electron-accepting conditions (for a review see Foght, 2008). However, anaerobic benzene degradation is usually slow and associated with long lag-times. Benzene is considered to be more persistent under anoxic conditions than its alkylated derivatives toluene, ethylbenzene and xylene isomers and the reasons for the recalcitrance of benzene are not yet clear. Co-contaminants have been shown to inhibit anaerobic benzene degradation (Edwards *et al*., 1992; Cunningham *et al*., 2001; Ruiz-Aguilar *et al*., 2003; Da Silva and Alvarez, 2007) – and other studies suggest that anaerobic benzene degraders

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are not ubiquitous in subsurface sediments (Kazumi *et al*., 1997; Nales *et al*., 1998; Weiner and Lovley, 1998a; Phelps and Young, 1999). As we outline in this review, benzene can be degraded by syntrophic consortia which may require narrow and specific environmental conditions for optimal functioning, possibly explaining why benzene is not or only slowly degraded at some field sites or in laboratory enrichment cultures.

The number of laboratory enrichment cultures capable to degrade benzene under anoxic conditions has increased in the last 10 years, but is still low compared with the number of cultures described for anoxic degradation of other hydrocarbons, e.g. toluene. Notably, only four benzene-degrading pure cultures were described, and all of them use nitrate as electron acceptor (Coates *et al*., 2001; Kasai *et al*., 2006). Under strictly anoxic conditions, pure benzene-degrading strains have been not yet isolated, although some enrichment cultures seem to be dominated by specific organisms. The lack of capable laboratory cultures and the slow growth of the available cultures might be the main reasons why the pathway for anaerobic benzene degradation is still not elucidated.

This review summarizes the current knowledge about the organisms involved in anaerobic benzene degradation and the possible mechanisms by which the benzene ring is activated and transformed in the absence of oxygen, including innovative experimental results published by different research groups in the last 5 years. The reader may find additional information regarding anaerobic benzene degradation also in previously released excellent reviews (Lovley, 2000; Coates *et al*., 2002; Foght, 2008; Weelink *et al*., 2010).

Benzene degradation in laboratory microcosms – electron acceptors and 'key players'

Benzene-mineralizing laboratory microcosms have been established under several electron-acceptor conditions; in most cases, successful enrichments of anaerobic benzene degraders were established by using anoxic sediment or soil taken from petroleum contaminated sites (for an overview see Table 1). The major part of microorganisms living in the subsurface are attached to sediment or soil particles (Harvey *et al*., 1984; Kölbel-Boelke *et al*., 1988; Hazen *et al*., 1991; Alfreider *et al*., 1997; Griebler *et al*., 2002; Anneser *et al*., 2010). Correspondingly, microcosms prepared from sediment usually show higher degradation rates and shorter lag-phases compared with microcosms prepared from groundwater (Holm *et al*., 1992). For most of the enrichment cultures, however, long lag-phases (sometimes more than 100 days) were observed before anaerobic benzene degradation was detectable. Initial degradation rates were low, indicating that the specific rate of anaerobic benzene degradation is generally rather low. Nevertheless, long lag-phases and low degradation rates seem to be not the only reasons for the limited number of anaerobic benzene-degrading cultures successfully enriched so far. On the one hand, several authors observed that anaerobic benzene degraders could not be detected at all at distinct sites (e.g. Langenhoff *et al*., 1996; Kazumi *et al*., 1997; Nales *et al*., 1998; Weiner and Lovley, 1998a; Phelps and Young, 1999), indicating that anaerobic benzene degraders may not be ubiquitous. On the other hand, it has been shown that the majority of bacteria cannot be cultured in the laboratory yet (Rappe and Giovannoni, 2003; Keller and Zengler, 2004). These yet uncultured organisms might include anaerobic benzene degraders. Possibly, 'not detected' means 'beyond the detection limit' in some cases. In our laboratory, we have set up anaerobic enrichment cultures from different contaminated sites with ^{13}C-labelled benzene as substrate, and observed in some (but not all) cultures a small but continuous release of ^{13}C-CO_2, demonstrating that benzene is mineralized in those cultures, but for long incubation times only detectable for highly sensitive gas chromatographic isotope ratio mass spectrometers (Carsten Vogt, unpubl. results). Similar results were obtained by Morasch and colleagues (2007).

Interestingly, the growth behaviour of benzene-degrading cultures seems to be not correlated to the amount of potential energy available by the electron acceptor. The biomass yields of benzene-degrading nitrate-reducing pure and enrichment cultures were reported to be low and comparable to the yield of methanogenic benzene-degrading enrichment cultures (Coates *et al*., 2001; Ulrich and Edwards, 2003). This is surprising as the standard free energy for benzene mineralization with nitrate or ferric iron as electron acceptor is more than an order of magnitude higher compared with the acceptors sulfate and carbon dioxide (Table 2).

At some sites, enrichment cultures could be established using different electron acceptors [e.g. Ponca City, USA: ferric iron (Caldwell *et al*., 1999; Caldwell and Suflita, 2000), sulfate (Anderson and Lovley, 2000), carbon dioxide (Weiner and Lovley, 1998a); Toronto Gas Station: nitrate, sulfate, carbon dioxide (Nales *et al*., 1998; Ulrich and Edwards, 2003)]. A few cultures were also described to switch from sulfate to carbon dioxide as electron acceptor and vice versa (Ulrich and Edwards, 2003). The latter examples support the hypothesis of syntrophic processes governing anaerobic benzene degradation, which are discussed in more detail below.

Benzene degradation under methanogenic conditions

Benzene degradation under methanogenic conditions was occasionally observed in laboratory microcosms (Wilson *et al*., 1986; Grbic-Galic and Vogel, 1987; Kazumi

Table 1. Overview about anaerobic benzene-degrading microcosms described so far: sources, microcosm preparation, dominant organisms and putative degradation pathway.

Source of microorganisms	Electron acceptor	Set-up of laboratory microcosms[a]	Dominant phylotypes in enrichment culture	Suggested degradation pathway	Reference
Cartwright gasoline station (Toronto, Canada)	NO_3^- (nitrate reduction to nitrite)	Soil + anoxic mineral salt medium Enrichment culture established	*Betaproteobacteria* (*Azoarcus*/*Dechloromonas*), *Chlorobi*	**Methylation** (metabolite study; compound-specific isotope analysis)	Nales *et al.* (1998) Burland and Edwards (1999) Mancini *et al.* (2003) Ulrich and Edwards (2003) Ulrich *et al.* (2005) Mancini *et al.* (2008)
Non-contaminated swamp (Ontario, Canada)	NO_3^-	Soil + anoxic mineral salt medium Enrichment culture established	*Pelotomaculum*, *Chlorobi*, *Betaproteobacteria*	**Methylation** (compound-specific isotope analysis)	Nales *et al.* (1998) Ulrich and Edwards (2003) Mancini *et al.* (2008)
Petroleum-contaminated aquifer (Bemidji, USA)	Fe^{3+}	Sediment + anoxic groundwater Enrichment culture established	*Geobacteraceae* (*Geobacter* sp.)	n.d.	Baedecker *et al.* (1993) Cozzarelli *et al.* (1994) Anderson *et al.* (1998) Anderson and Lovley (2000) Rooney-Varga *et al.* (1999)
Petroleum refinery site (Ponca City, USA)	Fe^{3+}	Sediment Enrichment culture established	n.d.	Phenol and benzoate as metabolites	Caldwell *et al.* (1999) Caldwell and Suflita (2000)
Landfill site (the Netherlands)	Fe^{3+}	Sediment + anoxic mineral salt medium or groundwater + anoxic mineral salt medium Enrichment culture established	*Geobacteraceae*; syntrophy assumed	Phenol and benzoate as metabolites	Botton and Parsons (2006; 2007) Botton *et al.* (2007)
Coal gasification site (Gliwice, Poland)	Fe^{3+}	Soil + anoxic mineral salt medium Enrichment culture established	*Peptococcaceae*, *Desulfobulbaceae*, *Actinobacteria*; syntrophy assumed	**Carboxylation** (metabolite study, proteomic analysis)	Kunapuli *et al.* (2007; 2008) Laban *et al.* (2010)
San Diego Bay (San Diego, USA)	SO_4^{2-}	Sediment + mineral salt medium Repeated benzene degradation in microcosms	n.d.	No metabolites detected	Lovley *et al.* (1995)
National park (Empire, USA)	SO_4^{2-}	Sediment + groundwater or mineral salt medium Enrichment culture established	n.d.	Phenol and benzoate as metabolites	Kazumi *et al.* (1997) Caldwell and Suflita (2000)
Guaymas Basin (Gulf of Mexico)	SO_4^{2-}	Sediment + mineral salt medium Enrichment culture established	Different phylotypes; *Deltaproteobacterium* (*Desulfobacteraceae*) assimilates benzene	Conversion to benzoate (no clear evidence for carboxylation with HCO_3^-)	Phelps *et al.* (1996; 1998; 2001) Oka *et al.* (2008)
Marine sediment	SO_4^{2-}	Sediment + synthetic seawater Enrichment culture established	*Deltaproteobacterium* (*Desulfobacteraceae*)	Conversion to benzoate (activity tests in dense cell suspensions)	Musat and Widdel (2008)
Coal gasification site (Gliwice, Poland)	SO_4^{2-}	Sediment + mineral salt medium Enrichment culture established	*Pelotomaculum*-related	Benzoate, phenol, 4-hydroxybenzoate as intermediates; hydroxylated aromatics probably abiotically produced	Laban *et al.* (2009)
Benzene contaminated aquifer (Zeitz, Germany)	SO_4^{2-}	Coarse sand + groundwater or mineral salt medium; pre-enrichment in sand-filled columns percolated with anoxic groundwater Enrichment culture established	Different phylotypes; phylotypes belonging to the *Peptococcaceae* and *Epsilonproteobacteria* assimilated benzene; syntrophy assumed	Analogous activation mechanisms as described for other methanogenic or sulfate-reducing enrichment cultures (compound-specific isotope analysis)	Vogt *et al.* (2007) Fischer *et al.* (2008; 2009) Herrmann *et al.* (2010) Kleinsteuber *et al.* (2008)

Table 1. *cont.*

Source of microorganisms	Electron acceptor	Set-up of laboratory microcosms[a]	Dominant phylotypes in enrichment culture	Suggested degradation pathway	Reference
Deep aquifer formation (France)	SO_4^{2-}	Filtered biomass from groundwater + mineral salt medium Repeated benzene degradation	*Pelobacter*-related *Thermotogales*	n.d.	Berlendis *et al.* (2010)
Sewage sludge	HCO_3^-	Ferulic acid-degrading methanogenic consortia (enriched for 5 years) as starting material	n.d.	**Hydroxylation from water**	Vogel and Grbic-Galic (1986) Grbic-Galic and Vogel (1987)
National park (Empire, USA)	HCO_3^-	Aquifer sediment + groundwater or mineral salt medium Enrichment culture established	n.d.	Benzoate and phenol as metabolites	Kazumi *et al.* (1997) Caldwell and Suflita (2000)
Gasoline station (Toronto, Canada)	HCO_3^- or SO_4^{2-}	Soil + anoxic mineral salt medium Enrichment culture established	*Desulfobacterium,* unclassified phylotype, *Desulfotomaculum, Spirochaetes*	n.d.	Nales *et al.* (1998) Ulrich and Edwards (2003) Mancini *et al.* (2008)
Oil refinery site (Oklahoma, USA)	HCO_3^- (first SO_4^2)	Soil + anoxic mineral salt medium Enrichment culture established	*Desulfosporosinus, Desulfobacteraceae*, *Methanosarcinales*, *Methanomicrobiales*, *Methanobacteriales*	Benzoate, toluene and phenol as metabolites	Nales *et al.* (1998) Ulrich and Edwards (2003) Da Silva and Alvarez (2007) Mancini *et al.* (2008)
Petroleum refinery site (Ponca City, USA)	HCO_3^-	Sediment + anoxic groundwater	n.d.	Phenol, propionate and acetate putative intermediates (inhibition study)	Weiner and Lovley (1998b)
Baltimore harbour (Baltimore, USA)	HCO_3^-	Sediment + anoxic mineral salt medium Repeated benzene degradation	Several phylotypes	n.d.	Chang *et al.* (2005)
Lotus field	HCO_3^-	Soil + distilled water Enrichment culture established	*Deltaproteobacterium* assimilated benzene (SIP), *Firmicutes*, *Methanosarcinales*, *Methanomicrobiales*; syntrophy assumed	n.d.	Sakai *et al.* (2009)
Coal-tar waste-contaminated site (Glens Falls, USA)	Unknown	Surface sediments + anoxic mineral salt medium, SO_4^{2-}- or NO_3^--amended	*Pelomonas, Ralstonia, Pseudomonas, Propionibacterium*	n.d.	Liou *et al.* (2008)

a. The microcosms were incubated with benzene as sole source of carbon and energy.

Table 2. Stoichiometric equations and standard free energy changes ($\Delta G^{0\prime}$) for benzene oxidation with different electron acceptors.

Electron acceptors (oxidized/reduced)	Stoichiometric equation	$\Delta G^{0\prime}$ (kJ mol^{-1})
CO_2/CH_4	$C_6H_6 + 6.75\ H_2O \rightarrow 2.25\ HCO_3^- + 3.75\ CH_4 + 2.25\ H^+$	–116[a]
SO_4^{2-}/H_2S	$C_6H_6 + 3\ H_2O + 3.75\ SO_4^{2-} \rightarrow 6\ HCO_3^- + 1.875\ H_2S + 1.875\ HS^- + 0.375\ H^+$	–185[b]
Fe^{3+}/Fe^{2+}	$C_6H_6 + 18\ H_2O + 30\ Fe^{3+} \rightarrow 6\ HCO_3^- + 30\ Fe^{2+} + 36\ H^+$	–3070[a]
NO_3^-/N_2	$C_6H_6 + 6\ NO_3^- \rightarrow 6\ HCO_3^- + 3\ N_2$	–2978[c]
NO_3^-/NO_2^-	$C_6H_6 + 15\ NO_3^- + 3\ H_2O \rightarrow 6\ HCO_3^- + 15\ NO_2^- + 6\ H^+$	–2061[c]
ClO_3^-/Cl^-	$C_6H_6 + 5\ ClO_3^- + 3\ H_2O \rightarrow 6\ HCO_3^- + 5\ Cl^- + 6\ H^+$	–3813[c]
O_2/H_2O	$C_6H_6 + 7.5\ O_2 + 3\ H_2O \rightarrow 6\ HCO_3^- + 6\ H^+$	–3173[c]

a. Burland and Edwards (1999).
b. Kleinsteuber and colleagues (2008).
c. Weelink and colleagues (2007).

et al., 1997; Nales *et al*., 1998; Weiner and Lovley, 1998b; Ulrich and Edwards, 2003; Chang *et al*., 2005; Sakai *et al*., 2009), but also verified *in situ* by means of push-pull experiments (Reinhard *et al*., 2005). The first demonstration of anaerobic benzene mineralization was reported by Grbic-Galic and Vogel (1987) who detected a small production (less than 10%) of ^{14}C-CO_2 from ring-labelled ^{14}C-benzene in a methanogenic culture which was originally prepared from sewage sludge and pre-enriched with ferulic acid as substrate. Complete mineralization of benzene to equal amounts of carbon dioxide and methane was shown later in microcosms set up with sediment samples taken from a benzene-contaminated aquifer and using ^{14}C-labelled benzene as substrate (Weiner and Lovley, 1998b). Added acetate (1 mM) or propionate (100 μM) strongly inhibited benzene mineralization, indicating that these compounds were fermentatively produced during benzene degradation. Two cultures initially enriched with sulfate as electron acceptor could switch to carbon dioxide; notably, degradation rates were significantly higher under methanogenic conditions (Ulrich and Edwards, 2003). In these cultures, phylotypes affiliated to the genera *Desulfobacterium* and *Desulfosporosinus* as well as aceticlastic methanogens were identified as dominant members of the community after prolonged incubation with benzene (Ulrich and Edwards, 2003; Da Silva and Alvarez, 2007; Mancini *et al*., 2008). Recently, a deltaproteobacterium distantly related to the *Syntrophaceae* was identified by stable isotope probing of DNA (DNA-SIP) and real-time PCR as a key player of anaerobic benzene degradation in a methanogenic enrichment culture set up from non-contaminated soil (Sakai *et al*., 2009). The archaeal clone library constructed from this enrichment culture was dominated by phylotypes belonging to the *Methanosarcinales* and *Methanomicrobiales*. The authors concluded that benzene was sequentially degraded by a consortium of fermenters, aceticlastic methanogens and hydrogenotrophic methanogens. Such syntrophic relationships likely exist in all benzene-degrading cultures under methanogenic conditions, as methanogens have a narrow organic substrate spectrum restricted to simple low-molecular-weight organic compounds, and are not known for degrading aromatic compounds. Thus, methanogens are assumed to consume hydrogen, acetate or other small molecules released by fermenting organisms. Such syntrophic relationships were also described for the degradation of long-chain alkanes under methanogenic conditions (Zengler *et al*., 1999).

Benzene degradation under sulfate-reducing conditions

Sulfate-dependent benzene mineralization was demonstrated for the first time in sediment microcosms from freshwater and marine origin (Lovley *et al*., 1995; Phelps *et al*., 1996). These studies verified a previous observation of benzene mineralization where the electron acceptor was assumed to be sulfate (Edwards *et al*., 1992); actually, this study was the first stating 'complete' anaerobic mineralization of benzene. *In situ* anaerobic benzene degradation at the Ponca City site (USA) could be stimulated by adding sulfate (Anderson and Lovley, 2000), indicating its preferred use as electron acceptor for anaerobic benzene degradation at this site. Sediment laboratory microcosms from this site degraded benzene also with carbon dioxide or ferric iron as electron acceptor (Weiner and Lovley, 1998a; Caldwell *et al*., 1999).

In the last 15 years, several cultures degrading benzene under sulfate-reducing conditions were enriched and the dominant organisms were described. The marine culture enriched by Phelps and colleagues (1996) contained 12 different phylotypes after incubation for 3 years with benzene as sole source of carbon and energy (Phelps *et al*., 1998). Four clones belonged to the *Desulfobacteraceae*, the other phylotypes were affiliated to *Thiomicrospira* (*Gammaproteobacteria*), *Sulfurovum* (*Epsilonproteobacteria*), *Bellilinea* (*Chloroflexi*), *Exiguobacterium* (*Bacilli*) as well as several members of the *Clostridia* and *Bacteroidetes* (according to the RDP Classifier; Wang *et al*., 2007). After more than 10 years of incubation, a phylotype belonging to the *Desulfobacteraceae* ('clone SB-21') was identified by DNA-SIP for

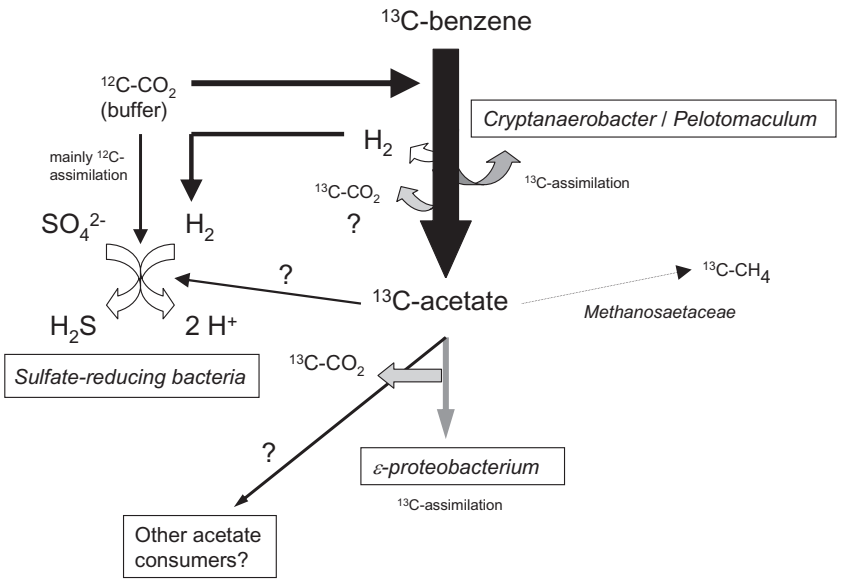

Fig. 1. Proposed degradation pathway for sulfate-dependent syntrophic benzene mineralization, based on previous observations (Vogt *et al*., 2007; Kleinsteuber *et al*., 2008; Herrmann *et al*., 2010; J. Rakoczy, K.M. Schleinitz, N. Müller, H.-H. Richnow, unpubl. data). T-RFLP and SIP data suggest that the *Cryptanaerobacter/ Pelotomaculum* (CP) phylotype assimilates the majority of benzene within the consortium. During benzene oxidation to acetate by the CP phylotype, hydrogen is formed and consumed by various sulfate reducers, driving the syntrophic process. Benzene-derived acetate might be consumed by several ecophysiologically different organisms, but especially by an epsilonproteobacterium. The majority of the produced carbon dioxide is thought to be formed from the acetate released by the CP phylotype. Small amounts of acetate are aceticlastically converted to methane.

assimilating benzene in this culture (Oka *et al*., 2008). Notably, a phylotype closely related to clone SB-21 was found to be the dominant organism in a benzene-mineralizing sulfate-reducing culture enriched also from marine sediment (Musat and Widdel, 2008). Furthermore, phylotypes related to the *Desulfobacteraceae* were detected in higher abundances in two benzene-degrading freshwater enrichments able to use sulfate or carbon dioxide as electron acceptor (Ulrich and Edwards, 2003; Mancini *et al*., 2008). These observations suggest that members of the *Desulfobacteraceae* are key players of benzene degradation under sulfate-reducing conditions. However, despite enrichment with benzene for several years, all of these cultures contained still other phylotypes, and a single benzene-degrading *Desulfobacteraceae*-like organism could not be isolated from any of the described cultures. Therefore, it is possible that benzene is mineralized in these cultures by synergetic or syntrophic relationships of *Desulfobacteraceae* and other organisms; this can be at least assumed for the enrichment cultures which can use both sulfate and carbon dioxide as electron acceptors.

In addition to phylotypes belonging to the Gram-negative *Desulfobacteraceae*, Gram-positive members of the family *Peptococcaceae* seem to be key players of sulfate-dependent anaerobic benzene degradation. The enrichment culture of Laban and colleagues (2009) was dominated by a phylotype related to the genus *Pelotomaculum*; the authors assumed that this organism may assimilate benzene solely using sulfate as electron acceptor. In our own culture enriched from groundwater-percolated sand columns at the field site Zeitz (Vogt *et al*., 2007), a phylotype affiliated to the *Cryptanaerobacter/ Pelotomaculum* group within the *Desulfotomaculum* subcluster Ih of the *Peptococcaceae* (Imachi *et al*., 2006) was shown to be crucial for sulfate-dependent benzene mineralization, as T-RFLP peak abundances of this organism increased after prolonged incubation with benzene and decreased during growth on other substrates (Kleinsteuber *et al*., 2008). Moreover, this phylotype and also an *Epsilonproteobacterium* distantly related to the genus *Sulfurovum* assimilated carbon from ^{13}C-labelled benzene in a subsequent DNA-SIP experiment (Herrmann *et al*., 2010). In this experiment, small amounts of ^{13}C-labelled benzene were converted to ^{13}C-labelled methane, and members of the aceticlastic *Methanosaetaceae* were detected in an archaeal 16S rRNA gene clone library. In addition, several sulfate-reducing *Deltaproteobacteria* were detected but not found to be ^{13}C-labelled in spite of prolonged incubation with benzene (Kleinsteuber *et al*., 2008; Herrmann *et al*., 2010). It was further shown that benzene mineralization could be reversibly inhibited by addition of hydrogen or low amounts of acetate (0.3 mM), indicating that both compounds are intermediates during anaerobic benzene fermentation (Rakoczy *et al*., unpubl. data). It was therefore suggested that benzene is mineralized by a consortium consisting of syntrophic fermenters, hydrogenotrophic sulfate reducers, aceticlastic methanogens and other acetate-consuming bacteria (Fig. 1). Contaminated aquifers might be a specific ecological niche for syntrophic benzene degradation, as syntrophs are versatile regarding the terminal electron acceptor and specifically adapted to a life at the thermodynamic limit. At the Zeitz site, anaerobic benzene degradation, likely with sulfate as electron acceptor, has been verified also in the flow path of the benzene plume by compound-specific stable isotope analysis (CSIA) (Fischer *et al*., 2007; 2009). This method, which is explained in more detail below, has generally great potential to verify anaerobic benzene degradation in

contaminated aquifers due to the strong hydrogen isotope fractionation linked to anaerobic benzene degradation.

Recently, a sulfate-reducing benzene-degrading culture was enriched from groundwater sampled from an underground gas storage area (Berlendis *et al*., 2010). The abundant phylotypes in this enrichment culture were distantly related to *Pelobacter*, *Thermotogales* and *Methanolobus*, indicating that benzene is syntrophically degraded.

Benzene degradation under iron-reducing conditions

Several benzene-mineralizing iron-reducing cultures were successfully established using amorphous iron hydroxide [$Fe(OH)_3$] as electron acceptor (Kazumi *et al*., 1997; Villatoro-Monzon *et al*., 2003; Jahn *et al*., 2005; Botton and Parsons, 2006; Kunapuli *et al*., 2007). Iron-dependent benzene mineralization could also be stimulated by adding nitriloacetic acid (NTA) or other iron-chelating compounds (Lovley *et al*., 1994; 1996; Caldwell *et al*., 1999). Presumably one of the best-investigated aquifers in which iron-driven benzene degradation takes place is the Bemidji site in Minnesota (USA), where a crude oil pipeline ruptured in 1979 and contaminated the adjacent aquifer (Essaid *et al*., 2011). Benzene degradation or mineralization in anaerobic microcosms prepared with sediment and groundwater from the iron-reducing zone of this aquifer was repeatedly reported (Baedecker *et al*., 1993; Cozzarelli *et al*., 1994; Anderson *et al*., 1998). Recently, benzene degradation was also verified directly in the anoxic iron-reducing zone of the plume by an *in situ* microcosms approach (Cozzarelli *et al*., 2010). Rooney-Varga and colleagues (1999) investigated the community structure of iron-reducing benzene-mineralizing enrichment cultures and sediment samples of the Bemidji site. MPN-PCR revealed an increase of *Geobacter*-related 16S rRNA gene copies in benzene-mineralizing sediments and enrichment cultures, indicating an involvement of *Geobacteraceae* in benzene mineralization under iron-reducing conditions.

Geobacteraceae were also dominant in iron-reducing benzene-degrading enrichment cultures set up from a landfill site in the Netherlands (Botton and Parsons, 2007). In contrast, no *Geobacteraceae* were identified in a highly enriched iron-reducing culture originating from a contaminated site in Poland (Kunapuli *et al*., 2007; Laban *et al*., 2010). Here, a phylotype affiliated to the *Peptococcaceae* was most abundant and assimilated ^{13}C-benzene in a DNA-SIP experiment (Kunapuli *et al*., 2007). Phylotypes affiliated to the *Desulfobulbaceae* and members of the *Actinobacteria* were also prominent. The authors suggested that benzene is syntrophically mineralized in this culture, with the *Peptococcaceae* phylotype as the primary benzene oxidizer.

Benzene degradation under nitrate-reducing conditions

Major and colleagues (1988) demonstrated first benzene degradation dependent on nitrate as electron acceptor, using microcosms set up from hydrocarbon-contaminated sediments and anoxic groundwater. Nitrate was shown to be reduced to dinitrogen during benzene degradation; the electron balance of reduced nitrate and degraded benzene indicated that benzene was mineralized, although carbon dioxide production from benzene was not experimentally confirmed. Benzene degradation under nitrate-reducing conditions was later observed in microcosms made of soil and anoxic groundwater taken from three different sites (Nales *et al*., 1998). Afterwards, stable benzene-degrading enrichment cultures were established and further examined. As demonstrated for one culture in experiments with ^{14}C-labelled benzene, more than 90% of the benzene's carbon was released as CO_2; benzene degradation appeared to be coupled to nitrate reduction to nitrite (Burland and Edwards, 1999). Notably, the biomass yield of the nitrate-reducing cultures was in the range observed for sulfate-reducing or methanogenic benzene-degrading enrichments (Ulrich and Edwards, 2003), despite the high redox potential of the nitrate/nitrite couple (Table 2). Dominant phylotypes in the cultures, determined after being incubated for several years in the laboratory, belonged to the *Betaproteobacteria* (genera *Azoarcus* and *Dechloromonas*), the *Peptococcaceae* (genus *Pelotomaculum*) and *Chlorobi* (Ulrich and Edwards, 2003; Mancini *et al*., 2008). Analogously to cultures enriched with ferric iron, sulfate or carbon dioxide as electron acceptor, it was apparently not possible to isolate a single benzene degrader out of these cultures, despite the long laboratory incubation time.

Benzene degradation with electron acceptors other than nitrate, sulfate, ferric iron or carbon dioxide

Manganese(IV) was shown to be used as electron acceptor for anaerobic benzene degradation in sediment microcosms and columns. The benzene degradation rates were significantly higher for manganese(IV) compared with ferric iron, indicating that this process is relevant in the environment (Villatoro-Monzon *et al*., 2003; 2008). Recently, Zhang and colleagues (2010) showed that benzene was mineralized by sediment microorganisms using a graphite anode as electron acceptor.

Pure cultures of anaerobic benzene degraders

Notably, the only pure cultures described to mineralize benzene using nitrate as electron acceptor were not isolated by the 'classical approach' from benzene-degrading enrichment cultures. The strains JJ and RCB, belonging

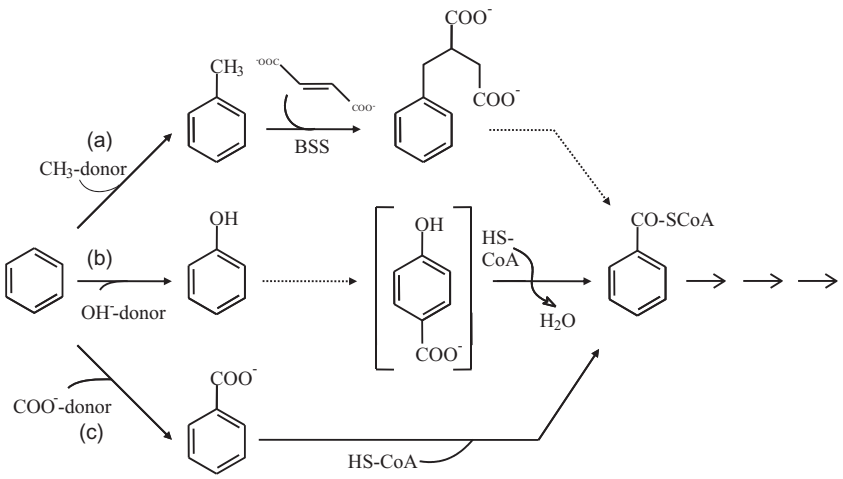

Fig. 2. Possible anaerobic benzene activation steps and further transformation reactions to benzoyl-CoA as central metabolite (modified from Foght, 2008 and Laban *et al*., 2010). (a) Methylation; (b) hydroxylation; (c) carboxylation. Benzoyl-CoA can be further reduced by ATP-dependent or -independent benzoyl-CoA reductases (Kung *et al*., 2009).

to the genus *Dechloromonas*, were originally isolated using humic substances and nitrate (JJ), or chlorobenzoate and chlorate (RCB) as electron donors and acceptors respectively (Coates *et al*., 2001). Kasai and colleagues (2006) identified a phylotype affiliated to the genus *Azoarcus* for assimilation of benzene with nitrate as electron acceptor in an RNA-SIP experiment targeting gasoline-contaminated groundwater. The authors than isolated several pure strains out of the groundwater using a non-selective medium and were able to identify two *Azoarcus* strains as nitrate-reducing benzene degraders in subsequent experiments with ^{14}C-labelled benzene (Kasai *et al*., 2006).

Tan and colleagues (2006) and Weelink and colleagues (2007) enriched a chlorate-reducing benzene-degrading culture. Later, a strain belonging to *Alicycliphilus denitrificans* was isolated that was able to mineralize benzene with chlorate as electron acceptor (Weelink *et al*., 2008). However, in the last step of the chlorate reduction pathway, chlorite dismutates to oxygen and chloride. Biochemical and physiological data suggest that this bacterium uses the oxygen released during chlorate reduction as a co-substrate for the initial attack and ring cleavage of benzene; thus it cannot be regarded as a true anaerobic benzene degrader. Benzene degradation by chlorate reduction is excellently and extensively described in the recent review of Weelink and colleagues (2010).

Mechanisms of anaerobic benzene activation

The question how benzene is activated in the absence of oxygen is still not convincingly answered, although several attempts have been made since the end of the 1980s to elucidate the activation mechanism. In principle, the benzene molecule is thermodynamically very stable due to the symmetric π-electron system of the aromatic ring and the lack of potentially destabilizing or reactive substituents.

Many alkylated aromatic hydrocarbons, e.g. toluene and xylenes, are activated under anoxic conditions by a reaction sequence in which fumarate is added to the alkyl side-chain of the aromatic ring (for a review see Heider, 2007). In the first step of this reaction, a relatively stable benzyl radical is thought to be formed. One might suggest that benzene is activated by a similar mechanism; however, the formation of a phenyl radical as a reactive intermediate for a subsequent methylation or other reaction is rather unlikely for energetic reasons. The abstraction of a hydrogen atom from benzene would need an activation energy of more than 460 kJ mol^{-1} which is roughly 100 kJ mol^{-1} more compared with the formation of a benzyl radical from alkylated benzene derivatives (Widdel and Rabus, 2001; Musat and Widdel, 2008). Thus, three other activation mechanisms have been intensively discussed: (i) an anaerobic hydroxylation of benzene yielding phenol, (ii) a Friedel-Crafts-type methylation of benzene yielding toluene and (iii) a carboxylation of benzene yielding benzoate (Fig. 2). We will discuss the pros and cons for all three possible activation reactions. Generally, isotope-based methods have been used to elucidate the reaction mechanism, either by CSIA, or by detection of ^{13}C- or ^{14}C-labelled metabolites which are formed during transformation of ^{13}C- or ^{14}C-labelled benzene. Due to the lack of pure cultures, most studies aiming to elucidate the reaction mechanism were performed with enrichment cultures.

Benzene hydroxylation

Benzene hydroxylation by hydroxyl ions stemming from water is an attractive model for benzene activation as the redox potential for this reaction is comparably low ($E^{\circ\prime} = -0.09$ V; Musat and Widdel, 2008) and hence likely possible even under sulfate-reducing or methanogenic conditions. Phenol itself has been shown to be mineralized by facultative and strictly anaerobic bacteria; the

degradation pathway proceeds via a carbon dioxide-dependent carboxylation of the aromatic ring to 4-hydroxybenzoate and further transformation to benzoyl-CoA (Bak and Widdel, 1986; Knoll and Winter, 1987; Tschech and Fuchs, 1987; Lovley and Lonergan, 1990; Zhang and Wiegel, 1994; Qiu *et al*., 2008; Schleinitz *et al*., 2009).

Hydroxylation of benzene had been already suggested as an initial reaction mechanism for benzene activation in one of the first reports regarding anaerobic benzene degradation (Vogel and Grbic-Galic, 1986). Addition of ^{18}O-labelled water resulted in the formation of ^{18}O-labelled phenol, indicating that the introduced hydroxyl group originated from water. In subsequent reports, phenol was often detected in enrichments as metabolite of benzene degradation under different electron acceptor conditions: in iron-reducing cultures (Caldwell and Suflita, 2000; Botton and Parsons, 2007; Kunapuli *et al*., 2008), sulfate-reducing cultures (Caldwell and Suflita, 2000; Laban *et al*., 2009) and methanogenic cultures (Weiner and Lovley, 1998b; Caldwell and Suflita, 2000; Ulrich *et al*., 2005). In some studies, benzoate was concomitantly detected with phenol (Caldwell and Suflita, 2000; Ulrich *et al*., 2005; Kunapuli *et al*., 2008).

It is noteworthy that it has recently been observed that phenol can be abiotically formed from benzene in culture media from iron and sulfate reducers by contact with air after sampling (Kunapuli *et al*., 2008). Hydroxyl radicals were likely generated by oxidation of iron in the sample during work up, which then reacted rapidly with benzene producing small amounts of phenol before sample analysis. The formation of 2-hydroxybenzoate and 4-hydroxybenzoate from benzoate was also explained by these mechanisms (Laban *et al*., 2009). These results indicate that it is generally problematic to distinguish between biotic and abiotic phenol formation in strongly reduced culture samples, probably preventing any clear evidence for benzene hydroxylation under strictly anoxic conditions by means of metabolite analysis. Hence, other methodological approaches are needed in addition. Substrate consumption tests with two highly enriched sulfate-reducing benzene-degrading cultures revealed that phenol is either not consumed (Laban *et al*., 2009) or only consumed after a certain lag-phase (Musat and Widdel, 2008), strongly indicating that phenol is unlikely to be an intermediate during benzene degradation in these cultures.

Chakraborty and Coates (2005) suggested that *Dechloromonas* strain RCB hydroxylated benzene to phenol, which was subsequently transformed to benzoate; the reaction was dependent on the presence of nitrate as electron acceptor. Here, the authors did not report phenol formation or benzene degradation in anoxic, nitrate-free control cultures amended with benzene. The origin of the introduced hydroxyl group could not be identified. When cells degraded benzene in $H_2{}^{18}O$-enriched mineral salt medium, the formed phenol was only slightly enriched with ^{18}O, suggesting that the hydroxyl group did not originate from water. On the other hand, hydroxyl free radical scavengers strongly inhibited benzene degradation and phenol formation, indicating that hydroxyl radicals were the source of the hydroxyl group in phenol. In an additional study, Chakraborty and colleagues (2005) showed that strain RCB could degrade benzene and several other aromatic hydrocarbons with nitrate, chlorate or oxygen as electron acceptor. Surprisingly, none of the known genes for anaerobic degradation of aromatic compounds could be found in the genome of strain RCB, which was recently sequenced (Salinero *et al*., 2009). Due to the lack of these genes, anaerobic benzene degradation in strain RCB 'remains enigmatic' (Salinero *et al*., 2009). On the other hand, strain RCB encodes several aerobic pathways for aromatics degradation. In the presence of chlorate, this organism releases oxygen during chlorate respiration allowing aromatics degradation by means of oxygenases even in the initial absence of oxygen in the culture medium, as shown also for the chlorate-reducing benzene degrader *Alicycliphilus denitrificans* (Weelink *et al*., 2008). Interestingly, it has been recently demonstrated that oxygen can be released during reduction of nitric oxide (NO) (Ettwig *et al*., 2010), an intermediate of the classical nitrate reduction pathway to dinitrogen. As also suggested by Weelink and colleagues (2010), strain RCB may contain this enzyme, allowing the use of oxygen for the initial attack of benzene and other aromatics even under nitrate-reducing conditions, explaining the apparently inconsistent physiological and genetic data. Unfortunately, the oxygen-releasing enzyme has not been characterized and can therefore not yet be identified in the genome of strain RCB. The genome of strain RCB contains the genes for the classical nitrate reduction to dinitrogen pathway, including those for nitric oxide reductase which catalyses nitric oxide reduction to nitrous oxide (N_2O) (Salinero *et al*., 2009).

Benzene methylation

Benzene methylation via Friedel-Crafts-type reaction is exergonic using the unique biological methyl donors *S*-adenosyl-methionine or methyl-tetrahydrofolate (Coates *et al*., 2002), which open the doors for another hypothesis for anaerobic benzene activation. Actually, *S*-adenosyl-methione-dependent alkylation of benzene (and substituted aromatics) has been observed in bone marrow (Flesher and Myers, 1991). Methylation has been also proposed for the anaerobic activation of the non-substituted aromatic hydrocarbon naphthalene (Safinowski and Meckenstock, 2006). If benzene is methylated

by anaerobes, the reaction product toluene could be further activated by addition of fumarate to the methyl group of toluene catalysed by the enzyme benzylsuccinate synthase (BSS), leading to the characteristic compound benzylsuccinate as intermediate. BSS has been detected in several anaerobic toluene-degrading pure and mixed cultures, and fumarate addition seems to be a unique activation mechanism for anaerobic toluene degradation (for an overview see Heider, 2007). PCR primers for the gene encoding the protein subunit which contains the reactive centre, *bssA*, have been also developed (Winderl *et al*., 2007). Thus, reasonable strategies for verifying benzene activation by methylation are detecting the intermediates toluene and benzylsuccinate or detecting the presence or expression of *bssA*-like genes or the induction or activity of BSS.

Ulrich and colleagues (2005) detected [*ring*-^{13}C]-labelled toluene and [*ring*-^{13}C]-labelled benzoate as intermediates in [$^{13}C_6$]-benzene-spiked nitrate-reducing and methanogenic enrichment cultures. The formation of [$^{13}C_6$]-phenol was observed only in the methanogenic culture. The nitrate-reducing culture degraded toluene rapidly and at higher rates than benzene, supporting the hypothesis that toluene might be an intermediate during benzene degradation in this culture. In contrast, toluene was only slightly degraded by the methanogenic culture. The authors concluded that two degradation pathways exist: (i) a methylation pathway leading to toluene with subsequent transformation to benzoate operating in the nitrate-reducing and methanogenic culture, and (ii) a hydroxylation pathway leading to phenol with subsequent formation of benzoate operating only in the methanogenic culture. This hypothesis was supported by studies in which CSIA was used for characterizing the initial step of benzene activation in different cultures (Mancini *et al*., 2003; 2008; Fischer *et al*., 2008). Mancini and colleagues (2008) showed that the ratio of hydrogen isotope fractionation ($\Delta\delta^2$H) versus carbon isotope fractionation ($\Delta\delta^{13}$C) – a value defined as lambda: $\Lambda = \Delta\delta^2H/\Delta\delta^{13}C$ (Fischer *et al*., 2008) – for anaerobic benzene degradation was significantly higher for the methanogenic culture ($\Lambda = 39 \pm 5$) compared with the nitrate-reducing enrichment culture ($\Lambda = 16 \pm 2$) investigated by Ulrich and colleagues (2005). Simplified, the lambda value can be seen as a biochemical fingerprint of a given biochemical reaction. Concordantly, other nitrate-reducing cultures showed lambda values in the range between 8 and 19, whereas for other methanogenic or sulfate-reducing cultures lambda values between 28 and 31 were determined (Mancini *et al*., 2003; 2008; Fischer *et al*., 2008). Thus, the CSIA data indicate that benzene activation under nitrate-reducing conditions is different from benzene activation under sulfate-reducing and methanogenic conditions. Nevertheless, 'different reaction mechanism' means that either the reactions are truly different (different products are formed), or the reactions are similar on paper (same products are formed) but proceed via different reaction mechanisms catalysed by different enzymes or cofactors leading to different fractionation patterns; the latter has been recently shown for toluene activation by benzylsuccinate synthase (Vogt *et al*., 2008; Herrmann *et al*., 2009). Hence, further research is needed to conclusively demonstrate that benzene can be methylated under nitrate-reducing conditions. Some highly enriched strictly anaerobic benzene-degrading cultures cannot degrade toluene (Kunapuli *et al*., 2008; Musat and Widdel, 2008; Laban *et al*., 2009), probably excluding biomethylation of benzene to form toluene as activation mechanism.

Benzene carboxylation

Similar to hydroxylation or methylation of benzene, benzene carboxylation is slightly exergonic or close to the thermodynamic equilibrium depending on the carboxyl donor and thus feasible even in sulfate-reducing or methanogenic cultures (Musat and Widdel, 2008). Additionally, for some non-substituted aromatic compounds, e.g. naphthalene (Zhang and Young, 1997; Musat *et al*., 2009; DiDonato *et al*., 2010) or phenanthrene (Zhang and Young, 1997; Davidova *et al*., 2007), a carboxylation reaction was suggested for ring activation, indicating that carboxylation might be an important activation principle for the degradation of non-substituted aromatic compounds in nature. Indeed, benzoate has been detected as intermediate of anaerobic benzene degradation in sulfate-reducing (Caldwell and Suflita, 2000; Phelps *et al*., 2001; Laban *et al*., 2009), iron-reducing (Caldwell and Suflita, 2000; Kunapuli *et al*., 2008), nitrate-reducing (Ulrich *et al*., 2005) or methanogenic (Caldwell and Suflita, 2000; Ulrich *et al*., 2005) enrichment cultures, concomitantly with the intermediates phenol (Caldwell and Suflita, 2000; Ulrich *et al*., 2005; Kunapuli *et al*., 2008; Laban *et al*., 2009) or toluene (Ulrich *et al*., 2005). Phelps and colleagues (2001) found deuterated benzoate (D5) as sole intermediate of deuterated benzene (D6) degradation in their highly enriched marine sulfate-reducing culture. Interestingly, incubation in the presence of a ^{13}C-labelled bicarbonate buffer system did not lead to ^{13}C incorporation in the carboxyl group of the benzoate intermediate, indicating that the introduced carboxyl group did not originate from carbon dioxide. This was in accordance with results presented by Caldwell and Suflita (2000) for their benzene-degrading sulfate-reducing freshwater culture. Here, fully [$^{13}C_7$]-labelled benzoate was formed when the culture was spiked with fully [$^{13}C_6$]-labelled benzene, showing that the carboxyl group of benzoate was stemming from transformation products of [$^{13}C_6$]-benzene itself, but not from the non-labelled bicarbonate buffer

system. In contrast, Kunapuli and colleagues (2008) found both [$^{13}C_6$]-benzoate and [$^{13}C_7$]-benzoate in their iron-reducing enrichment culture during incubation with fully labelled [$^{13}C_6$]-benzene. Furthermore, the authors detected ^{13}C-carboxy group-labelled benzoate if cells were incubated in medium prepared with non-labelled benzene and ^{13}C-labelled bicarbonate buffer, favouring the hypothesis that the bicarbonate buffer was the carboxyl group donor for benzoate formation.

However, it is generally difficult to interpret all these observations since benzoyl-CoA, the activated form of benzoate, is a common intermediate within the anaerobic degradation pathways of several aromatic compounds including toluene and phenol (for an overview see Carmona *et al*., 2009; see also Fig. 2); in addition, benzoate has been reported as an excreted intermediate during phenol degradation under methanogenic conditions (Knoll and Winter, 1987; Kobayashi *et al*., 1989; Bechard *et al*., 1990; Karlsson *et al*., 2000). Hence, the metabolite benzoate might be formed directly from benzene by a carboxylation step, but could be formed as well in later steps during anaerobic benzene degradation pathways starting, e.g. with a methylation or hydroxylation step.

Lately, Laban and colleagues (2010) investigated their highly enriched iron-reducing benzene-degrading culture using an approach combining metagenomics and metaproteomics. Subcultures were grown with benzene, phenol or benzoate as sole substrates, and peptide sequences were subsequently identified based on the metagenome which had been sequenced before. Proteins similar to the phenylphosphate carboxylase subunits PpcA and PpcD of *Azoarcus* sp. strain EbN1 and to the benzoate-CoA ligase of *Geobacter metallireducens* were specifically expressed during anaerobic benzene degradation. Based on these results, the authors suggested that benzene is directly carboxylated by a putative anaerobic benzene carboxylase. The formed benzoate might be further activated by a benzoate-CoA ligase to benzoyl-CoA. However, an enzyme activity test for the putative anaerobic benzene carboxylase, ultimately proving this hypothesis, could not be established yet.

Outlook – what is needed for a better understanding of the mechanisms of anaerobic benzene degradation?

Isolating strictly anaerobic benzene-degrading bacteria is still important for a better understanding of anaerobic benzene degradation. Sequencing the genome of such an organism may allow revealing the upper and lower degradation pathway; also more controlled physiological experiments would be possible for elucidating the anaerobic benzene activation mechanism. However, the gene encoding the responsible enzyme might be not yet detectable within the genome due to the unknown enzyme properties. Establishing any *in vitro* or *in vivo* assay undoubtedly proving a specific activation mechanism is clearly needed.

For highly enriched benzene-degrading cultures, sequencing of the metagenome might be very helpful to clarify syntrophic or cooperative relationships, as, for example, recently shown for anaerobic methane oxidation (Meyerdierks *et al*., 2010). If a metagenome is available, mRNA expression or proteomic studies may follow for further functional characterization (Ram *et al*., 2005; Meyerdierks *et al*., 2010).

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