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REVIEW ARTICLE

Microorganisms involved in anaerobic benzene degradation

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Abstract Benzene is a highly toxic compound. Moreover, benzene-contaminated groundwater is a widespread problem caused mainly by the lack of oxygen in the subsurface. Long-term exposure may cause leukaemia. However, pure cultures of microorganisms with the ability to degrade benzene anaerobically have recently been isolated from novel enrichment cultures. The novel pure cultures include the hyperthermophilic archaeon *Ferroglobus placidus* and the *Geobacter* strains Ben and *metallireducens* which degrade benzene anaerobically coupled to the reduction of Fe(III). Syntrophic interactions have been suggested in enrichments where members of the *Peptococcaceae* within the class *Clostridia* are the dominant organisms and suggested as being responsible for the first attack on the benzene ring. Laboratory enrichment studies have also resulted in the development of consortia which degrade benzene, with different terminal electron acceptors supporting the syntrophy. Other benzene-degrading microorganisms have been identified under methanogenic conditions, involving the reduction of humic acids by, for example, the deltaproteobacterium Hasda-A and members of the *Betaproteobacteria*, *Gammaproteobacteria* and *Deltaproteobacteria*, respectively. This review focuses on the microorganisms involved in anaerobic benzene degradation under conditions involving several electron acceptors in recent years. Information related to the anaerobic degradation of benzene is critical to understanding and predicting the fate of this contaminant in groundwater.

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Introduction

Benzene is derived from fossil fuels, cigarette smoke and forest fires, among other sources. It is an important raw chemical used as an intermediate to produce a wide range of products, such as plastics, resins, nylon, lubricants and pesticides. Benzene is also highly toxic; it may cause acute myeloid leukaemia, secondary aplastic anaemia and damage to the reproductive system. It can enter the body by skin contact, inhalation or consumption of contaminated water. Benzene-contaminated groundwater is a widespread problem caused partially by its high solubility in water (1.8 g l^{-1} at 25°C) relative to other organic compounds and partly to spills at production sites and leaks in underground storage tanks. These leaks have been documented by studies of the environment agencies in the USA and UK (Rudolph 1996; Atlas 2005). Benzene is readily degraded under aerobic conditions (Alvarez and Vogel 1991; Werlen et al. 1996; Fairlee et al. 1997; Greene et al. 2000) by ubiquitous microorganisms, such as *Pseudomonas* sp. and *Rhodococcus* sp. (Fahy et al. 2006). However contaminated groundwater is usually under anaerobic conditions due to the rapid depletion of available oxygen in subterranean environments, which in turn decreases the redox potential, favouring the growth of denitrifying, sulphate-reducing, iron-reducing and methanogenic populations that may also degrade the contaminant. Therefore, informed knowledge of anaerobic microorganisms capable of benzene degradation is critical to understanding and predicting the fate of this contaminant.

Initial studies suggested the lack of benzene degradation under anaerobic conditions (Barker et al. 1987; Kuhn et al. 1988; Acton and Barker 1992; Barbaro et al. 1992; Patterson

et al. 1993). However, laboratory-based and field studies in the last 20 years have confirmed the degradation of this pollutant coupled to the reduction of nitrate (Burland and Edwards 1999; Coates et al. 2001; Chakraborty and Coates 2005; Ulrich et al. 2005; Kasai et al. 2006; Jakoncic et al. 2007), sulphate (Lovley et al. 1995; Phelps et al. 1996; Weiner and Lovley 1998a; Sublette et al. 2006; Musat and Widdel 2008; Berlendis et al. 2010), iron (Lovley and Woodward 1996; Kazumi et al. 1997; Rooney-Varga et al. 1999; Jahn et al. 2005; Kunapuli et al. 2007), manganese (Villatoro-Monzón et al. 2003, 2008), humic acids (Cervantes et al. 2011) and methanogenesis (Vogel and Grbicgalic 1986; Grbicgalic and Vogel 1987; Kazumi et al. 1997; Weiner and Lovley 1998b; Caldwell and Suflita 2000; Ulrich and Edwards 2003; Chang et al. 2005; Da Silva and Alvarez 2007; Sakai et al. 2009).

Moreover, recent studies on the novel pure cultures of *Geobacter* species and the archaeon *Ferroglobus placidus* have confirmed the degradation of benzene coupled to iron-reducing conditions and have also provided information on the metabolites produced during growth on benzene (Holmes et al. 2011; Zhang et al. 2012). In addition, syntrophic associations are also suggested in enrichments capable of benzene degradation where members of the *Peptococcaceae* are the key players (Taubert et al. 2012; van der Zaan et al. 2012). Other microorganisms involved in the degradation of benzene under methanogenic conditions or coupled to the reduction of humic acids include the deltaproteobacterium Hasda-A and two phylotypes of *Gammaproteobacteria*, respectively (Cervantes et al. 2011; Masumoto et al. 2012). In this review, we focus on the microorganisms which have been identified in anaerobic benzene degradation studies in recent years and the possible terminal electron acceptors. It should be noted that excellent reviews on the anaerobic degradation of hydrocarbons (Boll et al. 2002; Foght 2008; Fuchs et al. 2011; Meckenstock and Mouttaki 2011; Flanagan et al. 2013; Heider and Schühle 2013; Boll et al. 2014), anaerobic microbial communities in hydrocarbon-contaminated aquifers (Kleinsteuber et al. 2012), the use of stable isotope probing (SIP) to identify anaerobic benzene and toluene degraders (Cupples 2011) and the degradation of benzene under anaerobic conditions (Lovley 2000; Coates et al. 2002; Weelink et al. 2010; Vogt et al. 2011) have been published during the past 12 years.

Pure cultures capable of anaerobic benzene degradation

Until a few years ago there were only a few pure cultures able to degrade benzene anaerobically, and all of these were coupled to nitrate reduction: an *Azoarcus* strain DN11 and AN9 (Kasai et al. 2006), a *Bacillus cereus* strain (Dou et al. 2010) and *Dechloromonas* strains JJ and RCB (Coates et al. 2001). However, no anaerobic degradation genes were found in the *Dechloromonas aromatica* RCB genome (Salinero et al. 2009), and subsequent data suggests the involvement of oxygen under nitrate-reducing conditions (Ettwig et al. 2010; Zhang et al. 2013). Nevertheless, recent studies have reported the novel pure cultures archaeon *Ferroglobus placidus* (Holmes et al. 2011) and a *Geobacter metallireducens* strain named Ben (Zhang et al. 2012), both of which are capable of anaerobic benzene degradation. The hyperthermophilic archaeon *F. placidus* is able to degrade benzene anaerobically under iron-reducing conditions, producing benzoate as the main metabolite, suggesting the carboxylation of benzene to form benzoate (Holmes et al. 2011). The *Geobacter* strain Ben, isolated from sediments within the ferric ion [Fe(III)]-reducing zone in a petroleum-contaminated aquifer in Bemidji, USA, was found to degrade benzene with ferric iron and anthraquinone-2,6-disulfonate (AQDS) as the only electron acceptors. In addition, *G. metallireducens* is also able to degrade benzene coupled to the reduction of Fe(III); trace amounts of phenol were detected during the degradation of benzene, suggesting phenol as an intermediate. This finding is also supported by the inhibition of benzene degradation by the deletion of genes involved in the metabolism of phenol and by their upregulation during benzene oxidation (Zhang et al. 2012; Zhang et al. 2013).

Microorganisms identified in recent anaerobic benzene degradation studies

Genera of the family *Peptococcaceae* (phylum *Firmicutes*, class *Clostridia*) have been identified as the main benzene degraders with most of the terminal electron acceptors [sulphate, nitrate, Fe(III), chlorate], and syntrophic interactions are strongly suggested. The most recent study by the Vogt group involved a sulphate-reducing enrichment analysed by the protein-SIP technique, with their observations indicating syntrophy between a benzene-fermenting clostridial group, a sulphate-reducing deltaproteobacterial group that uses the metabolites generated during benzene fermentation and a putative scavenger group from *Bacteroidetes*/*Chlorobi* (Taubert et al. 2012). This result is consistent with that of a previous study on the same consortium where acetate and hydrogen were strongly suggested as intermediate metabolites in the anaerobic benzene degradation process (Rakoczy et al. 2011). Members of the *Cryptanaerobacter*/*Pelotomaculum* group are suggested to be the benzene-fermenting group, as has been indicated in other studies from the same group (Kleinsteuber et al. 2008; Herrmann et al. 2010). Moreover, the syntrophic interactions and the presence of the *Peptococcaceae* members are also consistent with other enrichments. Van der

Zaan and colleagues (2012) identified members of the *Rhodocyclaceae*, *Burkholderiaceae* but mainly the *Peptococcaceae* as benzene degraders in an enrichment capable of benzene degradation with several electron acceptors [nitrate, sulphate, Fe(III), chlorate]; degradation was inhibited by the injection of hydrogen. Kunapuli and colleagues suggested syntrophy in an iron-reducing enrichment derived from a former Polish coal gasification site where members of the *Peptococcaceae*, only distantly related to the cultured *Thermincola*, were suggested to be the main benzene oxidizers. Other microorganisms identified included uncultured *Deltaproteobacteria* and members of the *Actinobacteria* (Kunapuli et al. 2007). Later, anaerobic benzene degradation coupled to sulphate reduction was reported for the same enrichment in the Meckenstock group (Abu Laban et al. 2009), and the enzymes involved in iron-reducing conditions were revealed (Abu Laban et al. 2010). Another enrichment from an underground gas storage aquifer in France revealed phylotypes related to the *Pelobacter* (*Deltaproteobacteria*), *Thermotogales*, *Methanolobus* and species not previously involved in BTEX (benzene, toluene, ethylbenzene, xylenes) degradation (Berlendis et al. 2010). Moreover, members of the *Desulfobacteraceae* family have also been reported to be the dominant organisms in sulphate-reducing and methanogenic enrichments (Musat and Widdel 2008; Oka et al. 2008). Other *Deltaproteobacteria* reported under methanogenic conditions include *Synthrophus gentianae* (Sakai et al. 2009), which is consistent with previous results (Dojka et al. 1998; Aburto et al. 2009).

Recent studies have identified a deltaproteobacterium, Hasda-A, which was isolated from non-contaminated lotus field soil in Japan as one of the main benzene degraders under methanogenic conditions (Masumoto et al. 2012). Members of the *Comamonadaceae* were isolated from benzene-contaminated groundwater in Canada where benzene degradation coupled to nitrate and sulphate reduction was enhanced by the addition of non-activated persulphate (Xiong et al. 2012).

Two *Gammaproteobacteria* phylotypes have also been reported to be the dominant organisms in a benzene-degrading enrichment from Mexico that used AQDS (humic acid) as the terminal electron acceptor (Cervantes et al. 2011). Anaerobic benzene degradation was also observed in salt marsh sediment from the Louisiana coast after the 2010 BP oil spill, but no microorganisms were identified (Yu et al. 2012). Table 1 summarizes the microorganisms identified in successful anaerobic benzene degradation during the last years, and Fig. 1 shows their phylogenetic distribution. The degradation of benzene with the different electron acceptors and the microbial communities detected are discussed in greater detail in the following sections.

Microorganisms identified in nitrate-reducing conditions

Although benzene was thought to be recalcitrant, by the late 1990s an enrichment culture established using a groundwater and soil inoculum demonstrated benzene degradation with nitrate as the only electron acceptor. Nitrate was reduced as nitrite accumulated stochiometrically, and over 90 % of labelled benzene was recovered in the form of CO_2, thereby confirming benzene degradation coupled to nitrate reduction. However, benzene degraders were not identified (Burland and Edwards 1999).

Nevertheless, two pure culture strains capable of benzene oxidation coupled to the reduction of nitrate were later reported (Coates et al. 2001). The organisms were isolated from two different environments in the USA: strain JJ was isolated from Campus Lake sediments from the Southern Illinois University; strain RCB was isolated from Potomac River sediments. Both strains were assigned to the genus *Dechloromonas* within the *Betaproteobacteria*, and they were reported to oxidize a wide range of compounds (formate, acetate, propionate, butyrate, lactate, succinate, pyruvate, benzoate, toluene) coupled to nitrate reduction. In a subsequent experiment using strain RCB, Chakraborty et al. (2005) reported the recovery of CO_2 from labelled benzene, which supported the degradation of benzene by strain RCB in anoxic aquatic sediments. Moreover, these authors found that the remaining BTEX compounds were also degraded by this strain.

Two other pure cultures (DN11 and AN9) capable of benzene degradation were isolated from gasoline-contaminated groundwater; these belong to the *Azoarcus* family, also found within the *Betaproteobacteria* (Kasai et al. 2006). The isolation of these two pure cultures is consistent with the identification of a dominant microbial population closely related to *Azoarcus* species in a benzene-degrading, denitrifying enrichment (Ulrich and Edwards 2003).

In 2010, Dou et al. (2010) reported the bacteria *Bacillus cereus* to be another pure culture capable of anaerobic benzene degradation under nitrate-reducing conditions. The strain, which was isolated from gasoline-contaminated soil in China, was able to degrade benzene concentrations up to 150 mg l^{-1}, producing phenol and benzoate as metabolites. However, as the anaerobic degradation of benzene coupled to nitrate reduction seems to involve the presence of molecular oxygen generated intracellularly, the cultures isolated by these authors may not be considered as true anaerobic degraders (Salinero et al. 2009; Vogt et al. 2011).

A recent study by van der Zaan and colleagues (2012) confirmed the anaerobic degradation of benzene under denitrifying conditions by bacteria belonging to the *Rhodocyclaceae*, the *Burkholderiaceae* and the *Peptococcaceae*, the latter being the dominant group. However, degradation was suggested to occur via a syntrophic process since sulphate, chlorate and Fe(III) iron were also

Table 1 Microorganisms identified in successful anaerobic benzene degradation studies during the last five years

Source	Terminal electron acceptor	Dominant phylotypes	Intermediates/ Suggested pathway	Benzene degradation rate	Reference
Lovley group collection.	Fe(III)	*Geobacter metallireducens* (pure culture)	Phenol/hydroxylation	3.66 μM day^{-1}	Zhang et al. 2013
Petroleum-contaminated aquifer Bemidji, MN, USA	Fe(III), AQDS	*Geobacter* strain Ben (pure culture)	ND	Fe(III): 3.42 μM day^{-1} AQDS: 2.51 μM day^{-1}	Zhang et al. 2012
Benzene-contaminated aquifer Zeitz, Germany	SO_4^{2-} Syntrophy	*Pelotomaculum*/*Cryptanaerobacter* (*Peptococcaceae*)	Hydrogen and acetate		Taubert et al. 2012
		Desulfobacca (*Syntrophaceae*)			Rakoczy et al. 2011
		Rhodothermus		3.6–5.7 μM day^{-1}	Herrmann et al. 2010
		Epsilonproteobacteria			
		Desulfovibrio			
Chemostat enrichment	NO_3, SO_4^{2-} Fe(III), chlorate Syntrophy	*Peptococcaceae*	Hydrogen	K_{max}: 0.7 day^{-1} (Highest reported)	van der Zaan et al. 2012
		Rhodocyclaceae			
		Burkholderiaceae			
Benzene-contaminated groundwater, Canada	NO_3 O_4^{2-}	*Comamonadaceae*	ND	75.9–92.8 % degradation rate.	Xiong et al. 2012
		Algoriphagus spp. *Firmicutes*			
		Treponema			
		Desulfovibrio			
Non-contaminated lotus field soil,Tsuchiura, Japan	CH_4 Syntrophy	*Deltaproteobacterium* Hasda-A	ND	3.39 μM day^{-1}; 0.51 μM day^{-1}	Masumoto et al. 2012; Sakai et al. 2009
		Firmicutes			
DSMZ strain	Fe(III)	*Ferroglobus placidus*	Benzoate/ carboxylation	81.2 % of CO_2 recovered from benzene in 30 days.	Holmes et al. 2011
Sediment and soil enrichments, Mexico	AQDS, HPSHA	ML sediment: *Desulfobacca acetoxidans*, *Shewanella*, *Chloroflexi*	ND	ML: 0.115 μM day^{-1} PR: 0.017 μM day^{-1}	Cervantes et al. 2011
		PR: Phylum TM-7, *Pseudomonadaceae*			
Coal gasification site, Poland	Fe(III)		Carboxylation	ND	Abu Laban et al. 2010
Deep aquifer, France	SO_4^{2-}	*Pelobacter*	ND	0.066 ppm day^{-1}	Berlendis et al. 2010

Fe(III), Ferric ion; DSMZ, German Collection of Microorganisms and Cell Cultures (Deutsche Sammlung von Mikroorganismen und Zellkulturen); AQDS, anthraquinone-2,6-disulfonate; HPSHA, highly purified soil humic acids; ND not determined; PR, hydrocarbon-contaminated soil; ML, hydrocarbon-contaminated sediment

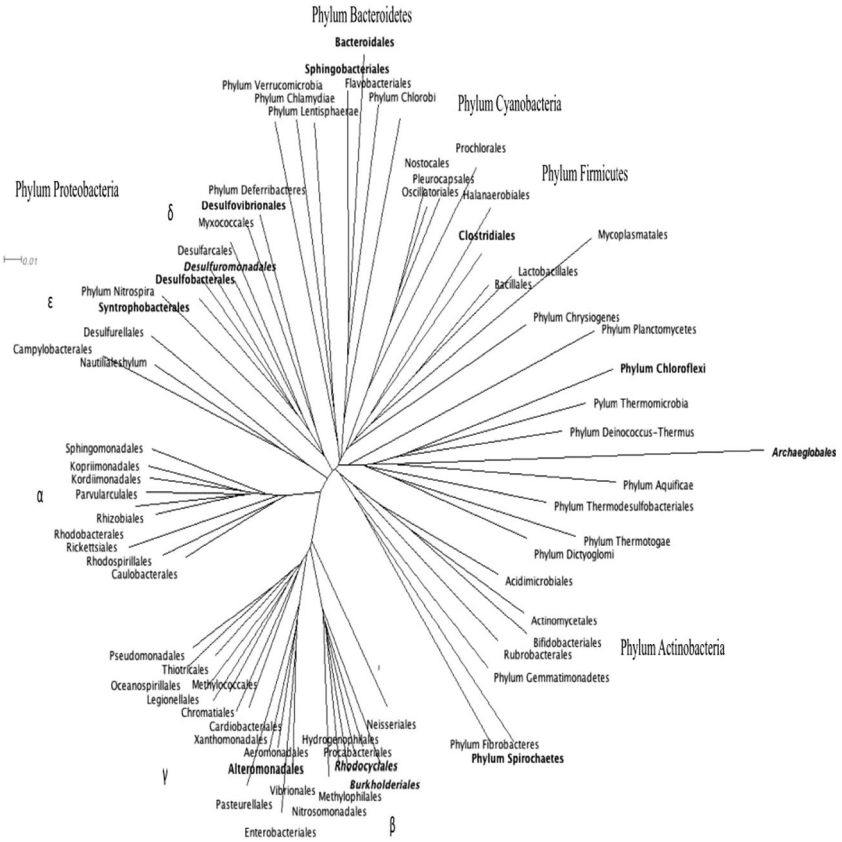

Fig. 1 Distribution of anaerobic benzene-degrading microorganisms based on 16S rRNA sequence comparisons. One representative organism from each order of each phylum was selected based on current bacterial taxonomy available from the online taxonomic outlines of prokaryotes available online (NCBI 2013), Jukes and Cantor distance, neighbour-joining method. *Names in bold* represent those orders from which organisms have been observed in anaerobic benzene degradation studies. *Names in bold and italics* represent orders from which anaerobic benzene degraders have been isolated. *Archeoglobales* belonging in the *Archaea* were included as the outgroup. *0.01* Changes per nucleotide position

used as electron acceptors, and the degradation was inhibited in the presence of hydrogen. In this study, t he degradation rate constant of benzene with nitrate was the highest among the electron acceptors and also higher than previously reported (van der Zaan et al. 2012). A later study reported the enhancement of benzene degradation under nitrate- and sulphate-reducing conditions by the addition of non-activated persulphate, which breaks down triethyl phosphate into ortophosphate and promotes nitrate and sulphate utilization (Xiong et al. 2012). The dominant microorganisms belonged to the *Comamonadaceae* family of the *Betaproteobacteria* and the *Algoriphagus* genus within the *Bacteroidetes* phylum, and nitrate and sulphite reductases have been reported for this genus (Yoon et al. 2005).

Microorganisms identified in iron-reducing conditions

Members of the *Geobacteraceae* within the *Deltaproteobacteria* are usually responsible for the degradation of BTEX coupled to iron reduction. An early study by Anderson and colleagues used sediments from the Fe(III)-reducing zone of a petroleum-contaminated aquifer in Bemidji, Minnesota to prepare microcosms spiked with benzene (Anderson et al. 1998). These authors did not observe any lag period during the degradation, which suggested the presence of adapted microorganisms in situ. The microorganisms were later identified as members of the *Geobacteriaceae*. A further study confirmed *Geobacter* to be the dominant organism in an enrichment prepared with the benzene-degrading sediments (Rooney-Varga et al. 1999). The Lovley group has studied in detail the degradation of benzene under iron-reducing conditions and found that it can be enhanced by the addition of Fe(III) chelators, such as nitrilotriacetic acid (Lovley et al. 1994). Insoluble Fe(III) oxides are solubilized by nitrilotriacetic acid, making them more accessible to Fe(III)-reducing microorganisms, thereby enhancing benzene degradation (Lovley and Woodward 1996). Other iron chelators, such as EDTA, ethanol diglycine, phosphates and humic acids, have also been found to stimulate the oxidation of benzene coupled to Fe(III) reduction in sediments from a petroleum-contaminated aquifer (Lovley et al. 1996b). The greatest degradation enhancement was observed with humic substances since they serve as intermediates in the reduction; the microorganisms transfer electrons to quinone moieties in the humic substances that are further transported to Fe(III) oxides, reducing them; this process regenerates the quinone moieties that can again receive electrons from Fe(III)-reducing microorganisms (Lovley et al. 1996a). Thus, multiple cycles of reduction and oxidation of humic substances can occur, and the reduction of iron can occur even at low humic acid concentrations. There is no need for contact between the

microorganisms and the Fe(III) oxide because humic substances accelerate the reduction of Fe(III); moreover, the latter they can reach Fe(III) oxides that the microorganisms can not and they react with more types of Fe(III).

Kunapuli and colleagues used SIP and a clone library to identify the microorganisms in a benzene-degrading enrichment coupled to iron-reducing conditions in soils from a former Polish coal gasification site (Kunapuli et al. 2007). The clones were related to the *Peptococcaceae* within the *Clostridia* (BF1 cluster) and the *Desulfobulbaceae* within the *Deltaproteobacteria*. The closest cultured relatives of the *Peptococcaceae*-related clones were *Thermincola carboxydiphila* and *T. ferriacetica*, while the *Desulfobulbaceae*-related clones were similar to other clones found in contaminated aquifers and in situ reactor clones monitoring monochlorobenzene degradation (Alfreider et al. 2002). A syntrophic mechanism was also proposed where the *Peptococcaceae* members were responsible for the first attack on the benzene ring, while the *Desulfobulbaceae* members consumed the electrons released by benzene and assimilated carbon from the media. Acetate or other fermentation products were discarded as potential metabolites since they were not labelled, and only iron could be used as the terminal electron acceptor.

A following study from the same group identified the enzymes responsible for anaerobic benzene degradation in the iron-reducing enrichment culture (Abu Laban et al. 2010). Proteins similar to the phenylphosphate carboxylase PpcA and PpcD of *Azoarcus* strain EbN1 and to the benzoate-CoA ligase of *Geobacter metallireducens* were expressed during benzene degradation, which suggested a direct carboxylation of the benzene ring by a putative anaerobic benzene carboxylase. Benzene degradation under iron-reducing conditions has also been observed in salt marsh sediments, but the microorganisms were not identified (Yu et al. 2012). To date, the hyperthermophilic *Ferroglobus placidus* is the only archaeon in pure culture reported to degrade benzene coupled to iron-reducing conditions (Holmes et al. 2011). *Ferrogloblus placidus* was able to degrade benzene, further accumulating benzoate but not phenol or toluene. Moreover, genes encoding the anaerobic degradation of benzoate were upregulated during the growth of this pure culture on benzene versus growth on acetate and a putative carboxylase was identified, suggesting a direct carboxylation of benzene to form benzoate.

More recently, two *Geobacter* strains have been shown to degrade benzene in pure cultures coupled to the reduction of iron. The *Geobacter* strain named Ben was isolated from a petroleum-contaminated aquifer in Minnesota and is most closely related to *Geobacter daltonii* (Zhang et al. 2012). The strain also grew with Fe(III) oxide, Fe(III) nitrilotriacetic acid, Fe(III) pyrophosphate and AQDS as electron acceptors. The authors also reported that the genome of strain Ben contains pathways for the anaerobic degradation of benzoate, toluene, phenol and *p*-cresol. Moreover, *Geobacter metallireducens* was also capable of benzene degradation coupled to iron reduction, confirming the suggested role of *Geobacter* species as hydrocarbon degraders in contaminated environments (Zhang et al. 2012). A further study by the same group on the *G. metallireducens* strain strongly suggested the presence of phenol as an intermediate since the genes coding for enzyme subunits for the first and second steps in phenol metabolism, *PpsA* and *PpcB*, respectively, were upregulated during growth on benzene and their deletion inhibited benzene degradation (Zhang et al. 2013). Moreover ^{18}O-labelled phenol was recovered from labelled water, suggesting that the phenol hydroxyl group was derived from water, as previously suggested by Vogel and Grbicgalic (1986), but for methanogenic cultures. Thus, these results provide evidence that phenol is a key intermediate in the degradation pathway and support hydroxylation as the first step in the anaerobic degradation of benzene by *G. metallireducens* (Zhang et al. 2013).

Microorganisms identified in sulphate-reducing conditions

The degradation of benzene coupled to sulphate reduction has been documented and the main microorganisms identified; however, no sulphate-reducing pure culture has been isolated (Edwards and Grbicgalic 1992; Chaudhuri and Wiesmann 1995; Lovley et al. 1995; Phelps et al. 1998; Weiner et al. 1998; Weiner and Lovley 1998a; Gieg et al. 1999; Sublette et al. 2006).

The first study to suggest the anaerobic oxidation of benzene under sulphate-reducing conditions involved enrichment cultures established with aquifer sediments from Seal Beach California and amended with sulphate; these cultures mineralized more than 90 % of the labelled benzene to CO_2, although no sulphate depletion was confirmed (Edwards and Grbicgalic 1992). A later study using sediments from San Diego Bay, California confirmed benzene oxidation coupled to sulphate reduction; in this study, benzene was metabolized within 55 days and new amendments increased its biodegradation (Lovley et al. 1995). These authors suggested a complete microbial oxidation to CO_2 since no extracellular intermediates were found. Moreover, the addition of molybdate (sulphate reduction inhibitor) completely stopped benzene degradation (Lovley et al. 1995).

Further studies on marine and freshwater sediments supported the oxidation of benzene under sulphate-reducing conditions (Phelps et al. 1996; Weiner and Lovley 1998a, b; Anderson and Lovley 2000). Moreover, the inoculation of a sulphate-reducing, benzene-oxidizing enrichment in sediments from a petroleum-contaminated aquifer initiated

successful anaerobic benzene degradation (Weiner and Lovley 1998a). Similarly, the addition of sulphate to some Oklahoma sediments and to a gasoline-contaminated aquifer in Bellingham, Washington significantly stimulated benzene and BTEX degradation, respectively (Weiner et al. 1998; Sublette et al. 2006). These observations were supported by an increase in the relative proportions of cyclopropyl fatty acids, which in turn suggest an increase in the sulphate-reducing *Desulfobacter* species (Sublette et al. 2006). The degradation of benzene in situ has also been successfully demonstrated by the addition of sulphate (Anderson and Lovley 2000; Cunningham et al. 2001). Moreover, the addition of sulphate to petroleum-contaminated aquifers has been proposed as a more cost-effective and efficient bioremediation technique due to the much greater solubility of sulphate compared with oxygen (Weiner et al. 1998; Anderson and Lovley 2000; Lovley 2001).

The first characterization of a sulphate-reducing marine consortium capable of anaerobic benzene degradation revealed a great diversity of microorganisms (Phelps et al. 1998). Phelps and colleagues characterized a consortium from Guaymas, Mexico via clone libraries and found members of the *Proteobacteria*, *Cytophagales* and Gram-positive bacteria. Four clones (SB-9, 21, 29 and 30) were found to be related to the *Desulfobacteraceae*, of which one was closely related to the aromatic degrader *Desulfobacula toluolica* strain Tol-2, while the other three were associated with the benzoate-degrader *Desulfosarcina variabilis*. Other clones were related to the sulphide oxidizer *Thiomicrospira* and to *Campylobacter* and *Wolinella*. Oka and colleagues (Oka et al. 2008) later used DNA-SIP to show that clone SB-21 incorporated most of the carbon from the ^{13}C-labelled benzene into its DNA. This is constant with results obtained with clone BznS295, which was the dominant organism in another sulphate-reducing, benzene-degrading marine culture and found to be closely related to clone SB-21 (Musat and Widdel 2008), suggesting that these members of the *Desulfobacteraceae* are important players in the degradation of benzene coupled to sulphate reduction.

Other studies on cultures from a benzene-contaminated aquifer near Zeitz, Germany identified members of the family *Peptococcaceae* to be important players in benzene oxidation coupled to sulphate reduction via clone libraries (Kleinsteuber et al. 2008). This result was later confirmed by DNA-SIP (Herrmann et al. 2010) These organisms were found to be related to the *Pelotomaculum* and *Cryptanaerobacter* group within *Desulfotomaculum* subcluster lh of the *Peptococcaceae* family within the *Clostridia* (Imachi et al. 2006). This phylotype along with an *Epsilonproteobacterium* incorporated carbon from ^{13}C-labelled benzene in a DNA-SIP experiment during the degradation of the contaminant (Herrmann et al. 2010). During benzene degradation, hydrogen and acetate were detected as intermediates in clone libraries during benzene degradation, as were sulphate-reducing *Deltaproteobacteria* and aceticlastic methanogens. Based on these observations, a degradation pathway was proposed where the *Cryptanaerobacter*/*Pelotomaculum* phylotype assimilates most of the benzene, while hydrogen is formed and consumed by the *Deltaproteobacteria* and the acetate produced is either converted to methane by aceticlastic methanogens or consumed by *Epsilonproteobacterium* (Herrmann et al. 2010; Vogt et al. 2011)

A later study identified phylotypes related to *Pelobacter*, *Thermotogales* and *Methanolobus* in an enrichment culture prepared with a sample from an underground gas storage aquifer with the ability to degrade BTEX (Berlendis et al. 2010). These authors also suggested syntrophy during the degradation of benzene. This was consistent with the results of a subsequent study on cultures derived from the Zeitz aquifer where syntrophic interactions were also strongly suggested since both acetate and hydrogen were found to be key intermediates in benzene mineralization (Rakoczy et al. 2011). In this latter study on the Zeitz aquifer enrichment, the authors used protein-SIP to analyse the carbon flux during the anaerobic degradation of benzene. Mass spectrometric data allowed quantification of the initial carbon source utilization and the metabolic intermediates. Members of *Clostridiales*, *Deltaproteobacteria* and *Bacteroidetes*/*Chlorobi* were identified as the functional groups of organisms in the quantitative analysis of carbon fluxes. These findings are consistent with previous results (Kleinsteuber et al. 2008). The *Clostridiales* were confirmed to be involved in benzene degradation, putatively fermenting benzene while fixing significant amounts of CO_2. The *Deltaprotebacteria* group used the metabolites released during the anaerobic fermentation and a putative scavenger group belonging to the *Bacteroidetes*/*Chlorobi* fed on dead cells (Taubert et al. 2012). The phylogenetic classification of the obtained proteins in the three groups revealed that almost 95 % are related to the genera *Desulfotomaculum* and *Pelotomaculum* within the *Peptococcaceae* family in the first group. The proteins in the *Deltaproteobacteria* group were found to be related to the *Desulfobacca* genus within the *Synthrophaceae* family, and the proteins in the *Bacteroidetes*/*Chlorobi* group are related to the *Rhodotermus* genus within the *Rhodothermaceae* family.

Microorganisms identified in methanogenic conditions

The process to produce methane involves a syntrophic association between proton-reducing bacteria and methanogenic archaeons that consume hydrogen to obtain energy. Methanogens also keep the hydrogen concentration sufficiently low for the reducing bacteria so they can still gain energy from BTEX oxidation.

The degradation of BTEX coupled to methanogenesis has been observed in several enrichment cultures derived from hydrocarbon-contaminated aquifers, sewage sludge and estuarine muds (Kazumi et al. 1997; Weiner and Lovley 1998b; Caldwell and Suflita 2000; Da Silva and Alvarez 2004). However, benzene degradation coupled to methanogenesis can be a lengthy process (Kazumi et al. 1997) unless an adapted consortium is present (Weiner and Lovley 1998b). Weiner and colleagues characterized a benzene-degrading methanogenic consortium composed of benzene- and phenol-degrading organisms, acetogenic organisms and aceticlastic methanogens (Weiner and Lovley 1998b). The latter belong to the *Methanosaeta* and *Methanosarcina* genera in the *Methanosarcinales* division of the *Archaea* and are thought to perform the last steps in the degradation of hydrocarbons since they have been constantly found in hydrocarbon-degrading consortia worldwide (Dojka et al. 1998; Ficker et al. 1999; von Wintzingerode et al. 1999; Watanabe et al. 2002; Da Silva and Alvarez 2004; Kasai et al. 2005; Struchtemeyer et al. 2005). The characterization of hydrocarbon-degrading consortia has also revealed members of the *Desulfotomaculum* within the *Peptococcaceae*, *Synthrophus* and aceticlastic methanogens (Dojka et al. 1998; Ficker et al. 1999). It has been proposed that the terminal steps of the hydrocarbon degradation are performed by *Syntrophus* species since they produce hydrogen and acetate during the anaerobic oxidation of organic acids together with the aceticlastic methanogens.

A later study by Ulrich and Edwards (2003) characterized a methanogenic benzene-degrading consortium that included members of the *Desulfosporosinus* (*Peptococcaceae*) and the *Desulfobacterium* (*Desulfobacteraceae*) genera, together with aceticlastic and hydrogenotrophic methanogens. These authors suggested that bacteria related to the sulphate reducers initiate the attack on benzene, while the *Desulfosporosinus*-related bacteria would use the intermediates and produce acetate, hydrogen and carbon dioxide. Toluene and phenol were found as metabolites, suggesting that methylation was the initial step in the benzene degradation although a hydroxylation step was not discarded (Ulrich et al. 2005). Recent studies from Japan involving DNA-SIP identified a deltaproteobacterium, Hasda-A, as a key consortium member in benzene degradation coupled to methanogenesis in a culture derived from non-contaminated soil (Sakai et al. 2009). The Hasda-A deltaproteobacterium was identical to the *Desulfobacterium* reported in the methanogenic consortium described earlier by Ulrich and Edwards (2003), leading Sakai et al. (2009) to suggest syntrophic associations since hydrogenotrophic and aceticlastic methanogens were also detected in the enrichment. No metabolites were identified, and benzene was degraded completely to methane and carbon dioxide, which was supported by the concurrent mineralization of amended toluene benzoate and phenol (Masumoto et al. 2012).

Microorganisms identified with other terminal electron acceptors

The degradation of benzene with the use of Mn(IV) as the terminal electron acceptor was first documented a decade ago (Villatoro-Monzon et al. 2003) and subsequently confirmed by Villatoro-Monzón et al. (2008) in enrichments derived from sediments of the Rhine river that had been shown in an earlier study by Cervantes et al. (2001) to be successful in terms of toluene degradation. BTEX and amorphous Mn(IV) oxide were amended to the enrichment as electron donor and acceptor, respectively, and benzene was the first of the BTEX compounds to be degraded. Other studies have reported the degradation of benzene using a graphite anode (Zhang et al. 2010) and humic acids as electron acceptors (Cervantes et al. 2011). The use of the humic acid model compound AQDS as electron acceptor during the degradation of benzene was tested in two enrichments derived from hydrocarbon-contaminated soil (PR) and sediment (ML) from two different locations in Mexico. The model compound enhanced the degradation in both consortia, and the microorganisms observed in PR soil included two *Gammaproteobacteria* phylotypes related to *Pseudoxanthomonas* and *Pseudomonas*, while members of the *Betaproteobacteria*, *Gammaproteobacteria* and *Deltaproteobacteria*, among others, were identified in the enrichment sediment ML (Cervantes et al. 2011). The *Geobacter* strain Ben was also able to degrade benzene coupled to the reduction of AQDS (Zhang et al. 2012).

Anaerobic benzene degradation pathway

Three mechanisms have been proposed as the initial steps of the anaerobic degradation pathway, namely, hydroxylation, carboxylation and methylation (Coates et al. 2002) (Fig. 2). Recent studies on pure cultures suggest that the anaerobic degradation pathway is dependent on the composition of the microbial community. An initial hydroxylation step in *Geobacter metallireducens* is supported by the studies of Zhang et al. (2012, 2013) on , carboxylation has been suggested for *Ferroglobus placidus* and the enrichment dominated by members of the *Peptococcaceae* from Zeitz, Germany (Abu Laban et al. 2009, 2010; Holmes et al. 2011) and methylation has been suggested for nitrate-reducing cultures (Ulrich et al. 2005). The studies providing experimental support for each of the mechanisms are discussed in greater detail in the following sections.

Hydroxylation

The metabolites phenol, cyclohexanone and propionate were detected under methanogenic conditions during the degradation of benzene in the mid-1980s, with water

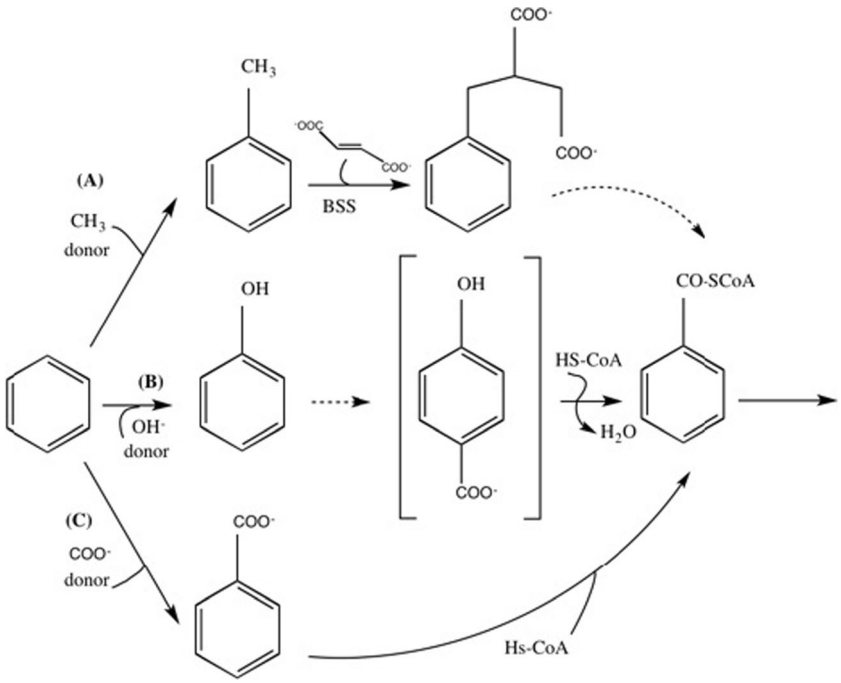

Fig. 2 Initial activation steps in the proposed anaerobic benzene degradation pathways that lead to benzoyl-CoA as the central metabolite. *(A)* Methylation, *(B)* hydroxylation, *©)* carboxylation (modified from Vogt et al. 2011)

being suggested as the source of oxygen for the initial benzene oxidation (Vogel and Grbicgalic 1986; Grbicgalic and Vogel 1987). Later studies reported phenol and benzoate under methanogenic (Ulrich et al. 2005), sulphidogenic (Chaudhuri and Wiesmann 1995; Phelps et al. 2001) and iron-reducing conditions (Caldwell and Suflita 2000). Ulrich and Edwards (2005) suggested two different initial steps depending on the electron acceptor, i.e. hydroxylation under methanogenic conditions and methylation under both nitrate-reducing and methanogenic conditions. Although phenol was reported as an intermediate for the *Dechloromonas* RCB strain (Chakraborty and Coates 2005), more recent studies strongly suggest that molecular oxygen is generated intracellularly from nitrate (Salinero et al. 2009; Weelink et al. 2010). In addition, phenol can also be produced abiotically if benzene-containing samples are in contact with atmospheric air (Kunapuli et al. 2008).

Nevertheless, Zhang and colleagues have suggested that phenol is an important metabolite during the growth of an isolated *Geobacter metallireducens* strain on benzene under iron-reducing conditions (Zhang et al. 2013). This mechanism is supported by the upregulation of genes for phenol metabolism during benzene degradation and its subsequent inhibition following the deletion of these genes, as mentioned above. In addition, these authors confirmed that water is the source of oxygen for the hydroxyl group. These results suggest that hydroxylation may be the initial step for benzene degradation where *Geobacter* species are considered to be the key players (Zhang et al. 2013).

Methylation

The first evidence of benzene methylation was observed in nitrate-reducing cultures spiked with [^{13}C]benzene (Ulrich et al. 2005). Labelled toluene and benzoate were recovered, leading the authors to suggest a pathway involving an initial methylation leading to toluene followed by a transformation to benzoate. This mechanism is supported by compound-specific isotope analyses (Zhang et al. 2002; Mancini et al. 2003; Fischer et al. 2008; Mancini et al. 2008).

Carboxylation

A direct carboxylation of benzene leading to benzoate has also been suggested as an initial step in benzene mineralization; benzoate has been observed as a metabolite in several studies of anaerobic benzene degradation (Caldwell and Suflita 2000; Phelps et al. 2001; Ulrich et al. 2005). The recovery of labelled benzoate from labelled benzene in freshwater and marine sulphate-reducing cultures suggests benzene transformation (Caldwell and Suflita 2000; Phelps et al. 2001). Also, a putative anaerobic benzene carboxylase was detected in protein extracts during the anaerobic degradation of benzene under iron-reducing conditions (Abu Laban et al. 2010). Furthermore, carboxylation has been suggested in the anaerobic benzene degradation by a pure culture of the hyperthermophilic archaeon *Ferroglobus placidus* under iron-reducing conditions (Holmes et al. 2011). There was an increased expression of genes for anaerobic benzoate degradation during growth on benzene and a gene for a putative carboxylase

that is homologous to the one mentioned above was also identified (Holmes et al. 2011).

Future work

The number and identification of consortia capable of anaerobic benzene degradation has increased in recent years worldwide. However, the isolation of new microorganisms remains critical in the quest to elucidate the degradation pathway of the contaminant, as has been shown for the archaeon *Ferroglobus placidus* and the *Deltaproteobacteria* strains. Detection of intermediates and analysis of gene expression favour an initial hydroxylation for the *Geobacter* species, while carboxylation by a putative carboxylase is suggested for the archaeon. Thus, future efforts should focus on isolating and elucidating the genome of more microorganisms in order to test them in specific assays. Studies involving metagenomics should also be carried out in consortia where isolation has not been possible in order to understand the function of each of the members of each consortium members.

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