Anaerobic benzene degradation by iron- and sulfate-reducing enrichment cultures

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Dedication

To the memory of my mother, to my wife

Anaerober Abbau von Benzol durch Eisen- und Sulfat-reduzierende Anreicherungskulturen

Nidal Abu Laban

Zusammenfassung

Trotz seiner hohen chemischen Stabilität kann Benzol unter anaeroben Bedingungen unter Verwendung verschiedener Elektronenakzeptoren biologisch abgebaut werden.
Allerdings ist unser Wissen über die initiale Aktivierungsreaktion und (über) die für den Abbau verantwortlichen Prokaryoten bisher sehr begrenzt. In der vorliegenden Studie wurden Bakterienkulturen angereichert, die unter sulfat-reduzierenden Bedingungen Benzol zu Kohlenstoffdioxid oxidieren.
Die bakterielle Gemeinschaft wurde anhand von T-RFLP, 16S rRNA-Gensequenzen und Fluoreszenz *in situ* Hybridisierung analysiert. Der dominierende Phylotyp (95 %) gehört zu der Gram-positiven Bakteriengattung *Pelotomaculum*, womit gezeigt werden konnte, dass Gram-positive, sulfat-reduzierende Bakterien am anaeroben Benzolabbau beteiligt sind.

Um erste Einblicke in den anfänglichen Aktivierungsmechanismus zu bekommen, wurde an einer sulfat-reduzierenden Kultur die Substratspezifität getestet. Fernerhin wurden Co-Metabolimus-Tests durchgeführt und nach möglichen Metaboliten gesucht.
Phenol, Toluol und Benzoat konnten von der Benzol-abbauenden Kultur nicht als alternative Kohlenstoffquellen verwendet werden. In der Anwesenheit von Phenol kam es bei Abbauexperimenten von Co-Metaboliten-Abbauexperimenten zu verlangsamten Benzol-Abbauraten, während Toluol keinen Einfluss auf den Benzol-Metabolismus zeigte. Phenol, 2-Hydroxybenzoat, 4-Hydroxybenzoat und Benzoate konnten als Metaboliten in der Anreicherungskultur identifiziert werden. Die abiotische Entstehung von hydroxylierten Aromaten konnte nachgewiesen werden. Daher unterstützt der Fund von Benzoat als Intermediat die Theorie der direkten Carboxylierung von Benzol als anfänglichen Aktivierungsmechanismus. Allerdings können weitere Benzoat-bildende Reaktionen nicht vollständig ausgeschlossen werden.

Zur Aufklärung des anaeroben Benzolabbaus wurde eine Metagenomanalyse der auf Benzol als Kohlenstoffquelle gewachsenen Eisen-reduzierenden Kultur BF durchgeführt, welche hauptsächlich aus *Peptococcaceae*-zugehörigen Bakterien besteht. Über 205 offene Leserahmen (ORF) zeigten starke Sequenzhomologien zu Genen, die für Transkriptionsregulatoren und Enzyme des anaeroben Abbaus aromatischer Kohlenwasserstoffe codieren.
Interessanterweise wurden in verschiedenen Regionen des Genoms Gencluster identifiziert, die für entsprechende Enzyme des Toluol- (*bssCABD* und *bbsCABEFD*), Phenol- (*ppsA* und *ppsBCAD*), 4-Hydoxybenzoat- (*pcmRST*) und Benzoat-Abbaus (*bamB-I*) codieren, was auf eine große metabolische Vielseitigkeit gegenüber aromatischen Verbindungen hinweist.
Des Weiteren wurden Gene entdeckt, die für ein Phenylphosphat Carboxylase-ähnliches Protein (PpcD und PpcA) sowie für Benzoat-CoA Ligase (BamY) und 3-Oktaprenyl-4-Hydroxybenzoat Carboxy-Lyase (UbiX) codieren und die in einer Operon-artigen Struktur liegen (~17 Kb). Dabei zeigten die für die Phenylphosphat Carboxylase codierenden Gene aber keine Homologien zu den zuvor beschriebenen *ppc*-Genen aus dem Phenol-Abbau-Cluster und wurden im späteren Verlauf der Arbeit als putative Benzol-Abbau-Gene charakterisiert.

In dieser Arbeit wurde zum ersten Mal das Proteom anaerob Benzol-abbauender Kulturen charakterisiert, wodurch die an der initialen Aktivierung von Benzol beteiligten Enzyme identifiziert werden konnten. Das gesamte Proteom der auf Benzol, Phenol und Benzoat gewachsenen Zellen der Eisen-reduzierenden Kultur BF wurde auf unterschiedlich exprimierte Proteine analysiert. Anschließend wurde das Proteom mit der Genomsequenz verglichen, um Gene zu identifizieren, die am anaeroben Benzol-Abbau beteiligt sind. Zusätzlich wurde die N-terminale Sequenz eines 60 kDa großen Proteins identifiziert, welches ausschließlich in Anwesenheit von Benzol exprimiert wurde. Der dadurch identifizierte ORF 138 der Kultur BF zeigte eine hohe Ähnlichkeit zu dem für die Phenylphosphat Carboxylase codierenden Gen (*ppcA*) aus *Aromatoleum aromatica* Stamm EbN1. Darüber hinaus wurden auch die angrenzenden ORFs 137 und 139 in der auf Benzol gewachsenen Kultur identifiziert, die für eine Phenylphosphat Carboxylase (*ppcD*) und eine Benzoat-CoA Ligase (*bamY*) codieren. Die hohe Ähnlichkeit der von ORF 137 und ORF 138 codierten Proteine zu Carboxylasen unterstützt die Schlussfolgerung aus zuvor durchgeführten Metabolitanalysen der initialen Carboxylierung von Benzol zu Benzoat. Im Anschluss findet der weitere Abbau von Benzoat über die von ORF 139 codierte Benzoat-CoA Ligase statt. Auf Grund ihrer Funktion wurden die von ORF 137 und ORF 138 codierten Proteine Anaerobe Benzol Carboxylase (Abc) genannt, die von den Genen *abcD* und *abcA* codiert wird. Interessanterweise konnten die Proteine AbcD und AbcA auch in der Benzol-gewachsenen, Sulfat-reduzierenden Kultur BPL nachgewiesen werden.

Nachdem durch die Proteomanalyse Enzyme identifiziert werden konnten, die wahrscheinlich die initiale Carboxylierung katalysieren, wurde im abschließenden Teil dieser Arbeit eine zweidimensionale Kohlenstoff- und Wasserstoffisotopenanalyse der Benzol-abbauenden Kulturen BF und BPL durchgeführt, um zu untersuchen, ob die initiale Reaktion in den verschiedenen Kulturen ähnlich ist. Die Steigungen Λ der Dual-Plots (17 ± 1 und 20 ± 2) aus dem Kohlenstoff-Wasserstoff-Isotopenverhältnis sind statistisch in dem gleichen Bereich, was auf einen gemeinsamen Abbaumechanismus der beiden Kulturen schließen lässt.

Auf Grund der Ergebnisse der physiologischen, Metabolom- und Proteomanalyse und der Doppel-Isotopenanalyse der Benzol-abbauenden Kulturen BF und BPL konnte schlussfolgernd in dieser Arbeit die direkte Carboxylierung von Benzol als gemeinsamer Mechanismus des anaeroben Abbaus in Eisen- und Sulfat-reduzierenden Kulturen gezeigt werden.

Anaerobic benzene degradation by iron- and sulfate-reducing enrichment cultures

Nidal Abu Laban

Abstract

Despite its high chemical stability, benzene is known to be biodegradable with various electron acceptors under anaerobic conditions. However, our understanding of the initial activation reaction and the responsible prokaryotes is limited. In the present study, we enriched a bacterial culture that oxidizes benzene to carbon dioxide under sulfate-reducing conditions. Community analysis using T-RFLP, 16S rRNA gene sequencing and fluorescence *in situ* hybridization revealed 95% dominance of one phylotype that is affiliated to the Gram-positive bacterial genus *Pelotomaculum* showing that sulfate-reducing Gram-positive bacteria are involved in anaerobic benzene degradation.

In order to get indications of the initial activation mechanism, we tested the substrate utilization, performed co-metabolism tests and screened for putative metabolites in a sulfate-reducing culture. Phenol, toluene, and benzoate could not be utilized as alternative carbon sources by the benzene-degrading culture. Co-metabolic degradation experiments resulted in retarded rates of benzene degradation in the presence of phenol whereas toluene had no effect on benzene metabolism. Phenol, 2-hydroxybenzoate, 4-hydroxybenzoate and benzoate were identified as metabolites in the enrichment culture. However, hydroxylated aromatics were shown to be formed abiotically. Thus, the finding of benzoate as an intermediate compound supports a direct carboxylation of benzene as the initial activation mechanism but additional reactions leading to its formation cannot be excluded definitely.

Towards resolving the biochemistry of anaerobic benzene degradation, we performed metagenomic analysis of an iron-reducing culture grown on benzene. The culture was mainly composed by *Peptococcaceae*-related microorganisms. About 205 ORFs showed sequence similarity to genes and transcriptional regulators involved in anaerobic aromatic hydrocarbon degradation. Interestingly, gene clusters containing toluene (*bssCABD* and *bbsCABEFD*), phenol (*ppsA* and *ppcBCAD*), 4-hydroxybenzoate (*pcmRST*), and benzoate (*bamb-I* and *bamY*) degradation catabolic genes were identified in different regions of the BF metagenome. Further operon-like organization of genes (~17 Kb) was discovered with genes encoding phenylphosphate carboxylase-like proteins (PpcD and PpcA), benzoate-CoA ligase (BamY), and 3-octaprenyl-4-hydroxybenzoate carboxy-lyase (UbiX). However, the *ppc* genes were not homologues to ones described in the phenol degradation cluster, they were later identified as a putative benzene degradation genes.

In this study for the first time, we described the proteome of an anaerobic benzene-degrading culture being responsible for the initial activation of benzene. The whole proteomes expressed in benzene-, phenol-, and benzoate-grown cells of the iron-reducing culture BF were compared by a shotgun proteomic approach and correlated to sequenced genome information of the culture BF to identify putative genes involved in anaerobic benzene degradation. Additionally, a specific protein band with a mass of 60 kDa driving from SDS gels, expressed only in benzene-grown cells of the culture BF, was subjected to N-terminal sequence analysis. Protein bands specifically expressed in benzene-grown cells corresponded to ORF 138 of the BF genome, which showed low sequence similarity (43% sequence identity) to a gene encoding phenylphosphate carboxylase (*ppcA*) in *Aromatoleum aromatica*

strain EbN1. More ORFs discovered close to ORF 138 were ORF 137 and ORF 139, which are similar to the genes encoding phenylphosphate carboxylase (*ppcD*) and benzoate-CoA ligase (*bamY*), respectively. The similarity of proteins encoded by ORF 137 and ORF 138 to carboxylase enzymes supports our recent metabolite analysis, which favored carboxylation of benzene to benzoate as initial activation mechanism, followed by a further degradation of benzoate via benzoate-CoA ligase that was encoded by ORF 139. We named the protein product of ORF 137 and ORF 138 putative anaerobic benzene carboxylase (Abc) enzyme being encoded by at least *abcD* and *abcA* genes. Interestingly, protein products of the *abcD* and *abcA* genes were also discovered in the proteome of the benzene-grown sulfate-reducing culture BPL.

Since the proteomic data identified proteins that might be involved in the carboxylation reaction, the last part of this study dealt with the carbon and hydrogen two-dimensional stable isotope analysis of benzene-degrading cultures BF and BPL in order to indicate whether the initial reaction of microorganisms utilizing different electron acceptors might be similar. The slopes Λ of dual-plots of carbon versus hydrogen isotope ratio studies were statistically in the same range (17 ± 1 and 20 ± 2) for the respective cultures, suggesting the existence of a common benzene degradation mechanism.

To summarise, combining the results of the physiological, metabolomic, proteomic, and dual-isotope fractionation analysis of the benzene-degrading cultures, this study strongly supports a direct carboxylation of benzene as a common anaerobic degradation mechanism for anaerobic iron- and sulfate-reducing cultures.

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1 General introduction

1.1 Aromatic compound contamination and fate in the subsurface environment

Anthropogenic activities have strongly influenced the natural chemical composition of groundwater, e.g. leaking of underground storage oil tanks, former gas plants, disposal of organic chemicals at industrial sites, land fill leachates and urban runoffs, leading to groundwater pollution. Polycyclic aromatic hydrocarbons (PAHs) and monoaromatic hydrocarbons like benzene, toluene, ethylbenzene, and xylene (BTEX) are among the most abundant toxic organic pollutants in contaminated aquifers (USEPA, 1998, Van Hamme*, et al.*, 2003). Due to their relatively high solubility and mobility, BTEX compounds are considered the most prevalent groundwater pollutants, frequently endangering the quality of drinking water supplies and representing a significant environmental threat (Anderson & Lovley, 1997, Chakraborty & Coates, 2004). The fate of the hydrocarbon contaminants depends on the hydraulic characteristics of the aquifer and the chemical properties of the pollutants. Mostly, they migrate with the groundwater flow and form contaminant plumes. In groundwater systems, where organic pollutants accumulate, a rapid depletion of oxygen occurs. Consequently, contaminated aquifers usually become anoxic, forming a redox gradient along the groundwater flow path (Christensen*, et al.*, 1994).

Natural attenuation is referred to intrinsic remediation occurring at contaminated sites by a variety of physical, chemical, and biological processes. BTEX compounds are barely degraded by natural physical and chemical processes. However, the natural attenuation of BTEX compounds by anaerobic microbial degradation has been shown under sulfate-, nitrate-, and iron-reducing conditions (Kuhn*, et al.*, 1985, Vogel & Grbic-Galic, 1986, Rabus & Widdel, 1995, Ball*, et al.*, 1996, Hess*, et al.*, 1997, Meckenstock*, et al.*, 1999, Rooney-Varga*, et al.*, 1999, Coates*, et al.*, 2001a). These findings have shown that the major fraction of groundwater contaminants such as BTEX can be degraded in the anoxic zone of plumes, implying anaerobic bacterial degradation as key process in natural attenuation of polluted sites (Christensen*, et al.*, 1994, Widdel & Rabus, 2001, Meckenstock*, et al.*, 2004b).

1.2 Anaerobic degradation of polyaromatic hydrocarbons

Polyaromatic hydrocarbons (PAHs) are compounds exhibiting multiple aromatic rings in their structure, including frequently found compounds such as naphthalene, anthracene, and more complex compounds such as pyrene and benzo(a)pyrene. PAHs containing 2- and 3-aromatic rings such as naphthalene, phenanthrene and biphenyl are potential groundwater contaminants, found in petroleum contaminated environments (Meckenstock*, et al.*, 2004b). PAHs were generally thought to be recalcitrant under anaerobic conditions. However, recent studies demonstrated that a wide variety of hydrocarbon contaminants can be removed from polluted aquifers by natural attenuation through microbial degradation processes utilizing different electron acceptors (Mihelcic & Luthy, 1988, Coates*, et al.*, 1996, Coates*, et al.*, 1997, Anderson & Lovley, 1999, Meckenstock*, et al.*, 2004b). The first successful microcosm experiment reporting PAH degradation showed the mineralization of naphthalene and acenaphathene under nitrate-reducing conditions (Mihelcic & Luthy, 1988). In later studies, anaerobic naphthalene degradation has been confirmed by measuring mineralization of ^{14}C-naphthalene in microcosms under nitrate-reducing conditions, containing material from a (chronically) diesel fuel-contaminated aquifer (Bregnard*, et al.*, 1996). In inocula from marine sediments under sulfate-reducing conditions, ^{14}C-naphthalene and phenanthrene were oxidized to $^{14}CO_2$ (Coates*, et al.*, 1996). In the Bemidji aquifer, naphthalene and benzene were degraded under iron-reducing conditions, without a lag period indicating that the microbial communities found in those sediments were adapted to the oxidation of these contaminants (Anderson & Lovley, 1999). Further investigations in the sediments revealed that methylnaphthalene, fluorine and fluoranthene were also anaerobically transformed to CO_2, while pyrene and benzo(a)pyrene could not be used (Coates*, et al.*, 1997). Moreover, Meckenstock *et al.* (2000) enriched the sulfate-reducing culture N47 from a tar-oil-contaminated aquifer growing on naphthalene and methylnaphthalene. Additional evidence was found by Chang *et al.* (2002) who followed the degradation of five 3-rings unsubstituted PAHs in soil under methanogenic, nitrate- and sulfate-reducing conditions. Very recently, naphthalene and 2-methylnaphthalene degradation was described using sulfate-reducing strains NaphS2, NaphS3, and NaphS6 (Musat*, et al.*, 2008).

1.3 Anaerobic degradation of BTEX compounds

Anaerobic biodegradation studies of BTEX (benzene, toluene, ethylbenzene, and xylene) mixtures from different sediment samples and pure bacterial cultures indicated the

degradation of these compounds with the use of different electron acceptors (Barbaro, *et al.*, 1992, Heider, *et al.*, 1999, Phelps & Young, 1999, Chakraborty, *et al.*, 2005). Toluene, the first model compound for anaerobic hydrocarbon degradation, can be degraded relatively quickly, allowing rapid growth of microorganisms under various electron-reducing conditions (Phelps & Young, 1999). The first evidence for toluene degradation was demonstrated in aquifer columns using nitrate as electron acceptor (Kuhn, *et al.*, 1985). Anaerobic toluene degradation was then repeatedly studied in tar-oil contaminated aquifers, enriched microcosms and pure cultures using various electron acceptors (Lovley, *et al.*, 1989, Dolfing, *et al.*, 1990, Evans, *et al.*, 1991, Rabus, *et al.*, 1993, Fries, *et al.*, 1994, Beller, *et al.*, 1996, Langenhoff, *et al.*, 1996). Also, toluene degradation has been investigated in methanogenic enrichment and fermentative syntrophic cultures (Meckenstock, 1999, Siddique, *et al.*, 2007).

Anaerobic degradation of the three *m*-, *p*-, and *o*-xylene isomers has been demonstrated with various electron acceptors. Studies based on sediments or enrichment cultures reported the degradation of both, *p*- and *m*-xylene, under nitrate-reducing conditions (Kuhn, *et al.*, 1985, Kuhn, *et al.*, 1988, Häner, *et al.*, 1995), *p*- and *o*-xylene under iron-reducing conditions (Jahn, *et al.*, 2005, Botton & Parsons, 2006), and of all xylene isomers after a significant lag period under sulfate-reducing conditions (Edwards, *et al.*, 1992, Morasch, *et al.*, 2004, Morasch & Meckenstock, 2005). Several subsequent studies found *m*-xylene being the most readily degraded isomer in mixed cultures (Beller, *et al.*, 1995). Its presence in some cultures as a co-substrate could inhibit *p*- and *o*-xylene degradation (Meckenstock, *et al.*, 2004a). However, *p*-xylene, which is often reported to be recalcitrant to degradation (Rabus & Widdel, 1995), was degraded by a mixed denitrifying *p*-xylene selective culture (Häner, *et al.*, 1995) and a sulfate-reducing enrichment culture in the presence of Amberlite XAD-7 resin, which provides very low substrate concentration in the culture medium (Morasch & Meckenstock, 2005). *o*-Xylene was found to be degraded by some cultures (Morasch, *et al.*, 2004), whereas Meckenstock *et al*. (2004a) reported the inhibition of anaerobic *o*-xylene degradation by toluene in sulfidogenic sediment columns and pure cultures. The later study showed that the differential degradation of BTEX compounds in contaminated aquifers could originate from a partial metabolic inhibition of xylene-degrading organisms by toluene.

In contrast to toluene and xylene isomers, relatively little is known about ethylbenzene degradation. The metabolism of ethylbenzene has been reported *in situ* and in enrichment cultures under sulfate- (Elshahed, *et al.*, 2001) and nitrate-reducing conditions (Reinhard, *et*

al., 1997). Villatoro-Monzón *et al*. (2003) observed that under iron-reducing conditions ethylbenzene was the most rapid degraded of all BTEX compounds, whereas Jahn *et al.* (2005) reported ethylbenzene degradation under iron-reducing conditions in enrichment cultures only after a relatively long lag phase. However, ethylbenzene was observed to be recalcitrant in microcosms degrading toluene and xylene under iron-reducing and methanogenic conditions (Botton & Parsons, 2006, Siddique*, et al.*, 2007).

Benzene is of major concern as a contaminant because of its stability, toxicity, and relatively high solubility (Coates*, et al.*, 2001a). It is considered to be the most recalcitrant compound among BTEX contaminants. Benzene-degrading enrichment cultures are rare and pure strains are even more scarce. Early evidence for anaerobic benzene-degradation was found in form of $^{14}CO_2$-, $^{14}CH_4$-, and ^{18}O-labeled phenol-formation in methanogenic enrichment cultures, amended with ^{14}C-benzene and $H_2{}^{18}O$ (Vogel & Grbic-Galic, 1986, Grbic-Galic & Vogel, 1987). However, the mass balance showed that less than 6% of ^{14}C-labeled benzene added was converted to $^{14}CO_2$. Many subsequent studies used carbon and hydrogen compound stable isotope fractionation, signature-metabolites analysis, 16S rRNA gene-targeted real time PCR, and DNA and RNA-based stable isotope probing approaches, provided evidences for substantial anaerobic benzene oxidation in petroleum-contaminated aquifers and in enrichment cultures deriving from aquatic sediments (Caldwell & Suflita, 2000, Phelps*, et al.*, 2001, Mancini*, et al.*, 2003, Andrea*, et al.*, 2005, Chakraborty & Coates, 2005, Geyer*, et al.*, 2005, Ulrich*, et al.*, 2005, Kasai*, et al.*, 2006, Da Silva & Alvarez, 2007, Fischer*, et al.*, 2007, Kunapuli*, et al.*, 2007, Fischer*, et al.*, 2008, Kunapuli*, et al.*, 2008, Liou*, et al.*, 2008). More comprehensive studies on benzene degradation have been reported in enrichment cultures under methanogenic (Vogel & Grbic-Galic, 1986, Grbic-Galic & Vogel, 1987, Kazumi*, et al.*, 1997, Weiner & Lovley, 1998, Mancini*, et al.*, 2003), sulfate-reducing (Edwards & Grbic-Galic, 1992, Lovley*, et al.*, 1995, Coates*, et al.*, 1996, Phelps*, et al.*, 1996, Kazumi*, et al.*, 1997, Anderson & Lovley, 2000, Caldwell & Suflita, 2000, Mancini*, et al.*, 2003, Griebler*, et al.*, 2004, Kleinsteuber*, et al.*, 2008, Musat & Widdel, 2008, Oka*, et al.*, 2008), ferric iron-reducing (Lovley*, et al.*, 1994, Lovley*, et al.*, 1996, Anderson*, et al.*, 1998, Anderson & Lovley, 1999, Caldwell*, et al.*, 1999, Kunapuli*, et al.*, 2007), and under nitrate-reducing conditions (Burland & Edwards, 1999, Coates*, et al.*, 2001a, Mancini*, et al.*, 2003, Kasai*, et al.*, 2007). Despite the availability of several enrichment cultures that showed potential toward anaerobic degradation of benzene, they showed to have a very complex community structure, the major factor limiting further understanding of anaerobic benzene degradation.

1.4 Microbial key-players in the contaminated subsurface environment

In the last two decades, several studies investigating microcosms enriched with contaminated soil and sediments from polluted aquifers showed anaerobic microorganisms related to *Beta-* and *Delta-proteobacteria* to be involved in xenobiotics substance degradation (Heider & Fuchs, 1997a, Heider*, et al.*, 1999, Widdel & Rabus, 2001, Zhang & Bennett, 2005). Attention has been focused on the characterization and isolation of anaerobic bacteria playing a role in the cleanup of widespread environmental pollutants, including BTEX and PAHs (Table 1.1).

Many anaerobic Gram-negative toluene-degrading bacteria have successfully been isolated: the *Beta-proteobacteria Thauera aromatica* strains T1 and K172, *Thauera* sp. DNT-1, *Azoarcus tolulyticus* strain Tol4, *Azoarcus evansii, Dechloromonas* strains RCB and JJ which are able to reduce nitrate during oxidation of toluene (Evans*, et al.*, 1991, Fries*, et al.*, 1994, Rabus & Widdel, 1995, Zhou*, et al.*, 1995, Leutwein & Heider, 1999, Song*, et al.*, 1999, Coates*, et al.*, 2001a, Shinoda*, et al.*, 2004), the *Delta-proteobacteria Geobacter metallireducens* GS-15 and *Geobacter grbiciae* strains TACP-2T and TACP-5 that couple iron reduction (Fe^{+3} to Fe^{+2}) to toluene oxidation (Lovley & Lonergan, 1990, Lovley*, et al.*, 1993, Coates*, et al.*, 2001b), and sulfate-reducing toluene-degrading isolates such as *Desulfobacula toluolica* Tol2, oXyS1, TRM1, and PRTOL1 strains (Beller*, et al.*, 1996, Harms*, et al.*, 1999, Meckenstock, 1999). Recently, anaerobic toluene-degrading microorganisms have been reported within *Clostridia* (Liu*, et al.*, 2004, Morasch*, et al.*, 2004). However, no pure strains of toluene-degrading *Firmicutes* have been isolated so far.

Several pure isolates of *m*-xylene-degrading microorganisms have been reported, including the nitrate-reducing *Beta-proteobacteria* strains *Azoarcus tolulyticus* Tol5, mXynN1, and M3 (Fries*, et al.*, 1994, Rabus & Widdel, 1995, Hess*, et al.*, 1997), the sulfate-reducing *Delta-proteobacterium* strain mXyS1 (Harms*, et al.*, 1999), and the *Firmicutes* strain *Desulfotomaculum* sp. OX39 (Morasch*, et al.*, 2004). The later strain and oXyS1 strain only two *o*-xylene-degrading organisms have been reported under sulfate-reducing conditions (Harms*, et al.*, 1999, Morasch*, et al.*, 2004). Most of the isolates often show narrow substrate specificity; therefore, no pure cultures utilizing *p*-xylene for the growth have yet been reported.

For ethylbenzene degradation, six pure cultures have been reported to date, of witch five are nitrate-reducing *Beta-proteobacteria*, including *Dechloromonas* strains RCB, *Aromatoleum*

aromaticum strain EbN1, *Azoarcus* sp. PbN1, EB1, and T strains (Dolfing*, et al.*, 1990, Rabus & Widdel, 1995, Coates*, et al.*, 2001a, Kloer*, et al.*, 2006), whereas *Delta-proteobacteria* strain EbS7 is the only sulfate-reducing, ethylbenzene-degrading pure culture reported to date (Kniemeyer*, et al.*, 2003).

Pure cultures of benzene-degrading denitrifying strains of the genera *Dechloromonas* and *Azoarcus* have been described (Coates*, et al.*, 2001a, Kasai*, et al.*, 2006). Strain RCB, now identified as *Dechloromonas aromatica* RCB (GenBank accession number CP000089), can also metabolize benzene using chlorate or oxygen as electron acceptors. Recently, Gram-positive bacteria belonging to the genus of *Clostridia* have been shown to play an important role in the degradation of benzene (Kunapuli*, et al.*, 2007, Fahy*, et al.*, 2008, Kleinsteuber*, et al.*, 2008, Abu Laban*, et al.*, 2009). However, there is no strictly anaerobic microorganism specifically identified to degrade benzene known so far.

For PAHs, only a few pure naphthalene-degrading cultures have been isolated, including the nitrate-reducing isolates from the marine sediments enrichment culture strains NAP-3-1 (phylogenetically related to *Pseudomonas stutzeri*) and NAP-4-1 (related to *Vibrio pelagius*) (Rockne*, et al.*, 2000), and the sulfate-reducing marine Delta-proteobacterium strain NaphS2 (Galushko*, et al.*, 1999). Furthermore, some PAHs-degrading bacterial stains have been isolated, but none of them was capable to perform a complete mineralization.

In contrast to the availability of anaerobic aromatic-degrading cultures, hydrocarbon-degrading bacteria have been found difficult to isolate (Mountfort & Bryant, 1982, Janke & Fritsche, 1985, Wallrabenstein*, et al.*, 1995, Meckenstock, 1999). For example, strains belonging to the genus of *Pelotomaculum* have been shown to degrade phthalate isomers only in a syntrophic co-culture with the hydrogenotrophic methanogens *Methanospirillum* (Qiu*, et al.*, 2006), and more recently, a benzene-degrading culture was proposed to be dependent on a syntrophic partner, hydrogenotrophic sulfate-reducing bacteria (Kunapuli*, et al.*, 2007, Kleinsteuber*, et al.*, 2008). In addition, it may be difficult to purify aromatic-oxidizing strains due to the toxicity of the organic substrate. Therefore, information about phylotypes and physiological types of microorganisms that might be responsible for anaerobic hydrocarbon degradation or remediation of the contaminated sites is still scarce.

Table 1.1. Major groups of anaerobic BTEX- and PAHs-degrading microorganisms isolated in pure cultures.

Compounds	Microorganism	Phylogeny	EA	Reference
BTEX				
Toluene	*Dechloromonas aromatica* RCB	*β-Proteobacteria*	NO_3^-	(Coates, *et al.*, 2001a)
	Dechloromonas sp. strain JJ	*β-Proteobacteria*	NO_3^-	(Coates, *et al.*, 2001a)
	Azoarcus tolulyticus Td15	*β-Proteobacteria*	NO_3^-	(Fries, *et al.*, 1994)
	Azoarcus sp. strain T	*β-Proteobacteria*	NO_3^-	(Dolfing, *et al.*, 1990)
	*Azoarcus tolulyticus*Tol4	*β-Proteobacteria*	NO_3^-	(Zhou, *et al.*, 1995)
	Azoarcus toluvorans	*β-Proteobacteria*	NO_3^-	(Song, *et al.*, 1999)
	Azoarcus toluclasticus	*β-Proteobacteria*	NO_3^-	(Song, *et al.*, 1999)
	Thauera aromatica K172	*β-Proteobacteria*	NO_3^-	(Anders, *et al.*, 1995)
	Thauera sp. strain DNT-1	*β-Proteobacteria*	NO_3^-	(Shinoda, *et al.*, 2004)
	Thauera aromatica T1	*β-Proteobacteria*	NO_3^-	(Evans, *et al.*, 1991)
	Strain ToN1	*β-Proteobacteria*	NO_3^-	(Rabus & Widdel, 1995)
	Strain T3	*β-Proteobacteria*	NO_3^-	(Hess, *et al.*, 1997)
	Magnetospirillum sps.	*α-Proteobacteria*	NO_3^-	(Shinoda, *et al.*, 2005)
	Strain mXyN1	*δ-Proteobacteria*	NO_3^-	(Rabus & Widdel, 1995)
	Geobacter metallireducens GS15	*δ-Proteobacteria*	Fe^{+3}	(Lovley, *et al.*, 1993)
	Geobacter grbiciae TACP5	*δ-Proteobacteria*	Fe^{+3}	(Coates, *et al.*, 2001b)
	Desulfobacula toluolica Tol2	*δ-Proteobacteria*	SO_4^{-2}	(Rabus, *et al.*, 1993)
	Strain PRTOL1	*δ-Proteobacteria*	SO_4^{-2}	(Beller, *et al.*, 1996)
	Desulfobacterium cetonicum	*δ-Proteobacteria*	SO_4^{-2}	(Harms, *et al.*, 1999)
	Strain TRM1	*δ-Proteobacteria*	SO_4^{-2}	(Meckenstock, 1999)
	Strain oXyS1	*δ-Proteobacteria*	SO_4^{-2}	(Harms, *et al.*, 1999)
	Desulfotomaculum sp. OX39	*Firmicutes*	SO_4^{-2}	(Morasch, *et al.*, 2004)
***m*-Xylene**	*Azoarcus* sp. strain T	*β-Proteobacteria*	NO_3^-	(Dolfing, *et al.*, 1990)
	Azoarcus tolulyticus Td15	*β-Proteobacteria*	NO_3^-	(Fries, *et al.*, 1994)
	Strain mXyN1	*β-Proteobacteria*	NO_3^-	(Rabus & Widdel, 1995)
	Azoarcus sps.	*β-Proteobacteria*	NO_3^-	(Kasai, *et al.*, 2006)
	Strain M3	*β-Proteobacteria*	NO_3^-	(Hess, *et al.*, 1997)
	Strain mXyS1	*δ-Proteobacteria*	SO_4^{-2}	(Harms, *et al.*, 1999)
	Desulfotomaculum sp. OX39	*Firmicutes*	SO_4^{-2}	(Morasch, *et al.*, 2004)
***o*-Xylene**	Strain oXyS1	*δ-Proteobacteria*	SO_4^{-2}	(Harms, *et al.*, 1999)
	Desulfotomaculum sp. OX39	*Firmicutes*	SO_4^{-2}	(Morasch, *et al.*, 2004)
Ethylbenzene	*Dechloromonas aromatica* RCB	*β-Proteobacteria*	NO_3^-	(Coates, *et al.*, 2001a)
	Aromatoleum aromaticum EbN1	*β-Proteobacteria*	NO_3^-	(Kloer, *et al.*, 2006)
	Azoarcus sp. strain PbN1	*β-Proteobacteria*	NO_3^-	(Rabus & Widdel, 1995)
	Azoarcus sp. strain EB1	*β-Proteobacteria*	NO_3^-	(Rabus & Widdel, 1995)
	Azoarcus sp. strain T	*β-Proteobacteria*	NO_3^-	(Dolfing, *et al.*, 1990)
	EbS7 strain	*δ-Proteobacteria*	SO_4^{-2}	(Kniemeyer, *et al.*, 2003)
Benzene	*Dechloromonas aromatica* RCB	*β-Proteobacteria*	NO_3^-	(Coates, *et al.*, 2001a)
	Dechloromonas sp. strain JJ	*β-Proteobacteria*	NO_3^-	(Coates, *et al.*, 2001a)
	Azoarcus sps.	*β-Proteobacteria*	NO_3^-	(Kasai, *et al.*, 2006)
PAHs				
Naphthalene	NAP-3-1, NAP-3-2, and NAP-4	*γ-Proteobacteria*	NO_3^-	(Rockne, *et al.*, 2000)
	NaphS2, NaphS3, NaphS6	*δ-Proteobacteria*	SO_4^{-2}	(Galushko, *et al.*, 1999)

1.5 Insights into anaerobic aromatic hydrocarbon activation mechanisms

Anaerobic biodegradation of aromatic hydrocarbons comprises novel biochemical reactions. In recent years, signature metabolites, functional genes and enzymes involved in the initial activation of several hydrocarbons have been identified. The initial activation of the aromatic ring is crucial for anaerobic hydrocarbon degradation. To overcome the high activation energy required in the initial reactions, different microorganisms employed novel enzymes catalyzing reactions of several hydrocarbons (Fig. 1.1).

The characterization of the metabolic pathways employed in oxygen-independent hydrocarbon degradation has resulted in the identification of various initial reactions dealing with inert and recalcitrant substrates, the most famous reactions namely fumarate-addition to methyl groups, catalyzed by a glycyl radical enzyme to yield aromatic-substituted succcinate such as in toluene, *o*-xylene degradation (Biegert*, et al.*, 1996, Beller & Spormann, 1997, Leutwein & Heider, 1999, Kniemeyer*, et al.*, 2003), or oxygen-independent hydroxylation of an alkyl substituent via dehydrogenase such as in ethylbenzene and *p*-cresol metabolism (Hopper*, et al.*, 1991, Ball*, et al.*, 1996). Methylation of unsubstituted aromatics was proposed for naphthalene and benzene metabolism (Ulrich*, et al.*, 2005, Safinowski*, et al.*, 2006, Safinowski & Meckenstock, 2006). An ATP-dependent carboxylation via phenylphosphate carboxylase is employed in phenol metabolism (Bisaillon*, et al.*, 1991, Schühle & Fuchs, 2004, Boll & Fuchs, 2005). Moreover, alternatively to methylation, direct carboxylation of unsubstituted aromatics like naphthalene, phenanthrene and benzene has been proposed (Zhang & Young, 1997, Meckenstock*, et al.*, 2000, Phelps*, et al.*, 2001, Kunapuli*, et al.*, 2008, Musat & Widdel, 2008, Abu Laban*, et al.*, 2009). These activation reactions of aromatic compounds degradation are generally divided into two-phase processes; in the first phase, the various aromatic substrates are converted to intermediates, which are eventually transformed to the central metabolite (e.g. benzoyl-CoA). The second phase involves dearomatization of benzoyl-CoA to cyclohex-1,5-diene-1-carboxyl-CoA using the common key enzyme benzoyl-CoA reductase, followed by the further oxidation of cyclohex-1,5-diene-1-carboxyl-CoA to acetyl-CoA and CO_2, in a sequence of reactions analogous to β-oxidation (Heider & Fuchs, 1997b).

Toluene biodegradation is considered as a model for the fumarate-addition reaction (Heider*, et al.*, 1999), a novel reaction for the degradation of alkyl aromatic and saturated hydrocarbons (Widdel & Rabus, 2001). The activation reaction of toluene is mediated by a

glycyl-radical like key enzyme (benzylsuccinate synthase), adding fumarate to the methyl group of toluene yielding benzylsuccinate, the signature metabolite of this process (Beller & Spormann, 1997, Beller & Spormann, 1999, Leutwein & Heider, 1999). Benzylsuccinate synthase is an oxygen sensitive heterohexamer ($\alpha_2\beta_2\gamma_2$) that consisting of three subunits BssCAB, two identical subunits (α and ά), containing the glycyl-radical site and two different small subunits (β and γ). The enzyme requires an adenosylmethionine-dependent activating enzyme BssD to introduce a free radical at the glycine residue (Leuthner*, et al.*, 1998). The *bssD* gene coding for such a potential-activating enzyme is located within the operon of the *bssCAB* genes, coding for the subunits of benzylsuccinate synthase (Kube*, et al.*, 2004). Similar fumarate-addition reactions were discovered to initiate anaerobic degradation of *p*-, *m*-, and *o*-xylene (Beller & Spormann, 1997, Krieger*, et al.*, 1999), 2-methylnaphthalene (Annweiler*, et al.*, 2000), *m*- and *p*-cresol (Müller*, et al.*, 1999, Müller*, et al.*, 2001), and ethylbenzene (Kniemeyer*, et al.*, 2003).

The oxygen-independent hydroxylation of ethylbenzene is considered another mechanism for the initial activation reaction of aromatic compounds (Kniemeyer & Heider, 2001). The reaction has been well described for ethylbenzene dehydrogenase, which catalyzes the hydroxylation of ethylbenzene to 1-phenylethanol (Johnson*, et al.*, 2001). This enzyme is a heterotrimer, consists of three subunits (αβγ), and is characterized as a molybdenum-cofactor containing-enzyme of the dimethyl sulfoxide (DMSO) reductase family (McDevitt*, et al.*, 2002). The α-subunit contains the catalytic center with a molybdenum held by two molybdopterin-guanine dinucleotides, the β-subunit contains four iron-sulfur clusters and is structurally related to ferredoxins, whereas the γ-subunit is the first known protein with a methionine and a lysine as axial heme ligands (Kloer*, et al.*, 2006). In addition, oxygen-independent hydroxylation has been described for *p*-cresol degradation. The reaction involves *p*-cresol methylhydroxylase that generates *p*-hydroxybenzyl alcohol by hydroxylation of the methyl group with water. Also, the oxidation of *p*-hydroxybenzyl alcohol to *p*-hydroxybenzyldehyde was supposed to be carried out by *p*-cresol methylhydroxylase, an NAD^+- dependent *p*-hydroxybenzyl alcohol dehydrogenase (Keat & Hopper, 1978, Cunane*, et al.*, 2000, Johannes*, et al.*, 2008). Similarly to ethylbenzene and *p*-cresol, hydroxylation has been proposed as an activation mechanism in benzene degradation (Grbic-Galic & Vogel, 1987, Chakraborty & Coates, 2005). However, information about the enzyme catalyzing of the proposed benzene hydroxylation is not available.

A biological Kolbe-Schmitt reaction has been described for phenol metabolism by the denitrifying bacterium *Thauera aromatica* (Boll & Fuchs, 2005). In the first step phenol is converted to phenylphosphate in a reaction catalyzed by a phenylphosphate synthase enzyme, consisting of three different subunits (proteins 1, 2 and 3) (Schmeling, *et al.*, 2004). Phosphorylation of phenol occurs at the conserved His-596 residue in protein 1. The reaction requires Mg-ATP, protein 2 resembles the N-terminal part of phosphoenolpyruvate synthase, and protein 3 which stimulates the reaction by unknown mechanism (Boll & Fuchs, 2005, Narmandakh, *et al.*, 2006). In the second step of phenol degradation, phenylphosphate is carboxylated in *para*-position, mediated by phenylphosphate carboxylase (Schühle & Fuchs, 2004). Phenylphosphate carboxylase is a member of a new family of carboxylase/decarboxylase enzymes that acts on phenolic compounds. It is an oxygen sensitive enzyme, does not contain biotin thiamine diphosphate, uses CO_2 as a substrate, and requires K^+ and a divalent metal cation (Mg^{+2} or Mn^{+2}) for activity. The enzyme consists of four different subunits (αβγδ), the α- and β-subunits products (54 and 53 kDa) show similarity to UbiD/UbiX aryl decarboxylases, which are involved in ubiquinone biosynthesis, whereas the γ- subunit product (10 kDa) belongs to the hydratase/phosphatase protein family (Schühle & Fuchs, 2004). The α-, β-, and γ- subunits were sufficient to catalyze the CO_2 exchange reaction, whereas δ-subunit (18 kDa) played a role in the restoration of the carboxylation reaction, and found alone catalyzes a very slow hydrolysis of phenylphosphate (Boll & Fuchs, 2005). Another carboxylation reaction involved in the degradation of the hydrocarbons occurs in anaerobic ethylbenzene degradation after the initial hydroxylation by ethylbenzene dehydrogenase and reduction to acetophenone (Rabus, *et al.*, 2002).

Direct methylation of an aromatic compound is a completely new type of initial reaction which has been proposed for degradation of unsubstituted aromatic compounds like naphthalene and benzene (Ulrich, *et al.*, 2005, Safinowski & Meckenstock, 2006). In this reaction, the microorganisms substitute the hydrogen in the aromatic ring with a methyl group from unknown methyl donors, which is further metabolized via fumarate addition and β-oxidation. However, recent studies on benzene and naphthalene degradation favored a direct carboxylation of the unsubstituted aromatic compound as initial activation mechanism (Kunapuli, *et al.*, 2008, Musat, *et al.*, 2008, Abu Laban, *et al.*, 2009).

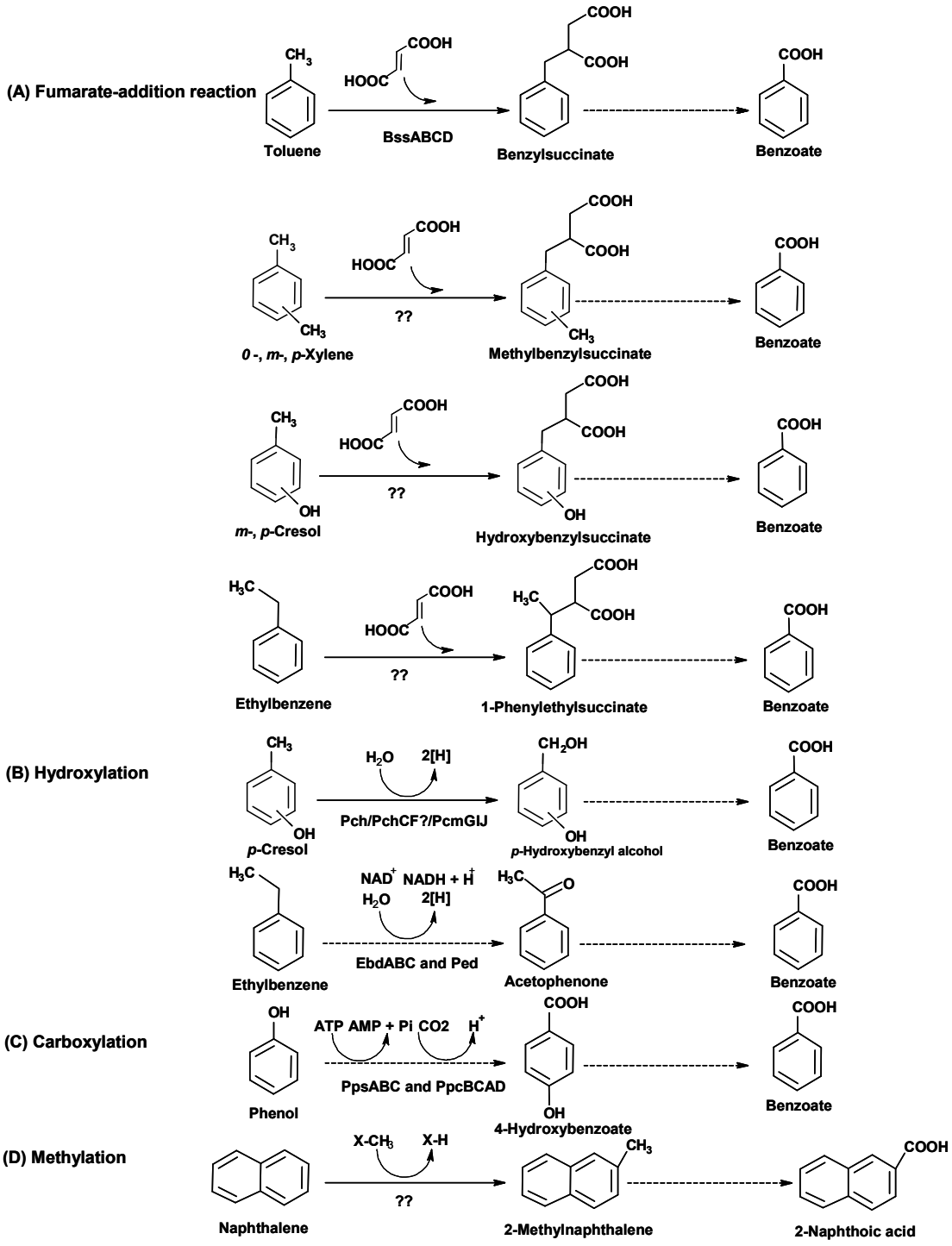

Figure 1.1. The initial reactions involved in the aromatic compounds degradation. (A) Fumarate-addition to methyl group, (B) oxygen-independent hydroxylation, (C) ATP-dependent carboxylation, and (D) methylation of the unsubstituted aromatic hydrocarbon. The names of the enzymes in the different organisms are BssABCD; benzylsuccinate synthase, Pch, PchCF, and PcmGIJ; *p*-cresol methylhydroxylase, EbdABC; ethylbenzene dehydrogenase, Ped; (*S*)-1-phenylethanol dehydrogenase, PpsABC; phenylphosphate synthase, and PpcBCAD phenylphosphate carboxylase. Broken arrows indicate multiple enzymatic steps. Modified from Carmona *et al.* (2009).

1.6 Major scope of the work

Today, benzene degradation is one of the most interesting biodegradation reactions, because the activation of an unsubstituted aromatic ring in the absence of molecular oxygen is considered to be extremely difficult and requires totally new biochemical reaction. The number of pure cultures performing benzene degradation is very limited and therefore the mechanism is only partially studied. Up to date three different mechanisms for the initial reaction of benzene degradation have been proposed (Fig. 1.2); benzene hydroxylation forming phenol (Vogel & Grbic-Galic, 1986, Caldwell & Suflita, 2000, Chakraborty & Coates, 2005), benzene carboxylation forming benzoate (Caldwell & Suflita, 2000, Kunapuli*, et al.*, 2008), and benzene methylation forming toluene (Coates*, et al.*, 2002, Ulrich*, et al.*, 2005). However, neither genes nor any of the expected key enzymes of the anaerobic benzene degradation could be identified. Furthermore, the initial activation reaction remains unclear.

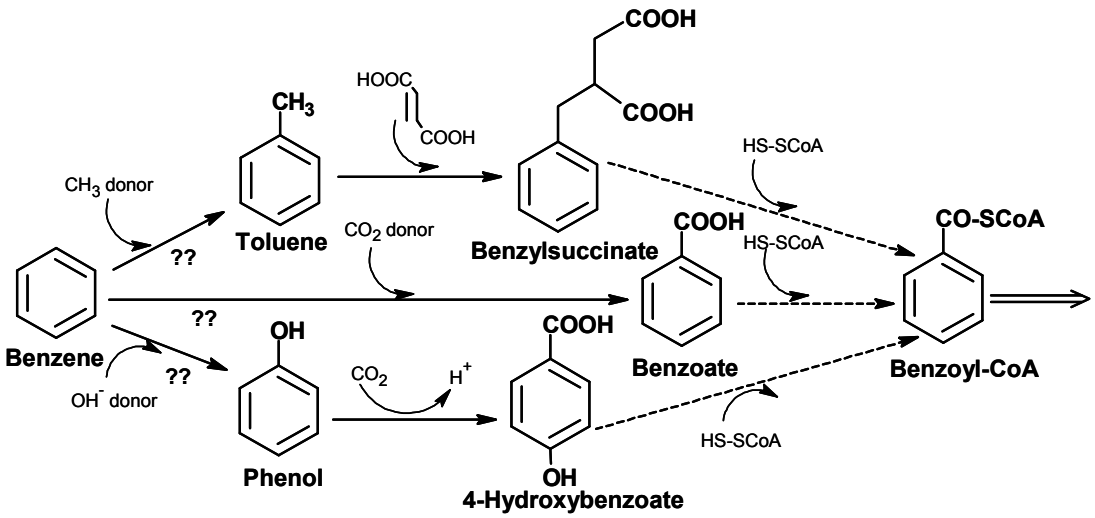

Figure 1.2. The three proposed mechanisms for initial activation of anaerobic benzene degradation. Broken arrows indicate multiple enzymatic steps and open arrows indicate further metabolism. Modified from Foght (2008).

The overall aim of the present thesis is to elucidate the mechanism of anaerobic benzene degradation using sulfate- and ferric iron-reducing enrichment cultures. In more detail, the following questions were raised:

- What are the anaerobic **benzene-degrading key-players** under sulfate-reducing condition?
- Which are **the signature-metabolites** of benzene-degrading sulfate-reducing bacteria?

- Which are the **functional marker genes** of anaerobic benzene degradation under iron- and sulfate-reducing conditions?
- What are the proteins and enzymes involved in anaerobic benzene degradation under iron- and sulfate-reducing conditions?
- Which **specific benzene-induced proteins** are catalyzing the initial activation reaction of anaerobic benzene degradation under iron- and sulfate-reducing conditions?
- Are the enzymatic reactions of anaerobic benzene degradation under iron- and sulfate-reducing conditions the same?

1.7 References

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2 Anaerobic benzene degradation by Gram-positive sulfate-reducing bacteria

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2.1 Introduction

Monoaromatic hydrocarbons such as benzene, toluene, ethylbenzene, and xylene isomers (BTEX) are frequent pollutants in contaminated groundwater systems. Compared to other aromatic hydrocarbons (Anderson & Lovley, 1997), BTEX compounds are highly water soluble and carcinogenic (Dean, 1978) and consequently are described as hazardous organic substances that endanger the quality of drinking water resources (USEPA, 1998). Benzene is rapidly biodegraded under aerobic conditions in soils and aquifers (Gibson & Parales, 2000). However, the input of heavy organic loads into saturated sediments results in the rapid depletion of available oxygen turning aquifers anoxic (Christensen*, et al.*, 1994). Based on field and batch studies, it could be shown that benzene can be effectively degraded under iron-reducing, (Anderson*, et al.*, 1998, Rooney-Varga*, et al.*, 1999, Kunapuli*, et al.*, 2007), sulfate-reducing (Lovley*, et al.*, 1995, Kazumi*, et al.*, 1997, Phelps*, et al.*, 1998, Caldwell & Suflita, 2000), denitrifying (Burland & Edwards, 1999, Coates*, et al.*, 2001, Ulrich & Edwards, 2003) and methanogenic conditions (Grbic-Galic & Vogel, 1987, Ulrich & Edwards, 2003, Chang*, et al.*, 2005).

Previous studies dealing with 16S rRNA gene based fingerprinting analysis of benzene-degrading enrichment cultures revealed the presence of phylogenetically diverse microorganisms (Ulrich & Edwards, 2003, Chang*, et al.*, 2005). Under iron-reducing conditions, bacterial members of the family *Geobacteraceae* were determined as dominant organisms (Rooney-Varga*, et al.*, 1999). Recently, Gram-positive bacteria related to *Clostridia* were discovered to play an essential role in benzene degradation with iron or sulfate as electron acceptor (Kunapuli*, et al.*, 2007, Kleinsteuber*, et al.*, 2008). In addition, members of the family *Desulfobacterecaea* have been identified as dominant organisms in several benzene-degrading cultures under sulfate-reducing and methanogenic conditions (Phelps*, et al.*, 1998, Ulrich & Edwards, 2003, Chang*, et al.*, 2005, Da Silva & Alvarez, 2007,

Musat & Widdel, 2008, Oka*, et al.*, 2008). In a highly enriched benzene-degrading culture, *Desulfobacterium* was identified as the prominent phylotype (Musat & Widdel, 2008). So far, only two denitrifying benzene-degrading strains of the genera *Dechloromonas* and *Azoarcus* have been described as pure cultures (Coates*, et al.*, 2001, Kasai*, et al.*, 2006). However, despite the availability of several enrichment cultures, repeated attempts to isolate obligate anaerobic pure cultures failed and consequently the physiological properties of these microorganisms are still largely unknown.

Our knowledge of the biochemical mechanism of anaerobic benzene degradation is still scarce. The analysis of metabolites in diverse benzene-degrading cultures led to the identification of phenol (Caldwell & Suflita, 2000, Chakraborty & Coates, 2005, Kunapuli*, et al.*, 2008), toluene (Ulrich*, et al.*, 2005), and benzoate (Caldwell & Suflita, 2000, Phelps*, et al.*, 2001, Kunapuli*, et al.*, 2008) as putative intermediates. Consequently, three different biochemical mechanisms were hypothesized for the initial activation reaction of benzene, including carboxylation to benzoate (Caldwell & Suflita, 2000, Phelps*, et al.*, 2001), hydroxylation to phenol (Vogel & Grbic-Galic, 1986, Grbic-Galic & Vogel, 1987, Caldwell & Suflita, 2000, Chakraborty & Coates, 2005), and methylation to toluene (Ulrich*, et al.*, 2005).

The present study delivers insights into physiological, phylogenetic and biochemical characteristics of an enrichment culture able to metabolize benzene under sulfate-reducing conditions. Here, the participation of Gram-positive bacteria in anaerobic benzene metabolism and carboxylation as the putative benzene activation mechanism are highlighted.

2.2 Materials and methods

2.2.1 Enrichment of the benzene-degrading culture and growth conditions

The anaerobic benzene-degrading culture BPL was enriched from soil at a former coal gasification site in Gliwice, Poland, with benzene as growth substrate in the presence of the adsorber resin Amberlite XAD-7 (Morasch*, et al.*, 2001) and 5 mM Na_2SO_4 as electron acceptor. The enrichment culture was transferred 15 times with 10% inoculum (v/v) into new 50 mL sediment-free medium. The enrichment culture was cultivated in bicarbonate-buffered (30 mM) freshwater medium (pH 7.2) under an anaerobic atmosphere of N_2/CO_2 (80:20 v/v) (Widdel & Bak, 1992). The freshwater medium was reduced with 1 mM Na_2S, and 3 mM $FeCl_2$ was added to scavenge produced sulfide. After repeated transfers (10% inoculum, v/v) into fresh medium, a stable anaerobic benzene-degrading enrichment culture was obtained. All culture bottles were incubated at 30°C in the dark.

2.2.2 Alternative substrate and electron acceptor utilization

The benzene-degrading enrichment culture BPL was tested for growth on different organic substrates. A 1 mM total concentration of the aromatic substrates biphenyl, toluene, phenol, 4-hydroxybenzoate, and benzoate were added separately as liquids with a glass syringe or as solid crystals to 120 mL serum bottles containing 50 mL of bicarbonate-buffered mineral medium and 0.3 g of Amberlite XAD-7 (Morasch*, et al.*, 2001). Bacterial growth on different substrates was checked visually by turbidity formation of the medium in triplicate incubations over a period of 3 months. For co-metabolism experiments, 500 µM of the respective aromatic substrates without Amberlite XAD-7 were added to the culture bottles. Formate, pyruvate, and lactate were added to the medium from autoclaved or filter sterilized aqueous stock solutions to a final concentration of 10 mM. H_2/CO_2 gas (30:70 v/v) was directly injected to the culture bottles. The tested electron acceptors included Na_2SO_3 (10 mM), $Na_2S_2O_3$ (10 mM), S^0 (1 g L^{-1}), $NaNO_3$ (5 mM) and $Fe(OH)_3$ (50 mM) respectively. Electron acceptors were autoclaved and added to the culture bottles from anoxic stock solutions. $Fe(OH)_3$ was prepared as described elsewhere (Lovley & Phillips, 1986).

2.2.3 Analytical procedures

For quantification of benzene and CO_2 in the electron and carbon balance experiments the culture was cultivated with 350 µM $^{13}C_6$-benzene (Sigma Aldrich, Steinheim, Germany, 99% purity) without Amberlite XAD-7 in 250 mL serum bottles and sealed with Viton rubber stoppers (Mag Technik, Dübendorf, Switzerland).

$^{13}C_6$-benzene, $^{12}C_6$-benzene, and toluene concentrations were determined by headspace analysis with gas chromatograph/mass spectrometer (GC/MS) (GC, Trace-DSQ; MS, Thermo Finnigan, San Jose, California, USA) in SIM mode selective ion monitoring with a fused-silica capillary column DB-5 (30 m length (L), 0.25 mm inside diameter (ID), 0.25 µm film thickness (T); Agilent, Palo Alto, USA). The injector temperature was 220°C, the carrier gas was helium (grade 5.0) at a flow rate of 1 mL min^{-1} and the split ratio was split /splitless 1:10. The temperature was held at 40°C for 1 min, raised to 200°C at 15°C min^{-1}, to 300°C at 25°C min^{-1} and held for 1 min. The total molar mass of benzene in the culture was calculated from benzene in the liquid phase and Henry's constant of benzene in the headspace as given by Peng & Wan (1997).

Culture samples for phenol quantification were taken inside an anoxic chamber and 25 µL of sample was analyzed directly using the auto injector (SIL-10A*i*, Shimadzu) of a Shimadzu

HPLC equipped with Shimadzu RF-10A XL fluorescence and Shimadzu SPD-10AVP UV diode array detectors at 30°C. The column was a C_{18} Prontosil Eurobond (250 mm L, 4 mm ID, 5 µm particle size; Bischoff, Leonberg, Germany). The mobile phase was (A) 1% acetic acid in water pH 2.7 and (B) 1% acetic acid in methanol at a flow rate of 0.19 and 0.20 mL min^{-1} respectively. UV absorption was measured at 270 nm; fluorescence excitation was at 265 nm and the emission wavelength 315 nm.

The total amount of $^{13}CO_2$ produced from $^{13}C_6$-benzene degradation was determined by measuring the $^{13}CO_2/^{12}CO_2$ isotope ratio in the headspace. 1 mL of the headspace sample was diluted in a 10 mL serum vial filled with helium and stored until analysis. 100 µL of the diluted gas samples were injected to a GC/C/IRMS system consisting of a TRACE GC Ultra gas chromatograph with split/splitless injector (GC) (Thermo Fisher Scientific Corporation, Milan, Italy) coupled to a Finnigan MAT 253 isotope ratio mass spectrometer (IRMS) via Finnigan GC combustion III interface (Thermo Fisher Scientific Corporation, Bremen, Germany). The GC was equipped with a DB-5 column (30 m L, 0.25 mm ID, 0.25 µm T). The temperature of the injector was held at 180°C isothermally with a split ratio 1:10 and column flow of 1.4 mL. Helium (grade 5.0) was used as carrier gas with a constant flow rate of 1.4 mL min^{-1}. The initial oven temperature was 50°C, ramped at a rate of 45°C min^{-1} to 100°C and held for 1.4 min. The $^{13}CO_2$-concentration was calculated from carbon isotope compositions of CO_2 expressed in δ-notation (‰) relative to the Vienna Pee Dee Belemnite standard (V-PDB):

$$\delta\,^{13}C\,[‰] = [(^{13}C/^{12}C_{\,sample} - {}^{13}C/^{12}C_{\,V\text{-}PDB\ standard})/(^{13}C/^{12}C_{\,V\text{-}PDB\ standard})] \times 1000 \quad (2.1)$$

$$^{13}CO_2/^{12}CO_{2\,sample} = [1 + (\delta^{13}CO_2/1000)] \times [^{13}CO_2/^{12}CO_{2\,V\text{-}PDB\ standard}] \quad (2.2)$$

$$^{13}CO_2\,[mM] = {}^{13}CO_2/^{12}CO_{2\,sample} \times \text{initial } ^{12}CO_2\,[mM] \quad (2.3)$$

The initial $^{12}CO_2$-concentration was calculated as the sum of $^{12}CO_2$ from the $NaHCO_3$ (30 mM) buffer in the liquid phase and 20% of $^{12}CO_2$ gas in the headspace of the culture bottles.

Sulfate concentrations were analyzed by ion chromatography using a Dionex 300 ion chromatograph (Dionex Corporation, Sunnyvale, USA) equipped with an IonPac AS14 analytical column (4 x 250 mm) (Dionex Corporation). The eluent was Na_2CO_3 (3.5 mM) and $NaHCO_3$ (1 mM) and the flow rate was 1.2 mL min^{-1}.

2.2.4 Cell counts by Fluorescence *in situ* Hybridization (FISH) and DAPI staining

Oligonucleotide probes used for FISH were: (1) EUB338 I-III mix specifically labelling all *Bacteria* (Amann*, et al.*, 1990, Daims*, et al.*, 1999), (2) DEM1164r targeting all *Desulfotomaculum* cluster I bacteria (Stubner & Meuser, 2000), (3) BET42a in combination with the unlabeled GAM42a oligonucleotide specifically labelling *Beta-proteobacteria* (Manz*, et al.*, 1992), and (4) GAM42a in combination with the unlabeled BET42a oligonucleotide used to detect *Gama-proteobacteria* (Manz*, et al.*, 1992). For the determination of cell numbers via FISH and DAPI staining, 1 mL of the benzene-degrading enrichment culture BPL and the control bacterium *Thiobacillus thiophilus* D24TNT (Kellermann & Griebler, 2009) were fixed for 1-3 h with 1 mL 100% ethanol. The cells were harvest by centrifugation at 6,000 x g for 5 min and the cells were stored in PBS-ethanol (1:1) at -20°C. The cells were filtered onto black 0.2 µm Nucleopore polycarbonate filters (Whatman, Brentford, UK) under vaccum pressure and dehydrated in 50%, 80%, and 100% ethanol for 3 min each. Hybridization of the filters was performed at 46°C for 1.5 h with 40 µL hybridization buffer (0.9 M NaCl, 20 mM Tris/HCl pH 8, 10% formamide, 0.1% SDS), 5 µL of Cy3-labelled probe (30 ng μL^{-1}) and 5 µL of fluorescein-labelled probe (50 ng μL^{-1}). The filters were treated 20 min with washing buffer (0.45 M NaCl, 20 mM Tris/HCl pH 8, 0.01% SDS) at 48°C, washed with distilled water and air dried. For staining with the DNA-binding dye 4,6-diamidino-2-phenylindole (DAPI) the hybridized polycarbonate filters were covered with 5 µL DAPI solution (1 mg mL^{-1}). Counting was performed with an Epifluorescence microscope (Zeiss Axioplan 2, Oberkochen, Germany) equipped with the filters Zeiss49 for DAPI, Zeiss9 for fluorescein and Zeiss43 for Cy3. For cell counting, two separate filters were prepared from duplicate incubations and 20 randomly selected fields were counted for each filter.

2.2.5 Metabolite analysis

Metabolite analysis was performed using a LC-ESI-MS-MS system with a method developed to measure 2-hydroxybenzoate, 4-hydroxybenzoate, benzoate, and benzylsuccinate. 15 mL samples from the cultures were spiked with 1 mL of a 100 µg L^{-1} fluorinated benzoate solution as internal standard, filtered (0.45 µm pore size; Millipore, Eschborn, Germany), acidified to pH 1 and stored at 4°C. Metabolites were extracted from the samples and pre-concentrated by solid phase extraction with ISOLUTE ENV^{+} (200 mg adsorbent; Biotage, Uppsala, Sweden). The analysis was performed using an HPLC Agilent 1100 (Agilent Technologies, Santa Clara, USA) equipped with binary pump, degasser, column oven, and

auto sampler which was coupled with an electrospray ionization source (TurboIon Spray, Applied Biosystems MDS/SCIEX, Foster City, USA) to the triple quadrupole mass spectrometer ABI 3000 (Applied Biosystems MDS/SCIEX) (Ohlenbusch*, et al.*, 2002). The separation column was a Purospher RP-18e (125 × 2 mm L, 5 µm particle size; Merck, Darmstadt, Germany). The column oven was set to 35 ± 2°C. The eluent consisted of (A) water with 0.1% acetic acid (v/v) and (B) 100% acetonitrile. The eluent B was raised from 20% to 90% within 10 min. The flow-rate was 0.3 mL min^{-1} and the sample injection volume was 50 µL. At the ESI interface the nebulizer gas flow was set to 1.5 L/min, the curtain gas flow to 1.6 L min^{-1} and the dry gas flow to 1.6 mL min^{-1}. The dry gas temperature was 450°C and the ion spray voltage of the ESI system was set to -5500 V. MS/MS measurements were performed in the negative multiple reaction mode (MRM) and the most abundant fragment ion was recorded after collision induced dissociation (CID). For benzylsuccinate a second but much less sensitive fragment (*m/z* 91) was used to confirm the detection of this analyte. The ratio of *m/z* 91 to *m/z* 163 is 0.07 (± 20%).

2.2.6 Molecular and phylogenetic analysis

Cells of 50 mL enrichment culture were harvested by centrifugation at 3,345 x g for 15 min at 4°C. The pellet was washed twice with 1 mL of 1 x PBS. Genomic DNA was extracted and purified by using the FastDNA Spin Kit for Soil according to the manufacture's protocol (MP Biomedicals, Illkirch, France).

Terminal restriction fragment length polymorphism (T-RFLP) was performed using the 16S rRNA gene primer set Ba27f-FAM/907r (Lane*, et al.*, 1985). T-RFLP analysis of benzene-grown cells was performed from two separate incubations. T-RFLP analyses of cells grown on other substrates were performed once. The T-RFLP analysis was carried out with 20 ng of the amplicon as described previously (Lueders*, et al.*, 2006).

16S rRNA gene sequences for construction of the clone library were amplified using the universal primers Ba27f/Ba1492r (Weisburg*, et al.*, 1991) resulting in almost full-length products. The PCR reaction, cloning and sequencing were conducted as described previously (Winderl*, et al.*, 2007). The CHECK_CHIMERA program of the ribosomal database project II (Biomedical and Physical Sciences Building, Michigan State University, USA) was used to check for chimeric sequences. The aligned sequences were compared with the closely related sequences available in the public database using BlastN program (http://ncbi.nlm.nih.gov/BLAST). The 16S rRNA gene sequences were added into a database

existing of about 25,000 small-subunit rRNA gene sequences (http://arb-home.de) (Ludwig, *et al.*, 2004). Phylogenetic analysis was performed by parsimony, maximum-likelihood, and verified by neighbour-joining as implemented in the ARB software package. The sequences determined in this study are available at GenBank under accession no. EU523065 to EU523097.

2.3 Results

2.3.1 Physiological properties of the benzene-degrading culture

Enrichment of benzene-degrading, sulfate-reducing bacteria was performed with contaminated soil of a former coal gasification site as inoculum and was since then transferred 15 times into new sterile, soil-free medium. The enrichment culture was able to tolerate benzene up to 1 mM without Amberlite XAD-7. Carbon and electron balances of the culture showed that the complete degradation of 350 µM $^{13}C_6$-benzene was coupled to the production of 1.8 mM $^{13}CO_2$ (equations 2.1-3; Fig. 2.1) and to the reduction of 1.2 mM sulfate (Fig. 2.1). These values correspond to an electron recovery of 88.8% ± 10.7% in the reduced sulfate for total reduction to HS^- and to the production of 83.6% ± 2.6% $^{13}CO_2$ from $^{13}C_6$-benzene mineralization. These experimental results were close to the theoretically stoichiometric values for the complete degradation of benzene to carbon dioxide (equation 2.4).

$$C_6H_6 + 3.75\ SO_4^{-2} + 3\ H_2O \rightarrow 6\ HCO_3^- + 3.75\ HS^- + 2.25\ H^+ \qquad (2.4)$$

There was no decrease of $^{13}C_6$-benzene or sulfate in autoclaved control cultures. The addition of 5 mM molybdate (Na_2MoO_4), a specific inhibitor of sulfate reduction, at day 38 after inoculation, caused a complete inhibition of $^{13}C_6$-benzene degradation and $^{13}CO_2$-production (Fig. 2.1). To prevent a potential toxic effect of sulfide on the microorganisms in the enrichment culture BPL, ferrous chloride was added to the culture medium and consequently a solid iron sulfide mineral (greigite) precipitated.

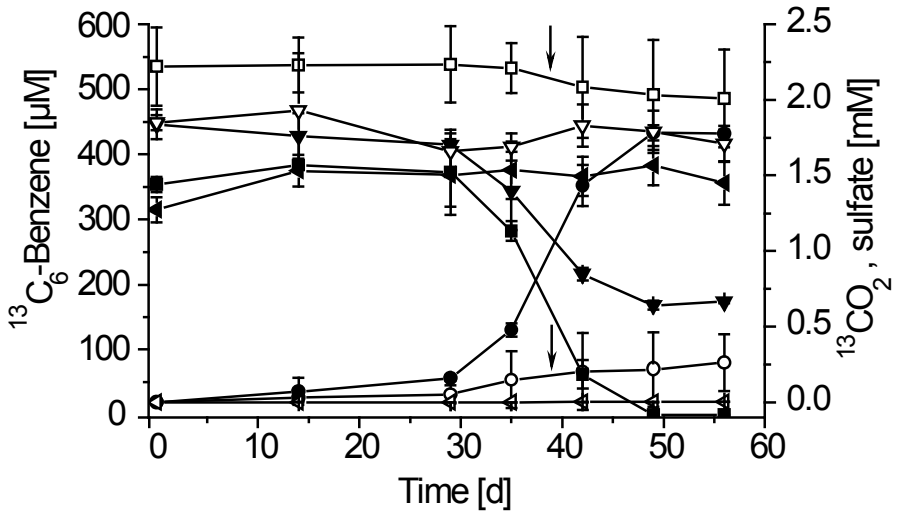

Figure 2.1. Degradation of $^{13}C_6$-benzene by the sulfate-reducing enrichment culture BPL. Carbon balance between $^{13}C_6$-benzene degradation (squares), $^{13}CO_2$ evolution (circles), and sulfate reduction (inverted triangles) in the active cultures. Concentrations of $^{13}C_6$-benzene in the autoclaved control (left triangles) and molybdate inhibited cultures open squares), $^{13}CO_2$ in the autoclaved control (open left triangles), and molybdate inhibited cultures (open circles), and sulfate in the autoclaved control cultures (open inverted triangles). 5 mM Na_2MoO_4 was added after 38 days (arrows). Data are the means of triplicate incubations and error bars represent standard deviations.

The enrichment culture BPL was tested for utilization of several monoaromatic, polycyclic and non-aromatic compounds as carbon source with sulfate as electron acceptor. Besides benzene, the culture was able to grow with biphenyl, whereas benzoate, 4-hydroxybenzoate, phenol, and toluene could not be utilized. The enrichment culture was also tested for its capability to use phenol and toluene as co-substrates (Fig. 2.2). Here, neither phenol nor toluene could be degraded co-metabolically. Interestingly, the rate of benzene degradation was retarded in the presence of phenol (Fig. 2.2a) whereas toluene had no effect on the rate of benzene degradation (Fig. 2.2b). Additionally, growth of microorganisms in the culture BPL could be observed with the organic acids formate, lactate, and pyruvate as well as H_2/CO_2 (30:70 v/v) as evaluated by turbidity formation of the growth medium. None of the tested electron acceptors sulfite, thiosulfate, elemental sulfur, nitrate, or ferrihydrite was utilized with benzene as carbon source.

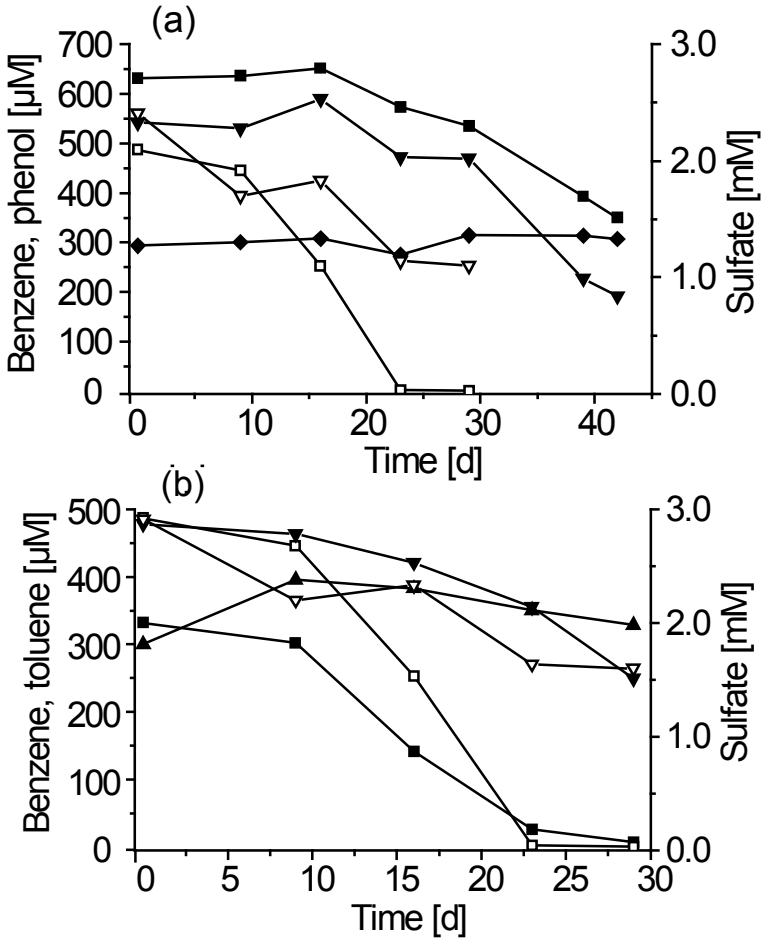

Figure 2.2. Degradation of benzene (squares) by the enrichment culture BPL in the presence of (a) phenol (diamonds) and (b) toluene (upright triangles) as co-substrates using sulfate (inverted triangles) as an electron acceptor. The open symbols represent the degradation of benzene (open squares) and sulfate reduction (open inverted triangles) in the control cultures without co-substrates. Data are the means of duplicate incubations.

2.3.2 Community fingerprinting and phylogenetic analysis

The bacterial community composition of the enrichment culture BPL grown on different substrates was assessed by T-RFLP analysis of 16S rRNA gene sequences. The T-RFLP profile of benzene-grown cells was clearly dominated by 16S rRNA gene sequences forming a 141 bp T-RF (Fig. 2.3a). *In silico* analysis considered the occurrence of an additional T-RF of 152 bp length to be a pseudo-T-RF (Fig. 2.3a) which was verified by T-RFLP analysis of specific 16S rRNA clone sequences. Pseudo T-RFs are formed when the restriction enzyme failed to cut at the first recognition site and thus a longer T-RF is formed by restriction at the second site (Egert & Friedrich, 2003). Thus, the T-RFs of 141 bp and 152 bp have derived from the same 16S rRNA gene sequence. The 141 bp T-RF could also be identified as a dominant peak in the biphenyl-grown culture together with an additional T-RF of 164 bp in

length (Fig. 2.3b). A 164 bp T-RF was continuously present in all growth cultures (except lactate) tested for alternative electron donors (Fig. 2.3c-f).

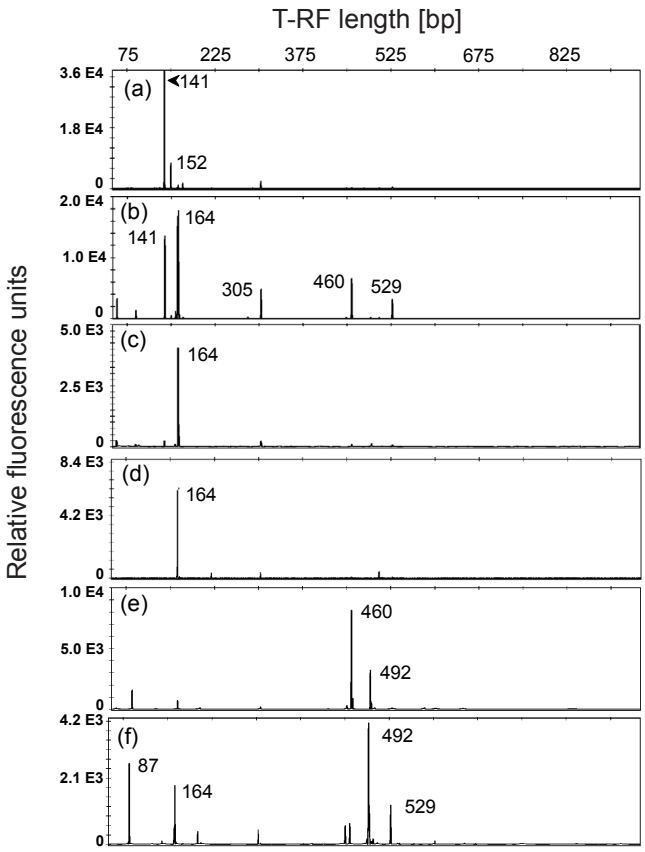

Figure 2.3. T-RFLP fingerprinting profiles of 16S rDNA gene sequences of the enrichment culture BPL grown on (a) benzene, (b) biphenyl, (c) formate, (d) pyruvate, (e) lactate, and (f) H_2/CO_2 gas. Dominant based paris are laballed with with lengh (bp).

To get insights into the phylogenetic affiliation of the microorganims in the benzene-grown culture we performed cloning and sequencing of almost full-length 16S rRNA gene sequences. The analysis clearly showed a dominance of 16S rRNA gene sequences (designated as BpP) that accounted for 30 out of 33 analysed small-subunit sequences and that could be correlated with the 141 bp T-RF. These highly abundant sequences were affiliated with the genus *Pelotomaculum* within the Gram-positive family *Peptococcaceae* (Fig. 2.4) and grouped to a cluster that was related to *Pelotomaculum isophthalicum* JI (95% sequence similarity). Moreover, two closely related 16S rRNA gene sequences (BpC52 and BpC43; represented by the 305 bp T-RF) were detected that were affiliated to the family *Clostridiaceae*. The 16S rRNA gene sequence of the clone BpD108 was affiliated to the

Delta-proteobacterial family *Syntrophaceae* exhibiting a similarity value of 93.3% to the dehalogenating bacterium *Desulfomonile tiedjei.*

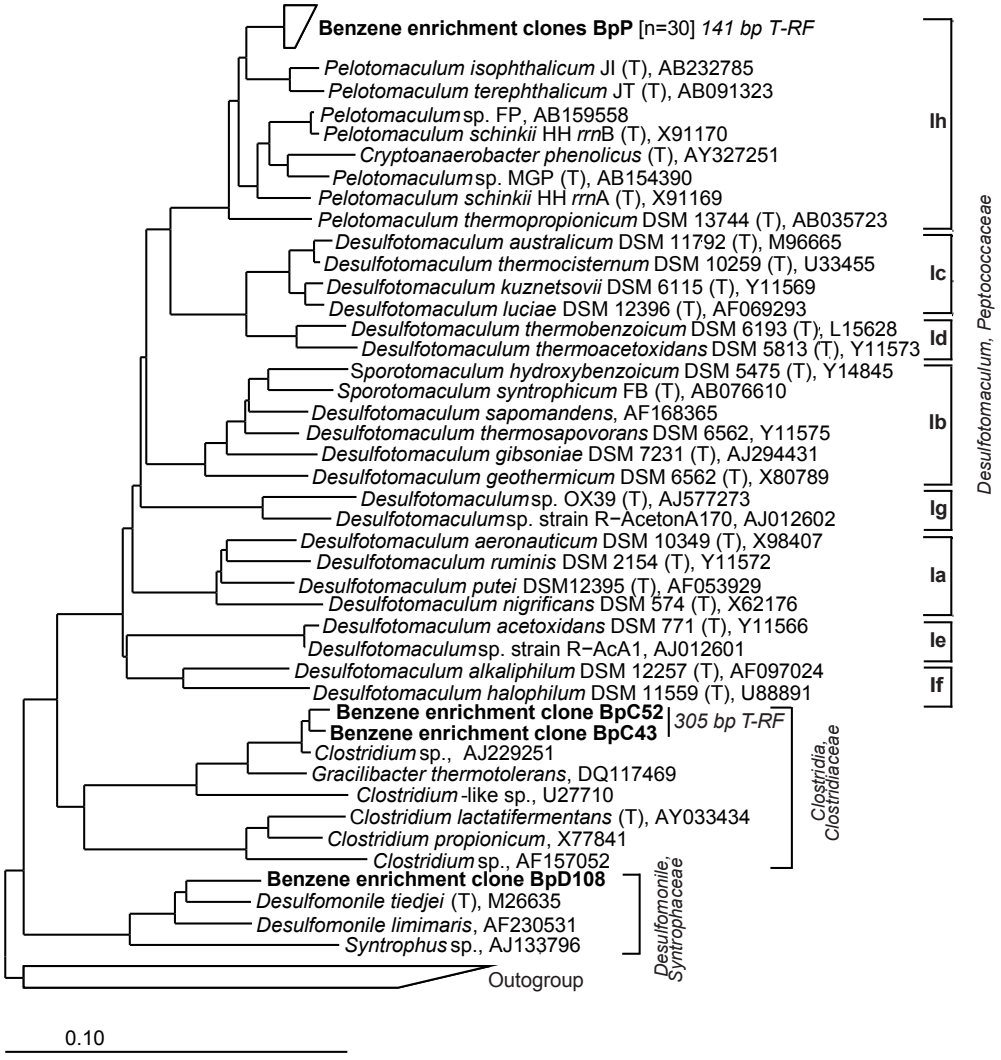

Figure 2.4. Phylogenetic tree showing the affiliation of the 16S rRNA gene sequences amplified from the enrichment culture BPL. 16S rRNA clone sequences from the enrichment culture BPL are shown in bold. Subdivisions Ia-Ih of the *Desulfotomaculum* cluster I are listed. An encompassing collection of organisms representing all major lineages of the *Archaea* and *Bacteria* was used as outgroup for tree calculations. The scale bar represents 10% sequence divergence.

2.3.3 Cell counts determined by FISH and DAPI-staining

In order to demonstrate that bacteria with *Pelotomaculum*-related 16S rRNA gene sequences (BpP) are dominant and their cell numbers are increasing during the full time course of benzene degradation, *in situ* hybridization with the probes DEM1164r, specific for *Desulfotomaculum* cluster I bacteria comprising *Pelotomaculum* sp., and EUB338 I-III mix (*Bacteria*) was performed (Fig. 2.5). No hybridization to 16S rRNA gene sequences of the

enrichment culture BPL could be observed with the negative control probes BET42a and GAM42a. However, the probe BET42a but not DEM1164r hybridized to the control organism *Thiobacillus thiophilus* D24TNT. During the time course of benzene degradation the average percentage of DEM1164r-labelled cells was 94.5 (n = 9 time points) of the EUB338 I-III mix-positive cells. The portion of the bacterial cells stained with DEM1164r and EUB338 I-III mix constituted 86.6 (n = 9) and 91.8 (n = 9) of the total DAPI-positive cells (Fig. 2.6).

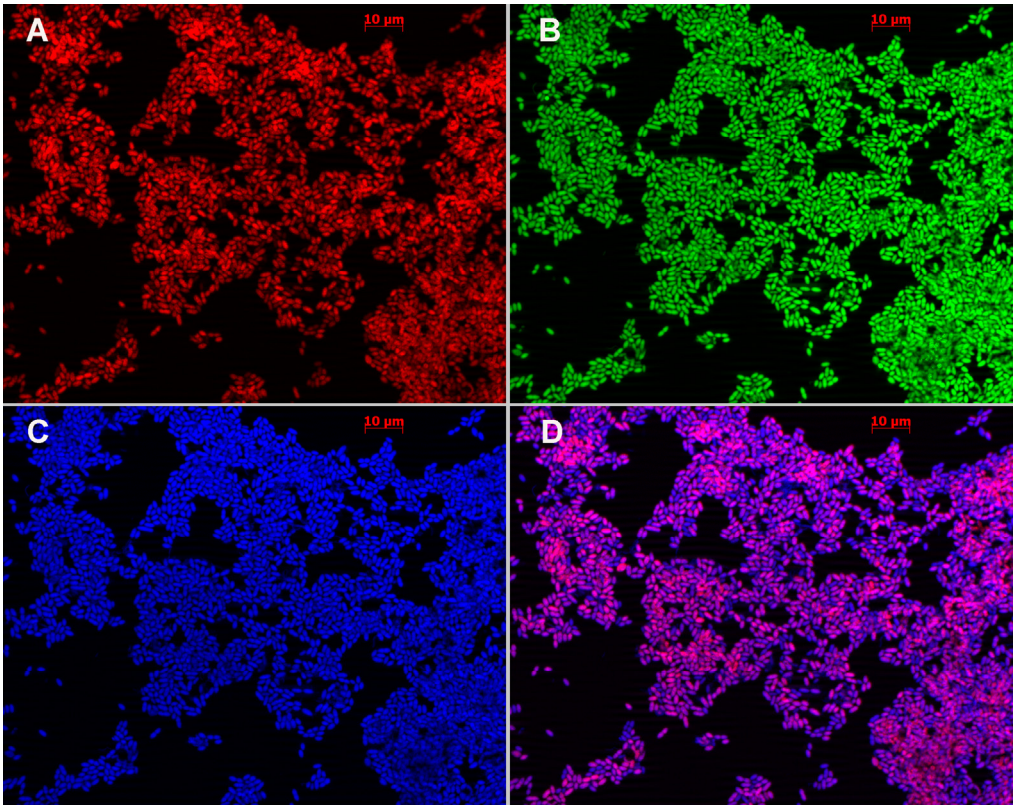

Figure 2.5. Micrographs of cells from the culture BPL grown with benzene and sulfate as electron acceptor. (a) Specific labeling of Des*ulfotomaculum* subcluster I cells with the Cy3-labeled probe DEM1164r, (b) General labeling with the Fluos-labeled probe mixture EUB338 I-III, (c) DAPI-staining of all bacteria, and (d) labeling of cells with the specific probe DEM1164r and DAPI resulting in overlays of the probe and DAPI signals; cells targeted by the specific probe appear in magenta and non-targeted cells in blue.

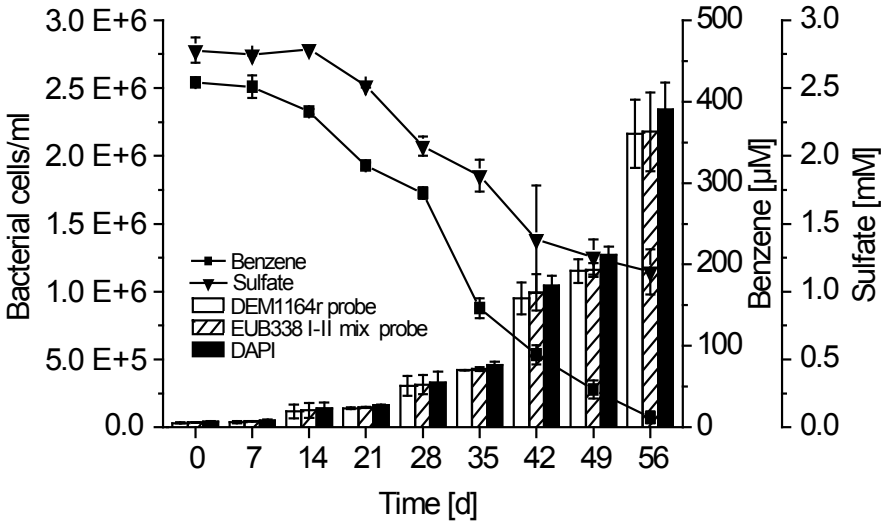

Figure 2.6. Relative quantification of bacterial cells detected by *in situ* hybridization with the specific probe DEM1164r (*Desulfotomaculum* cluster I) or EUB338 I-II mix (*Bacteria*) and DAPI-staining during the full time course of benzene degradation by the enrichment culture BPL. Data are the means of duplicate incubations with two separate filters counting each and error bars represent SDs.

2.3.4 Metabolite analysis

Metabolites were screened and quantified with LC-MS-MS and HPLC/UV fluorescence analysis during benzene degradation (54 days). Benzoate, 4-hydroxybenzoate, and phenol were detected in active as well as in autoclaved control cultures (Fig. 2.7). The phenol formation increased concomitant to benzene degradation in the active culture reaching a maximum concentration of 1.8 µM at day 28. Interestingly, the phenol concentration in the autoclaved control culture was comparably high reaching the maximum value of 1.6 µM at day 47. The amount of benzoate in the active culture was highest at day 47 increasing to 0.4 µM, whereas its concentration in the autoclaved culture remained constantly low (0.06 µM). Additionally, at the end of the time course of benzene-degradation 4-hydroxybenzoate was identified in the active culture at 0.14 µM. 2-hydroxybenzoate was detected at maximum concentration of 2.9 nM from autoclaved control cultures and with a slightly higher concentration (7.9 nM) in the active culture (data not shown). Benzylsuccinate could not be identified.

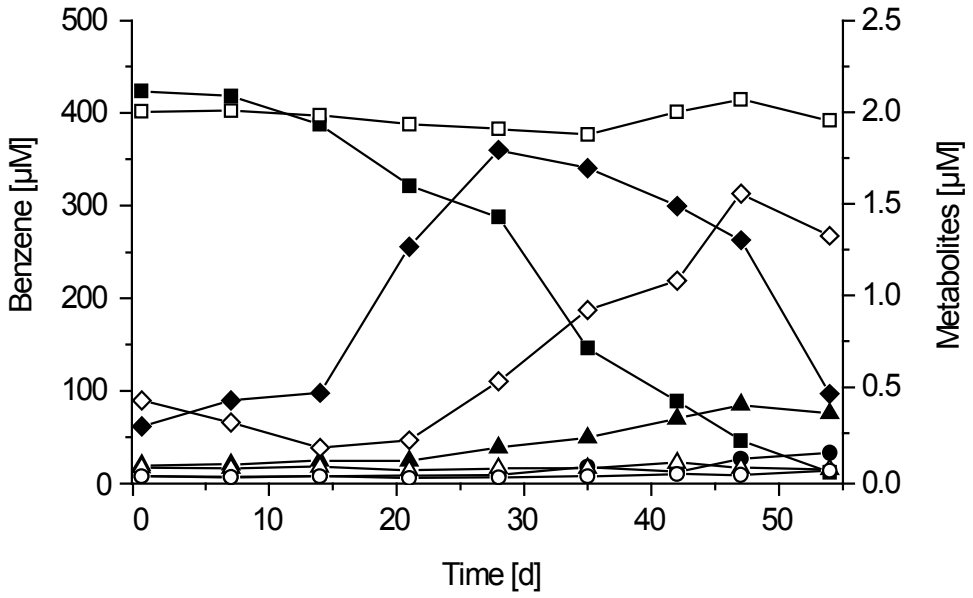

Figure 2.7. Metabolite analysis from active and autoclaved control cultures under sulfate-reducing conditions during the full time course of benzene degradation. The concentration of benzene (squares), phenol (diamonds), 4-hydroxybenzoate (circles), and benzoate (triangles) in the active cultures are given over time. Open symbols represents the concentration benzene and metabolites in the autoclaved controls. Data are the means of duplicate incubations.

2.4 Discussion

In the present study, we report on anaerobic benzene degradation by a sulfate-reducing enrichment culture obtained from soil at a former coal gasification site. Electron and carbon balances with $^{13}C_6$-benzene indicated a total oxidation of the aromatic hydrocarbon to CO_2 coupled to sulfate reduction.

2.4.1 Bacterial community structure of the benzene-degrading culture

Based on the quantitative determination of the putative benzene degrader by *in situ* hybridization and the analysis of 16S rRNA gene sequences, we have provided evidence leading to the hypothesis that organisms phylogenetically related to the Gram-positive genus *Pelotomaculum* play an important role in benzene degradation. However, the 16S rRNA based sequence similarity to the next cultivated representative constitutes only 95%. Thus, these sequences could be clustered between the genera *Desulfotomaculum* and *Pelotomaculum*. *P. isophthalicum*-related 16S rRNA gene sequences were detected in benzene-contaminated groundwater and *in situ* reactor columns degrading benzene under sulfate-reducing and denitrifying conditions (Kasai*, et al.*, 2007, Kleinsteuber*, et al.*, 2008). Kleinsteuber *et al.*

(2008) hypothesized that *Pelotomaculum* syntrophically ferments benzene to acetate under sulfate-reducing conditions. The similarity of the sequences of the *Pelotomaculum*-related phylotypes described by Kleinsteuber *et al*. (2008) to those identified in the present study range from 88.8% to 95.7% indicating that these phylotypes are totally different. Based on the results with our highly enriched culture, where only one dominant organism is present, we propose that bacteria with *Pelotomaculum*-related 16S rRNA sequences oxidize benzene directly with sulfate as electron acceptor. However, members of the genus *Pelotomaculum* were not reported to carry out dissimilatory sulfate reduction so far (Imachi*, et al.*, 2002). Recently, Imachi *et al.* (2006) proposed that *Desulfotomaculum* subcluster Ih bacteria have lost their ability to reduce sulfate in an evolutionary process and thus are restricted to methanogenic environments for syntrophic oxidation of organic substrates. Such a syntrophic benzene degradation could be definitely excluded for the presented culture because neither methane nor methanogenic archaea have been detected (data not shown). Additionally, the *in situ* hybridization and T-RFLP analysis of the benzene-grown enrichment culture clearly showed the presence of only one dominant genotype. Thus, we can show here that bacteria that are phylogenetically affiliated to *Pelotomaculum* but physiologically strongly resemble *Desulfotomaculum* are utilizing benzene as sole carbon source with sulfate as electron acceptor. This hypothesis is supported by the identification of the gamma subunit of the dissimilatory sulfite reductase (key enzyme of dissimilatory sulfate reduction) by proteomic analysis of the benzene-grown culture (data not shown). The identified peptide is closely related to the dissimilatory sulfite reductase of *P. thermopropionicum* (Imachi*, et al.*, 2006).

Our knowledge of the role and importance of Gram-positive bacteria in degradation of aromatic hydrocarbons is quite limited. Recently, an iron-reducing enrichment culture was described where benzene degradation was performed by *Clostridia*-related bacteria in association with members of *Desulfobulbaceae* (Kunapuli*, et al.*, 2007). In addition, few Gram-positive sulfate-reducers were successfully isolated in pure cultures that degrade aromatics (Cord-Ruwisch & Garcia, 1985, Tasaki*, et al.*, 1991, Kuever*, et al.*, 1999, Morasch*, et al.*, 2004). Interestingly, the majority of these bacterial strains are phylogenetically restricted to the *Desulfotomaculum* cluster I. Morasch *et al.* (2004) isolated the strict anaerobic bacterium *Desulfotomaculum* sp. Ox39 that metabolizes the aromatic hydrocarbons toluene, *m*-xylene and *o*-xylene. *D. sapomandens* (Cord-Ruwisch and Garcia, 1985)*, D. thermobenzoicum* (Tasaki*, et al.*, 1991) and *D. gibsoniae* (Kuever*, et al.*, 1999) exhibit a great versatility in the kind of aromatic electron donors they can use for growth including benzoate,

phenol, and 4-hydroxybenzoate. Molecular based investigations of benzene-degrading communities at contaminated sites and in enrichment cultures in the presence of different electron acceptors showed that they were mostly affiliated to *Beta*-, *Delta*-, and *Gama-proteobacteria* (Phelps*, et al.*, 1998, Rooney-Varga*, et al.*, 1999, Coates*, et al.*, 2001, Musat & Widdel, 2008, Oka*, et al.*, 2008). However, several studies identified *Firmicutes*-related 16S rRNA gene sequences that so far have not been brought into direct correlation with contaminant degradation (Phelps*, et al.*, 1998, Ulrich & Edwards, 2003, Chang*, et al.*, 2005). Nevertheless, the large diversity of the microbial communities was not enough to emphasize the key players and their role in benzene degradation. So far, only a limited number of sulfate-reducing benzene-degrading enrichment cultures have been purified to an extend that allowed the identification of the key degraders. *Delta-proteobacterial* phylotypes related to *Desulfobacterium* were found to be abundant in a benzene-degrading sulfate-reducing culture (Qiu*, et al.*, 2006, Musat & Widdel, 2008). Furthermore, members of the family *Desulfobacteraceae* have been identified as significant bacteria in a sulfidogenic benzene-degrading culture by DNA stable isotope probing (Oka*, et al.*, 2008). More recently, bacteria affiliated to the genera *Sulfurovum*, *Desulfovibrio*, and *Cryptanaerobacter/Pelotomaculum* have been characterized to be the most prominent bacteria in sand-filled columns percolated with groundwater from a benzene-contaminated aquifer whereas the proportion of the latter one increased after repeated benzene-spiking indicating a role in benzene degradation (Kleinsteuber*, et al.*, 2008). The cultivation of our *Pelotomaculum*-related bacterium further supports our hypothesis that Gram-positive microorganisms have been largely overlooked as being important in degradation of benzene (Kunapuli*, et al.*, 2007, Kunapuli*, et al.*, 2008). Especially Gram-positive sulfate-reducing bacteria may play an up to date underestimated role, because their ability of spore-formation allows this group to survive periods of environmental fluctuating conditions such as alternating oxic and anoxic conditions (Nielsen*, et al.*, 2005).

2.4.2 Initial reaction mechanism of anaerobic benzene degradation

In previous studies, three possible mechanisms have been proposed for the initial reaction of anaerobic benzene degradation. Hydroxylation of benzene to phenol has been hypothesized as one putative pathway under methanogenic and denitrifying conditions (Vogel & Grbic-Galic, 1986, Weiner & Lovley, 1998, Caldwell & Suflita, 2000, Chakraborty & Coates, 2005). In the present study, phenol and the specific intermediate of anaerobic phenol degradation 4-hydroxybenzoate were detected in the active enrichment culture. These results might indicate

that benzene is initially hydroxylated to phenol. If the degradation would proceed via hydroxylation to phenol, the benzene-grown culture should have the ability to utilize phenol immediately. However, phenol as sole carbon source was not utilized by the enrichment culture BPL. In addition, benzene degradation was retarded when phenol was added as a co-substrate. It seems that phenol decreased the activity of the benzene degraders or that the benzene degraders had to adapt to the presence of phenol, which may exhibit a toxic effect. This observation is congruent with results obtained from a sulfate-reducing culture enriched from marine sediment where the rate of benzene degradation was also slightly slower in the presence of phenol (Musat & Widdel, 2008).

In addition, 2-hydroxybenzoate has been identified as a putative metabolite in the benzene-grown cultures, which is not known to be a specific intermediate compound of anaerobic degradation of monoaromatic hydrocarbons, so far. Thus, it might indicate that the detected hydroxylated aromatic compounds are formed abiotically due to contact of reduced compounds with oxygen during sampling. This is strongly supported by the detection of phenol in our autoclaved control cultures. Kunapuli *et al.* (2008) showed that benzene is readily autohydroxylated by hydroxyl ($OH^{\bullet}$) radicals formed from oxygen during sampling, which might also have produced the hydroxylated monoaromatic compounds in our culture. Another evidence for this abiotic process is the formation of 4-hydroxybenzoate concomitant to benzoate evolution. The medium was supplemented with 3 mM of $FeCl_2$ to scavenge the produced sulfide. As a result of this reaction, reduced iron is produced that is considered to be a catalyst of Fenton's reaction. During sampling, ferrous ion gets in contact with oxygen which results in the formation of highly reactive hydroxyl $OH^{\bullet}$ radicals (Kavith & Palanivelu, 2004, Mortazavi*, et al.*, 2005). These hydroxyl $OH^{\bullet}$ radicals readily react with organic compounds such as benzene and benzoate forming hydroxylated products (Kunapuli*, et al.*, 2008).

Methylation is proposed as an alternative mechanism for anaerobic degradation of non-substituted aromatic compounds (Ulrich*, et al.*, 2005, Safinowski & Meckenstock, 2006). Benzylsuccinate as the specific metabolite of anaerobic toluene degradation was not detected in our benzene-grown culture. Similarly to recent studies (Musat & Widdel, 2008, Oka*, et al.*, 2008), toluene could not be used as substrate by our culture and the presence of toluene in the medium had no effect on the rate of benzene degradation. This is in contrast to investigations where other authors clearly showed that toluene inhibits the rate of benzene degradation (Da

Silva & Alvarez, 2007). Thus, methylation could probably be excluded as initial degradation mechanism in the investigated enrichment culture.

Another proposed pathway of anaerobic benzene degradation involves direct carboxylation of benzene to benzoate (Caldwell & Suflita, 2000). Previous studies with sulfate-reducing enrichment cultures using labeled benzene revealed the presence of ^{13}C-benzoate and d_5-benzoate as intermediate compounds and showed that the carboxyl group derived from benzene (Caldwell & Suflita, 2000, Phelps*, et al.*, 2001). However, *Kunapuli et al.* (2008) showed for an iron-reducing culture that the carboxyl group of benzoate stems from the carbonate buffer. In the present study, benzoate was identified as putative intermediate compound, but we could not prove that the benzene-degrading bacteria have the capability to utilize benzoate as substrate. As benzoate can not freely diffuse into cells, we assume that benzene-degraders in our culture lack an active transport system for benzoate as it is reported for *Pseudomonas putida* (Thayer & Wheelis, 1982). Furthermore, it might be possible that benzoate could not be activated to benzoyl-Co-enzyme A due to the lack of a benzoate-CoA ligase (Barragan*, et al.*, 2004). Nevertheless, our findings would tentatively favour a direct carboxylation of benzene as the initial activation reaction in the sulfate-reducing enrichment culture BPL.

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3 Identification of enzymes involved in anaerobic benzene degradation by two strictly anaerobic enrichment cultures

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3.1 Introduction

The aromatic hydrocarbon benzene is a component of crude oil and gasoline and is intensively used in the chemical industry. Due to its high solubility (1.78 g L^{-1} in water at 25°C), chemical stability (ΔG_{f° = +124.4 KJ mol^{-1}), and impact on human health, benzene is considered as one of the most hazardous pollutants of groundwater and sediments (Johnson*, et al.*, 2003). Therefore, it is of great interest to understand the fate and the biodegradability of benzene in the environment.

For aerobic degradation of benzene well studied pathways are known (Gibson*, et al.*, 1968). Furthermore, enrichment cultures and microcosm studies indicated that benzene can be effectively degraded in the absence of molecular oxygen under iron-reducing (Lovley*, et al.*, 1996, Anderson*, et al.*, 1998, Rooney-Varga*, et al.*, 1999, Kunapuli*, et al.*, 2008), sulfate-reducing (Lovley*, et al.*, 1995, Kazumi*, et al.*, 1997, Phelps*, et al.*, 1998, Caldwell & Suflita, 2000, Abu Laban*, et al.*, 2009), denitrifying (Burland & Edwards, 1999, Coates*, et al.*, 2001, Ulrich & Edwards, 2003), and methanogenic (Ulrich & Edwards, 2003, Chang*, et al.*, 2005) conditions. So far, only a two benzene-degrading strains were isolated to purity that were described as denitrifying members of the genera *Dechloromonas* and *Azoarcus* (Coates*, et al.*, 2001, Kasai*, et al.*, 2006). Despite the cultivation of benzene-degrading cultures, a profound knowledge of the biochemical mechanism and the enzymes and genes of anaerobic benzene degradation is still lacking.

In the presence of molecular oxygen, benzene is activated by oxygenases introducing one or two hydroxyl groups from O_2 yielding phenol, benzene epoxide, or catechol, respectively (Bugg, 2003). Under anaerobic conditions, the reactive oxygen is missing and degradation requires other biochemical reactions to overcome the extreme chemical stability of benzene.

Examples of anaerobic activation reactions of hydrocarbons include oxygen-independent hydroxylation of alkyl substituent via e.g. ethylbenzene dehydrogenase (Rabus & Widdel, 1995, Ball, *et al.*, 1996, Kniemeyer & Heider, 2001), or in *p*-cresol degradation (Hopper, *et al.*, 1991, Peters, *et al.*, 2007), fumarate addition by glycyl radical enzymes to methyl carbon atoms in toluene (Biegert, *et al.*, 1996, Beller & Spormann, 1998, Leuthner, *et al.*, 1998), *m*,*o*,*p*-xylene, (Krieger, *et al.*, 1999, Achong, *et al.*, 2001, Morasch, *et al.*, 2004, Morasch & Meckenstock, 2005) and *p*-cresol (Müller, *et al.*, 2001), and anaerobic carboxylation of phenol (biological Kolbe-Schmitt reaction) (Schmeling, *et al.*, 2004, Schühle & Fuchs, 2004, Narmandakh, *et al.*, 2006).

Although the mechanism of benzene activation under anaerobic conditions is still unclear, putative reactions were proposed based on metabolite detection using $^{13}C_6$-benzene, $H_2{}^{18}O$, or ^{13}C-labelled bicarbonate buffer. Such reactions included (1) hydroxylation of benzene to phenol (Vogel & Grbic-Galic, 1986, Grbic-Galic & Vogel, 1987, Caldwell & Suflita, 2000, Chakraborty & Coates, 2005), (2) direct carboxylation to benzoate (Caldwell & Suflita, 2000, Phelps, *et al.*, 2001, Kunapuli, *et al.*, 2008, Abu Laban, *et al.*, 2009), (3) or a methylation to toluene (Ulrich, *et al.*, 2005) (Fig. 3.1). Recent studies showed an abiotic formation of phenol from benzene in culture media for iron- and sulfate-reducing organisms by contact with air during sampling (Kunapuli, *et al.*, 2008, Abu Laban, *et al.*, 2009). This indicated that phenol as a putative intermediate of benzene degradation has to be interpreted with caution.

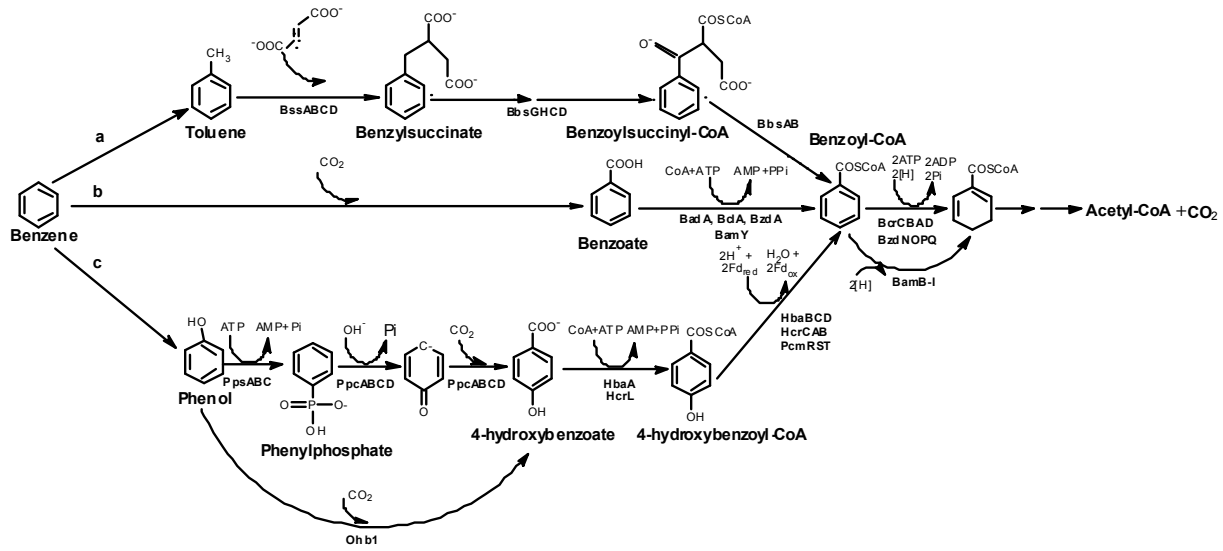

Figure 3.1. Proposed options for anaerobic biodegradation of benzene. (a) Methylation to toluene followed by fumarate addition to form benzylsuccinate that is subsequently metabolized to benzoyl-CoA. (b) Carboxylation to benzoate and ligation of CoA forming benzoyl-CoA. (c) Hydroxylation to phenol, and subsequent degradation via 4-hydroxybenzoate and benzoyl-CoA. The names of the enzymes in the different organisms are: BssABC,

Benzylsuccinate synthase; BbsEF, succinyl-CoA:(R)-benzylsuccinate CoA-transferase; BbsG, (R)-benzylsuccinyl-CoA dehydrogenase; BbsH, phenylitaconyl-CoA hydratase; BbsCD, 2-[hydroxy(phenyl)methyl]-succinyl-CoA dehydrogenase; BbsAB, benzoylsuccinyl-CoAthiolase; BadA, BclA, BzdA, and BamY, benzoate-CoA ligase; BcrCBAD, BzdNOQP, and BamB-I, benzoyl-CoA reductase; PpsABC, phenylphosphate synthase; PpcABCD, phenylphosphate carboxylase; HbaA, HcrL, 4-hydroxybenzoate-CoA ligase; and HbaBCD, HcrCAB, and PcmRST, 4-hydroxybenzoyl-CoA reductase.

Recently, the genomes of five anaerobic aromatics-degrading microorganisms including the phototrophic organism *Rhodopseudomonas palustris* strain CGA009 (Larimer*, et al.*, 2004), the denitrifying organisms *Magnetospirillum magneticum* strain AMB-1 (Matsunaga*, et al.*, 2005) and *Aromatoleum aromaticum* strain EbN1 (Rabus*, et al.*, 2005), the iron reducer *Geobacter metallireducens* strain GS-15 (Butler*, et al.*, 2007), and the fermenter *Syntrophus aciditrophicus* strain SB (McInerney*, et al.*, 2007) have been sequenced and several operons coding for enzymes of anaerobic aromatic hydrocarbon degradation have been identified (Carmona*, et al.*, 2009). The overall organization of anaerobic catabolic gene clusters was conserved across a wide variety of microorganisms. However, genus- and species-specific variations account for differences in gene arrangements, substrate specificities, and regulatory elements. Although the genome sequence of the benzene-degrading *Dechloromonas aromatica* strain RCB has been elucidated (accession number NC_007298) no genes coding for putative enzymes involved in anaerobic benzene degradation were indentified (Salinero*, et al.*, 2009).

In the present study, we describe for the first time a combined proteomic and genomic approach to elucidate the biochemical mechanism of anaerobic benzene degradation. Whole proteomes of benzene-, phenol-, and benzoate-grown cells were compared by a LC/ESI-MS/MS-based shotgun proteomic analysis and were correlated to the high-throughput sequenced metagenome information of the iron-reducing, benzene-degrading enrichment culture BF. Additionally, specific protein bands expressed in benzene-grown cells were subjected to N-terminal sequence analysis. A putative benzene carboxylase-related protein was identified as key-protein that might be responsible for the initial activation of benzene, and thus provide new evidence for carboxylation of benzene under iron- and sulfate-reducing conditions.

3.2 Materials and methods

3.2.1 Growth conditions of anaerobic benzene-degrading enrichment cultures

The iron-reducing enrichment culture BF (Kunapuli*, et al.*, 2007) and the sulfate-reducing culture BPL (Abu Laban*, et al.*, 2009) were cultivated as previously described. For differential protein expression analyses, cultivation was performed in 4 L growth medium. Inoculums (10%, v/v) from benzene-grown precultures of culture BF were transferred into separate bottles containing either benzene (1 mM), phenol (1 mM), or benzoate (1 mM) as growth substrates in the presence of XAD7 as substrate reservoir (Morasch*, et al.*, 2001) and 50 mM of ferrihydrite as electron acceptor. For culture BPL, inoculums (10%, v/v) of benzene-grown cells were transferred into new bottles containing 1 mM benzene without XAD7. All culture bottles were incubated at 30°C in the dark.

3.2.2 Microbial community structure analysis of the iron-reducing enrichment culture BF

The total genomic DNA of benzene-, phenol-, and benzoate-grown cells of the iron-reducing enrichment culture BF was extracted during the exponential growth phase with the FastDNA Spin Kit for Soil (MP Biomedicals, Illkirch, France) according to the manufacturer's protocol. The microbial community structure was analyzed by terminal restriction fragment length polymorphism (T-RFLP) analysis as previously described by Winderl *et al.* (2008). The analysis was carried out in three biological replicates for each aromatic substrate.

3.2.3 Shotgun genomic DNA sequencing of the iron-reducing culture BF

The whole metagenome of the iron-reducing culture BF grown on benzene was extracted with the FastDNA Spin Kit for Soil (MP Biomedicals) according to the manufacturer's protocol. Genomic information was obtained by shotgun sequencing using a GS FLX sequencer (performed by Eurofins MWG Operon, Ebersberg, Germany). The sequence reads have been automatically assembled into contigs. Automated annotation of the assembled sequences was performed using the PEDANT (PMID 18940859) software system. The prediction of coding sequences was performed with a combination of Genemark 2.6r (PMID 11410670) and Glimmer 3.02 (PMID 17237039). Homology to already published proteins in Uniref100 was used to decide about the best gene models and in order to decide about the best gene starts. Coding sequences were automatically assigned by PEDANT to functional categories according to the functional role catalogues FunCat (PMID 15486203) and Gene

Ontology (PMID 14681407). All sequence data from this study were deposited at GenBank under the accession numbers GU357855 to GU358059.

3.2.4 Protein extraction and SDS-PAGE

Cells were harvested from 4 L culture bottles by centrifugation at 9,000 x g for 15 min. The cell pellet was washed three times with 50 mM Tris-HCl buffer (pH 7.5) and incubated overnight at -20°C. One gram cell pellet was resuspended in one mL lysis buffer (9 M urea, 2% CHAPS, 1% DTT; GE Healthcare Europe GmbH, Freiburg, Germany). The cell extracts were treated with a 7 x stock solution (167 µL.mL^{-1} lysis buffer) of Complete Mini EDTA-free Protease Inhibitor Cocktail Tablet (Roche Diagnostics GmbH, Penzberg, Germany) and incubated at 15°C for 30 min. Extracts were transferred into matrix lysis tubes B (MP Biomedicals) and were treated in a bead beating FastPrep®Instrument 120 (MP Biomedicals) at 6.0 m sec^{-1} for 35 sec. After centrifugation for 1 min at 20,000 x g the supernatant was incubated with nuclease mix (1µL per 100 µL of supernatant; GE Healthcare) at 20°C for 30 min and again purified by centrifugation (20,000 x g, 1 h).

Quantification of protein concentrations was performed using 2-D Quant Kit according to the manufacturer's instructions (GE Healthcare). Proteins were precipitated with acetone for 10 min at -20°C, and collected by centrifugation at 15,000 x g for 30 min. 30 µg of proteins were resuspended in 20 µL lysis buffer and subsequently separated by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE; 4% stacking gel, 12 % separating gel) with a running buffer containing glycine 14.4 g L^{-1}, Tris-base 3 g L^{-1} (pH 8.3), and SDS 1 g L^{-1}. After electrophoresis, the gels were stained with colloidal Commassie Brilliant Blue (Zehr*, et al.*, 1989). Protein extracts were prepared from three biological replicates for each substrate. Three parallel gels for each protein extract were analyzed to account for technical variations in proteomic analysis.

3.2.5 Protein identification by ESI/LC-MS/MS

For identification of proteins from the SDS-PAGE of benzene-, phenol-, and benzoate-grown cells, the complete lane was cut into 10 slices, washed with H_2O for 30 min, reduced with 5 mM DTT (15 min, 20°C), and acetylated with 25 mM iodacetamide (15 min, 20 °C). Then, the gel slices were washed twice with 40% acetonitrile and once for 5 min with 100% acetonitrile. Subsequently, proteins were digested with trypsin (0.03 µg µL^{-1} of 50 mM ammonium bicarbonate) overnight at 37°C. The digested peptides were separated by reversed phase chromatography (PepMap, 75 µm id× 250 mm, LC Packings) operated on a nano-

HPLC (Ultimate 3000, Dionex) with a nonlinear 170 min gradient using 2% acetonitrile and 0.1% formic acid in water (A) and 0.1% formic acid in 98% acetonitrile (B) as eluents with a flow rate of 250 nL min^{-1}. The gradient settings were subsequently: 0-140 min: 2-30% B, 140-150 min: 30-60% B, 150-160 min: 60-99% B, 160-170 min: stay at 99% B. The nano-LC was connected to a linear quadrupole ion trap-Orbitrap (LTQ Orbitrap) mass spectrometer (ThermoElectron, Bremen, Germany) equipped with a nano-ESI source. The mass spectrometer was operated in the data-dependent mode to automatically switch between Orbitrap-MS and LTQ-MS/MS acquisition. Survey full scan MS spectra (from m/z 300 to 1500) were acquired in the Orbitrap with resolution R = 60,000 at m/z 400 (after accumulation to a target of 1,000,000 charges in the LTQ). The method used allowed sequential isolation of the most intense ions, up to five, depending on signal intensity, for fragmentation on the linear ion trap using collisionally induced dissociation at a target value of 100,000 charges. Target ions already selected for MS/MS were dynamically excluded for 30 seconds. General mass spectrometry conditions were: electrospray voltage, 1.25-1.4 kV; no sheath and auxiliary gas flow. The ion selection threshold was 500 counts for MS/MS. Furthermore, an activation Q-value of 0.25 and activation time of 30 ms were applied for MS/MS. The resulted peptide MS/MS spectra were identified by Mascot search (www.matrixscience.com). Mascot was searched with a fragment ion mass tolerance of 1.00 Da and a parent ion tolerance of 10.0 PPM. Iodacetamide derivatives of cysteine were specified in Mascot as a fixed modification. Protein identification was carried out by blast of peptides fragments against NCBI-BlastP (http://blast.ncbi.nlm.nih.gov/Blast.cgi) or against the translated metagenome sequence of the iron-reducing culture BF in a pedant database (Munich information center for protein sequences) by using the Scaffold 2.02.03 software (Proteome Software Inc., Portland, OR). Protein identification was accepted if the probability was greater than 95% and when at least two peptides were identified. Protein probabilities were assigned by the Protein Prophet algorithm (Nesvizhskii*, et al.*, 2003).

3.2.6 N-terminal sequence analysis

A 60 kDa protein band specifically expressed in benzene-grown cells was transferred from SDS gels onto a PVDF membrane using a semi-dry blotting apparatus (Bio-RAD Trans-Blot SD) at 1 mA cm^{-2} for 2 h. The PVDF membrane was stained with Coomassie Brilliant Blue and washed with 50% methanol. Coomassie-stained proteins were excised from the PVDF membrane and collected fractions were subjected to N-terminal sequence analysis using a 492-protein sequencer (Applied Biosystems, Darmstadt, Germany) according to the

manufacturer's instructions. The obtained N-terminal sequence was used for protein identification by search against the translated metagenome sequence of the iron-reducing culture BF. The analysis was carried out with two separate biological replicates.

3.3 Results

3.3.1 Microbial community structure of the benzene-degrading enrichment cultures

To ensure that the microbial composition of the iron-reducing enrichment culture BF did not change when the culture was transferred from benzene to a different substrate, the community structure of benzene-, phenol-, and benzoate-grown cultures was assessed by T-RFLP analysis when approximately 25 mM of ferrous iron were produced (Fig. 3.2). With all substrates, the analysis showed the same dominance of the *Peptococcaceae*-related microorganisms forming a T-RF of 289 bp (Fig. 3.3a-c).

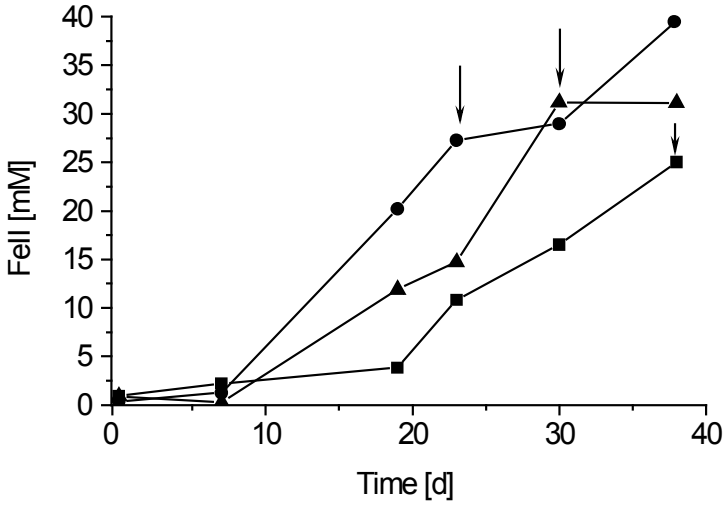

Figure 3.2. Formation of ferrous iron during growth of enrichment culture BF on benzene (■), phenol (●), and benzoate (▲). Arrows indicate the time points of sampling for proteomic analysis.

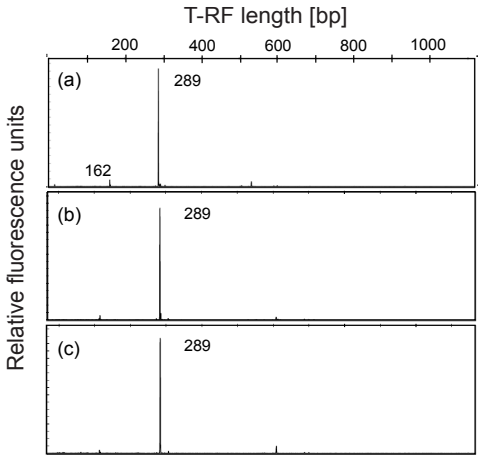

Figure 3.3. Terminal restriction fragment length polymorphism (T-RFLP) fingerprinting of the iron-reducing culture BF grown on (a) benzene, (b) phenol, and (c) benzoate. Numbers represent the length of major T-RFs in base pairs (bp).

3.3.2 The metagenome of the iron-reducing culture BF

The draft metagenomic sequences (10.13 Mb) of the iron-reducing culture BF contained 14270 open reading frames (ORFs) and 5832 contigs while the DNA sequence length of the contigs ranged between 0.064 - 537.470 Kb. Blastp search of the translated ORFs indicated that 21.3% of the total identified ORFs could be assigned with more than 50% identity to all genes with known functions in the NCBI non-redundant protein sequence database and around 40.7% of the total ORFs did not show any relevant identity to genes with known function. Details about general genomic features are summarized in Table A1.

About 205 genes were identified to have closest sequence similarity to genes encoding enzymes and transcriptional regulators known to be involved in anaerobic aromatic hydrocarbon degradation indicating the importance of aromatic compounds as carbon source for the growth of culture BF. Co-localized gene clusters were discovered for some aromatic hydrocarbon-degrading proteins. However, the majority of genes were scattered across 63 different contigs of the metagenome (Table A2). Moreover, around 90 genes were discovered to have sequence similarity to genes encoding proteins related to xenobiotics transporters e.g. ATP-binding cassette (ABC) and major facilitator superfamily MFS-1.

An interesting feature of the metagenome was the presence of about 112 ORFs similar to genes encoding phage-like proteins related to phage attachment and integration such as terminase, recombinase, integrase, and resolvase. In addition, 18 ORFs similar to genes encoding for transposable elements (e.g. transposase) indicating a potential mobility of the genetic elements within the metagenome.

3.3.3 Genes encoding proteins involved in anaerobic aromatic hydrocarbon metabolism in the metagenome of culture BF

Several ORFs with sequence similarity to genes encoding proteins of anaerobic degradation of toluene, phenol, 4-hydroxybenzoate, and benzoate were identified in the metagenome of the iron-reducing culture BF (Fig 3.4; Table A2).

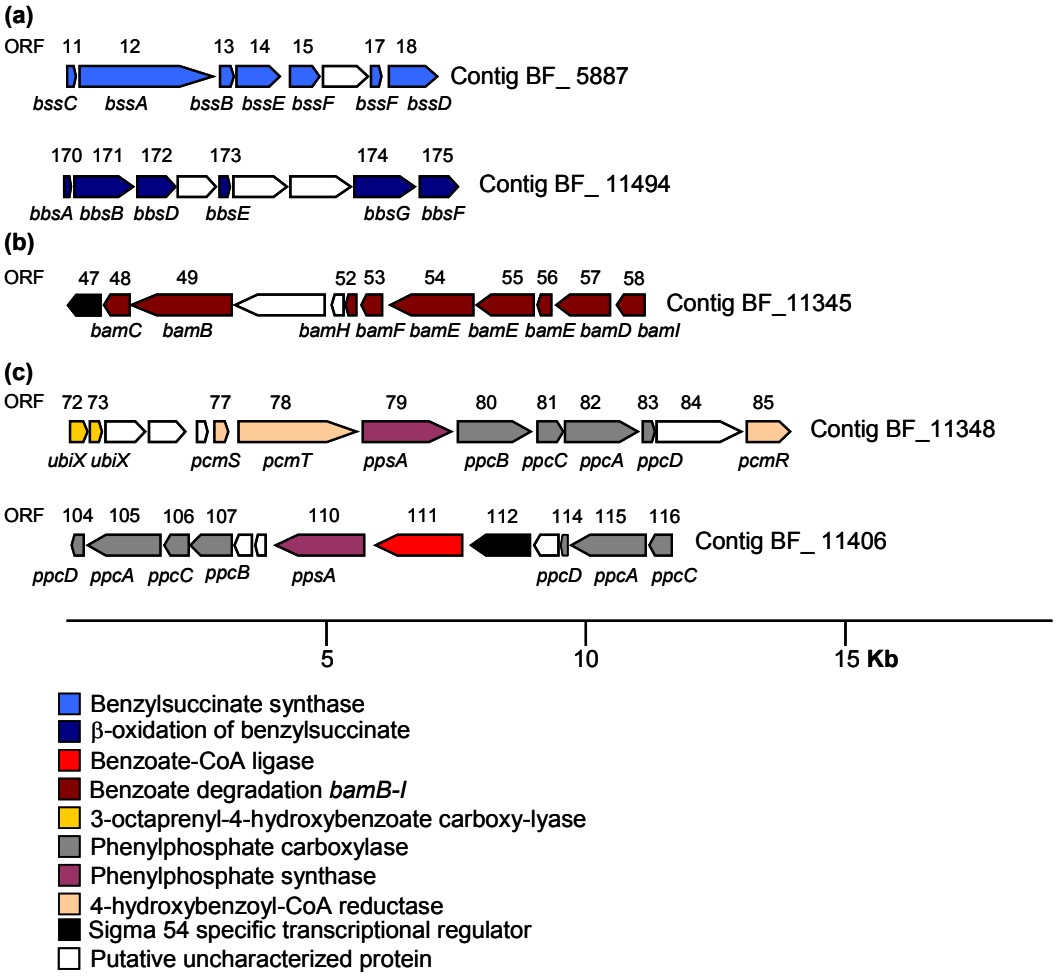

Figure 3.4. Putative genes identified in the metagenome of the iron-reducing culture BF that encode proteins of anaerobic aromatic hydrocarbon degradation: (a) toluene, (b) benzoate, and (c) phenol and 4-hydroxybenzoate. Predicted functions of ORFs were based on sequence similarities to known genes. The gene clusters were discovered on different contigs of the metagenome of culture BF (Table A2).

3.3.3.1 Toluene metabolism

ORFs (ORFs 11-13) similar to *bssCAB* toluene catabolic genes encoding benzylsuccinate synthase of *A. aromaticum* strain EbN1 (YP_158059 to YP_1580561) and *bssD* (ORF 18) encoding the activating enzyme were identified on one contig (BF_5902; Table A2) of the metagenome. These genes were part of a large toluene-like cluster which contained also ORFs

similar to *bssF* (ORFs 15 and 17) and *bssE* (ORF 14) encoding proteins with so far unknown functions in toluene degradation of *A. aromaticum* strain EbN1 (Fig 3.4a). In addition to the putative *bss* genes, a further contig (BF_11494; Table A2) containing 123 ORFs harboured putative *bbsCABEFD* genes whose products might be involved in the β-oxidation of benzylsuccinate (ORFs 170-175; Fig 3.4a).

3.3.3.2 Benzoate metabolism

ORFs homologous to putatively benzoate anaerobic metabolism (*bam*) genes that encode proteins involved in reductive dearomatization of benzoyl-CoA to a cyclic, conjugated diene during benzoate degradation in *G. metallireducens* (Wischgoll*, et al.*, 2005) were found to be scattered over 19 different contigs of the metagenome sequence (Table A2). A large contig with 561 ORFs (contig BF_11345) contained genes similar to *bamCBDEFHI* (ORFs 48-58; Fig 3.4b) encoding proteins responsible for benzoyl-CoA reduction in *G. metallireducens*. The predicted gene products showed a high sequence identity (> 60%) to the respective proteins described in *Geobacter* sp. FRC-32 (YP_002535701, YP_002535686, YP_002535687, YP_002535704, YP_0025-35705, and YP_002535691; Table A2). Furthermore, the metagenome of culture BF contained one gene cluster putatively encoding proteins catalyzing subsequent reactions in benzoate degradation. The formation of a hydrolytic ring cleavage product in *A. aromaticum* strain EbN1 involves the enoyl-CoA hydratase (BzdW), a short-chain alcohol dehydrogenase (BzdX), and the ring opening hydrolase (BzdY) which are probably encoded by ORFs 95-99 in culture BF (Table A2).

3.3.3.3 Phenol metabolism

ORFs coding for enzymes similar to phenylphosphate synthase and phenylphosphate carboxylase in *A. aromaticum* strain EbN1 were found in two different gene clusters located on separate contigs of the metagenome of culture BF (BF_11348 and 11406; Table A2). The respective contigs contained 72 and 60 ORFs, respectively. ORFs similar to the putative α-subunit of the phenylphosphate synthase (PpsA) were located in both clusters (ORFs 79 and 110; Fig. 3.4c) and contained the characteristic conserved His-522 residue which is the specific binding site for ATP. ORFs similar to genes coding for the phenylphosphate synthase β- and γ-subunits (PpsB and PpsC) were absent in both gene clusters. However, such sequences were identified on other contigs of the metagenome sequence (ORFs 36-37, 89, and 156; Table A2). Adjacent to ORFs 79 and 110 which are putatively encoding phenylphosphate synthase subunit A (PpsA), two clusters of ORFs were found respectively

that are similar to genes encoding phenylphosphate carboxylase *ppcBCAD* (ORFs 80-83 and 104-107; Fig 3.4c). The identified subunits of the putative phenylphosphate carboxylases showed more than 45% sequence identity to the respective proteins of *A. aromaticum* strain EbN1 (YP_158783 to YP_158786).

Degradation of 4-hydroxybenzoate as an intermediate compound of anaerobic phenol degradation requires 4-hydroxybenzoate-CoA ligase and 4-hydroxybenzoyl-CoA reductase producing benzoyl-CoA. No ORFs similar to genes encoding 4-hydroxybenzoate-CoA ligase could be identified in the shotgun metagenome of culture BF. However, ORFs similar to the gene coding for benzoate-CoA ligase (BamY) of *G. metallireducens* (YP_385097) were found within one cluster of putative phenol degradation genes mentioned above (ORF 111) and another gene cluster (ORFs 189-198) related to 4-hydroxybenzoate degradation (ORF 194). In general, amino acid sequences of aromatic CoA-ligases are very similar to each other and it is difficult to differentiate such ligases only based on the sequence (Butler*, et al.*, 2007, Peters*, et al.*, 2007). ORFs similar to genes *pcmRST* encoding three subunits of 4-hydroxybenzoyl-CoA reductase (ORFs 77-78, and 84; Fig 3.4c) were identified within one of the two putative phenol degradation gene clusters (contig BF_11348; Table A2) showing 73% (ORF 77: putative PcmS), 72% (ORF 78: putative PcmT) and 57% (ORF 85: putative PcmR) amino acid sequence identities to the respective proteins in *Geobacter* sp. strain FRC-32 (YP_002535582, YP_002535580, and YP_002535581). Moreover, like in *Geobacter* sp. strain FRC-32 the PcmR subunit of the putative 4-hydroxybenzoyl-CoA reductase lacks the insertion of 40 amino acids which carries the additional [4Fe-4S] cluster loop responsible for an inverted electron flow in facultative anaerobes. Additionally, several ORFs orthologous to 4-hydroxybenzoyl-CoA reductase encoding genes were discovered on 9 different contigs of the BF metagenome sequence (ORFs 35, 62, 63, 64, 103, 161, 162, 183, 186, 197, 198, 199, 201, 202, and 203, respectively; Table A2).

3.3.3.4 Putative benzene metabolism

ORFs coding for the putative subunits PpcD (ORF 137) and PpcA (ORF 138) of the phenylphosphate carboxylase were discovered on a 47 ORFs containing contig (BF_11418; Table A2). The two ORFs clustered together with genes encoding an UbiD/UbiX-like carboxylase (3-octaprenyl-4-hydroxybenzoate carboxy-lyase; ORFs 124, 133, and 140; Table A2). The predicted gene products of ORF 137 (14.7 kDa) and ORF 138 (57.4 kDa) displayed markedly reduced sequence identities (35% and 43%) to that of the other mentioned putative

ppcD- and *ppcA*-like genes (ORF 83 and ORF 82) of the putative phenol degradation gene cluster mentioned above. The adjacently located ORF 139 was very similar (56% sequence identity) to *bamY* coding for benzoate-CoA ligase in *G. metallireducens* (YP_385097). ORFs similar to phenylphosphate synthase genes *ppsABC* were not present in this gene cluster.

3.3.4 Differential comparative proteome analysis of benzene-, phenol-, and benzoate-grown cells under iron-reducing conditions

3.3.4.1 Identification of benzene-induced enzymes by N-terminal sequence of SDS-PAGE

Electrophoretic separation of benzene-, phenol-, and benzoate-expressed proteins revealed a very prominent protein band with a mass of about 60 kDa specifically expressed with benzene as growth substrate (Fig. 3.5). The N-terminal sequence of the first 31 amino acids of the protein in this band was determined by Edman sequencing and was identical to the amino acid sequence predicted from ORF 138 (Fig. 3.5; Table 3.l) The gene product of ORF 138 showed 43% sequence identity to genes coding for the α-subunit of phenylphosphate carboxylase (PpcA; Mw 57.4 kDa) in *A. aromaticum* strain EbN1 (YP_158784).

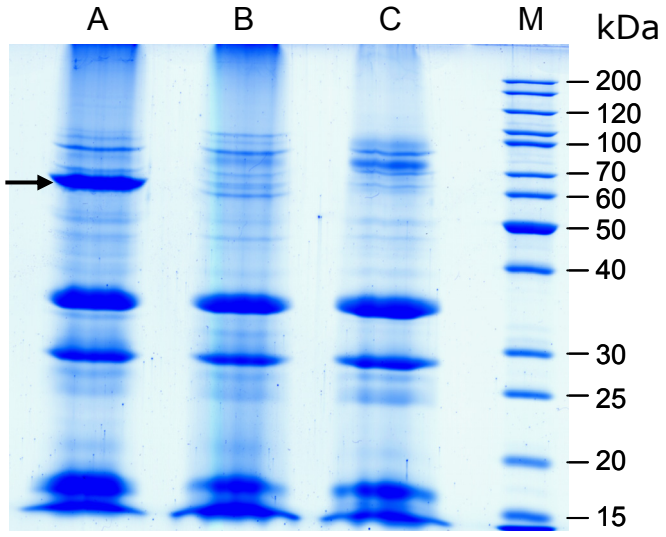

Figure 3.5. Coomassie-stained SDS-PAGE of proteins extracted from the iron-reducing culture BF grown on either benzene (lane A), phenol (lane B), or benzoate (lane C). The arrow indicates the specific benzene-induced protein of 60 kDa mass. Lane M represents the molecular mass standard.

3.3.4.2 Mass spectrometric analysis of proteins expressed in benzene-, phenol- and benzoate-grown cells

The soluble proteomes of benzene-, phenol-, and benzoate- grown cells of the iron-reducing culture BF were separated by SDS-PAGE and differentially identified by mass spectrometric analysis. In total, 4020 proteins were identified for all tested substrates. 276 proteins were exclusively expressed in benzene-grown cells, 28 of which could be assigned to aromatic hydrocarbon degradation pathways. Two proteins were considered as putative enzymes involved in initial activation of anaerobic benzene degradation (Table 3.1). In addition, proteomes of phenol-grown cells revealed the exclusive expression of 90 proteins, 6 of which were similar to proteins involved in phenol degradation (Table 3.1). Further 200 proteins were identified to be specific for benzoate-grown cells, 14 of which were similar to putative Bam and Bzd proteins involved in the anaerobic degradation of benzoate (Table 3.1).

Toluene metabolism

Although genes were identified in the metagenome that might encode the toluene degradation enzymes benzylsuccinate synthase (BssCABD; ORFs 11-18) and enzymes involved in the subsequent β-oxidation of benzylsuccinate (BbsCABEFD; ORFs 170-175), gene products could neither be identified from protein extracts of benzene-, nor phenol-, or benzoate grown cells.

Benzoate specific expression

In benzoate-grown cells different putatively *bam*-related ORFs probably encoding benzoate-CoA ligase and benzoyl-CoA reductase (ORFs 6, 53, 56, 68, 86, 101, 152, 155, 157, 159, 168, 178, and 194; Table 3.1) were expressed. The expressed *bam*-like genes were located on different contigs of the metagenome sequence. Some of these genes were also expressed in benzene- and phenol-grown cells (ORFs 33, 56, 86, 139, 155, 168, 178, and 194). In addition, specific genes probably encoding enzymes involved in the lower, ring-opening pathway of anaerobic benzoate degradation were found to be expressed in the culture BF. *bzdY*- and *bzdW*-like genes (ORFs 95-97) encoding a putative enoyl-CoA hydratase and ring-opening hydrolase were expressed in benzoate-grown cells, whereas the later gene (ORF 97) was induced with all substrates.

Table 3.1. Benzene-, phenol-, and benzoate-expressed ORFs of cultures BF and BPL related to known genes involved in anaerobic aromatic hydrocarbon degradation.

		Expressed proteins[a]						Related gene product (BLASTP annotation)[c]					
				MS/MS peptides identification[b]									
Gene product	Contig no.	Length (aa)	Size (kDa)	Benzene (BF)	Phenol (BF)	Benzoate (BF)	Benzene (BPL)	Gene	Protein	E-value	Identity (%)	Accession no.	Organism[d]
ORF 6	BF_5887	170	18.8	–	–	2 (24)	–	*bamM*	Acetyl-CoA dehydrogenase domain protein	3.00E-67	72	YP_517949	Deshd
ORF 10	BF_5887	185	21.7	2 (7)	–	–	–		Acetophenone carboxylase, gamma subunit	1.00E-38	50	ZP_03494850	Aliac
ORF 25	BF_5966	199	21.8	2 (5)	2 (5)	–	–		Probable UbiX-like carboxylase	2.00E-72	65	YP_158781	Azose
ORF 29	BF_5978	479	53.2	–	2 (6)	2 (5)	–		Phenylphosphate carboxylase, alpha subunit	4.00E-177	64	YP_158784	Azose
ORF 33	BF_5983	397	44.3	–	2 (10)	–	–	*bamI*	Formate dehydrogenase, alpha subunit	1.00E-130	61	ZP_04353073	N.A.
ORF 34	BF_6004	289	30.9	13 (50)	–	2 (11)	–	*pcmR*	Molybdopterin dehydrogenase, FAD-binding subunit	1.00E-84	58	YP_002535583	Geosf
ORF 35	BF_6004	114	12.2	2 (18)	–	–	–	*pcmS*	(2Fe-2S)-binding domain protein	9.00E-44	71	YP_385089	Geomg
ORF 53	BF_11345	143	15.9	–	–	2 (5)	–	*bamF*	Methyl-viologen-reducing hydrogenase, delta subunit	2.00E-45	56	YP_002535705	Geosf
ORF 56	BF_11345	76	8	–	2(5)	2 (5)		*bamE*	4Fe-4S ferredoxin, iron-sulfur binding protein	2.00E-26	85	YP_461442	Synas
ORF 68	BF_11345	259	27.6	–	–	2 (8)	–	*bamA*	Enoyl-CoA hydratase	3.00E-80	56	ZP_03557383	N.A.
ORF 70	BF_11345	326	37.6	2 (8)	–	–	–		Benzoyl-CoA reductase/2-hydroxyglutaryl-CoA dehydratase	1.00E-132	67	YP_001211338	Pelts
ORF 77	BF_11348	95	10.1	–	2 (16)	2 (16)	–	*pcmS*	(2Fe-2S)-binding domian protein	6.00E-33	73	YP_002535582	Geosf
ORF 83	BF_11348	82	9.4	–	2 (28)	–		*ppcD*	Phenylphosphate carboxylase, delta subunit	2.00E-14	44	YP_158783	Azose
ORF 86	BF_11348	652	74.2	2 (5)	–	2 (5)	–	*bamB*	Aldehyde ferredoxin oxidoreductase	0.00E+00	73	YP_002535685	Geosf
ORF 87	BF_11352	514	57	–	–	–	2 (5)	*bamY*	Benzoate-CoA ligase	2.00E-157	54	YP_385097	Geomg
ORF 90	BF_11370	587	66.8	–	2 (15)	2 (10)	–		UbiD-like carboxylase	0.00E+00	58	ZP_02849982	Dprot
ORF 95	BF_11393	376	42.4	–	–	2 (9)	–	*bzdY*	6-oxocyclohex-1-ene-1-carbonyl-CoA hydratase	3.00E-148	66	AAQ08805	Azoev
ORF 96	BF_11393	171	18.5	5 (43)	2 (12)	2 (44)	–	*bzdW*	Putative dienoyl-CoA hydratase	3.00E-42	51	CAD21636	Azoev
ORF 97	BF_11393	70	8	2 (36)	–	2 (36)	–	*bzdW*	Dienoyl-CoA hydratase	7.00E-03	35	CAD21628	Azoev
ORF 98	BF_11393	183	20.1	2 (15)	–	–	–		6-hydroxycylohex-1-ene-1-carboxyl-CoA dehydrogenase	4.00E-51	61	CAI78830	**N.A.**
ORF 99	BF_11393	138	15.2	2 (18)	–	–	–		6-hydroxycylohex-1-ene-1-carboxyl-CoA dehydrogenase	4.00E-32	45	CAI78830	**N.A.**
ORF 101	BF_11393	385	42	7 (22)	–	4 (14)	–	*bamM*	Acyl-CoA dehydrogenase domain protein	1.00E-160	69	ZP_03023273	Geosf
ORF 115	BF_11406	481	53.2	–	2 (5)	–		*ppcA*	Phenylphosphate carboxylase, alpha subunit	4.00E-160	59	YP_158784	Azose
ORF 117	BF_11411	199	21.7	2 (18)	–	–	–		3-polyprenyl-4-hydroxybenzoate decarboxy-lyase	8.00E-67	64	YP_160255	Azose

ORF 120	BF_11415	302	33.2	2 (9)	–	–	–		Probable UbiD-like carboxylase	6.00E-52	41	YP_158782	Azose
ORF 121	BF_11416	421	47.4	18 (38)	–	–	–	*ppcA*	Phenylphosphate carboxylase, alpha subunit	1.00E-45	31	YP_158784	**Azose**
ORF 122	BF_11416	206	23	5 (30)	–	–	–		UbiD (3-polyprenyl-4-hydroxybenzoate decarboxy-lyase)	1.00E-19	31	ZP_04376160	**N.A.**
ORF 123	BF_11416	286	32.5	7 (23)	–	–	–	*ppcA*	Phenylphosphate carboxylase, alpha subunit	7.00E-24	30	YP_158784	Azose
ORF 137	BF_11418	126	14.7	13 (85)	–	9 (48)	2 (5)	*ppcD*	Phenylphosphate carboxylase, delta subunit	2.00E-02	37	YP_158783	**Azose**
ORF 138	**BF_11418**	**508**	**57.4**	**30 (85)**	**–**	**15 (43)**	**2 (5)**	***ppcA***	**Phenylphosphate carboxylase, alpha subunit**	**2.00E-106**	**43**	**YP_158784**	**Azose**
ORF 139	BF_11418	541	60,7	7 (12)	–	–	–	*bamY*	Benzoate-CoA ligase	1.00E-172	56	YP_385097	Geomg
ORF 140	BF_11418	153	16.8	5 (29)	–	–	–		Probable UbiX-like carboxylase	2.00E-52	68	P57767	Thaar
ORF 152	BF_11447	102	11.5	–	–	2 (8)	–	*bamF*	Methyl-viologen-reducing hydrogenase, delta subunit	5.00E-09	50	YP_002535689	Geosf
ORF 155	BF_11452	654	74.3	17 (29)	–	8 (7)	–	*bamB*	Aldehyde ferredoxin oxidoreductase	0.00E+00	73	YP_002535685	Geosf
ORF 157	BF_11452	184	20.7	–	–	4 (30)	–	*bamC*	4Fe-4S ferredoxin, iron-sulfur binding protein	1.00E-54	57	YP_384757	Geomg
ORF 159	BF_11460	637	72.6	–	–	2 (10)	–	*bamB*	Aldehyde ferredoxin oxidoreductase	0.00E+00	70	YP_002535685	Geosf
ORF 161	BF_11460	289	30.9	–	–	5 (10)	–	*pcmR*	Molybdopterin dehydrogenase, FAD-binding subunit	1.00E-54	57	YP_384757	Geomg
ORF 163	BF_11460	405	45.2	2 (7)	–	–	–	*bamM*	Acyl-CoA dehydrogenase domain protein	1.00E-118	51	ZP_04150428	Bac11
ORF 168	BF_11492_	365	40.5	10 (32)	–	25 (31)	–	*bamI*	4Fe-4S ferredoxin, iron-sulfur binding protein	8.00E-38	29	YP_002430370	Geomg
ORF 178	BF_11515	732	82	22 (20)	–	25 (31)	–	*bamF*	Acetyl-CoA decarbonylase, beta subunit	0.00E+00	74	YP_360060	Carhz
ORF 184	BF_11550	339	36.6	–	2 (12)	–	–	*hbaD*	4-hydroxybenzoyl-CoA reductase, beta subunit	1.00E-47	37	NP_946025	Rhop2
ORF 186	BF_11550	543	58.7	–	–	–	4 (8)	*pcmT*	4-hydroxybenzoyl-CoA reductase, alpha subunit	3.00E-109	40	YP_385090	Geomg
ORF 194	BF_11554	521	57.7	10 (20)	–	2 (5)	–	*bamY*	Benzoate-CoA ligase family	7.00E-168	55	YP_002535674	Geosf
ORF 197	BF_11554	774	85	18 (32)	–	9 (12)	–	*pcmT*	4-hydroxybenzoyl-CoA reductase, alpha subunit	0.00E+00	77	YP_002535581	Geosf
ORF 199	BF_11555	167	17.8	2 (14)	–	–	–	*pcmS*	4-hydroxybenzoyl-CoA reductase, gamma subunit	2.00E-67	70	ZP_03023308	Geosf
ORF 201	BF_11556	773	84.9	2 (5)	–	–	–	*pcmT*	4-hydroxybenzoyl-CoA reductase, alpha subunit	0.00E+00	76	YP_002535581	Geosf

The specifically benzene-expressed protein of 60 kDa identified by the direct N-terminal sequencing from the SDS-gels is depicted in bold.

[a]aa, is the number amino acids; kDa, is the size of proteins in kiloDalton.

[b]Numbers without parenthesis indicate unique peptides. Numbers with parenthesis give the percentage of coverage (%) of the amino acid sequences of the identified proteins by the detected peptides. (BF), iron-reducing enrichment culture; and (BPL), sulfate reducing-enrichment culture.

[c]Hits were obtained from BLASTP comparison of predicted proteins with NCBI data bank (http://blast.ncbi.nlm.nih.gov/Blast.cgi). The E-value, gives expectation value.
Identity, gives the percentage of amino acids that are identical between the ORF and the next related protein in the data bank. Acc. no., the accession number from NCBI database.

[d]Species names are abbreviated according to the list of organism identification code (SWISS-PROT): Azose, *Aromatoleum aromaticum* strain EbN1; Pelts, *Pelotomaculum thermopropionicum* strain S1; Deshd, *Desulfitobacterium hafniense*; Geosf, *Geobacter* sp. strain FRC-32; Geomg, *Geobacter metallireducens* strain GS-15; Aliac, *Alicyclobacillus acidocaldarius*; Dprot, *Delta-proteobacterium;* Azoev, *Azoarcus evansii;* Carhz, C*arboxydothermus hydrogenoformans*; Rhop2, *Rhodopseudomonas palustri*; Synas, *Syntrophus aciditrophicus*; Thaar, *Thauera aromatica*; Bac11, *Bacillus* sp.; N.A, Name of the organism is not available in SWISS-PROT.

Phenol specific expression

The majority of proteins expressed in phenol grown-cells were related to phenylphosphate carboxylase (Ppc; ORFs 29, 83, and 115), 4-hydroxybenzoyl-CoA reductase (Hba/Pcm; ORFs 77 and 184), and 3-octaprenyl-4-hydroxybenzoate carboxy-lyase (UbiD/UbiX; ORF 90) enzymes. In addition, ORFs 77 and 184 encoding PcmS- and HbaD-like proteins were expressed. Noteworthy, ORFs 77, 83, and 115 were located in the putative phenol-degrading cluster that is described in Fig 3.4c.

Benzene specific expression

Benzene-grown cells specifically expressed phenylphosphate carboxylase-like proteins such as PpcA (ORFs 121-123, and 138), and PpcD (ORF 137). The mass spectrometric analysis of proteins in the SDS gels revealed peptides (Mw 57.4 kDa) that could be correlated to the gene product of ORF 138. Whereas no expression of ORF 138 could be detected in protein extracts from phenol-grown cultures, ORF 138 was found to be expressed as well with benzoate as growth substrate. Furthermore, a product of ORF 139 (Mw 60.7 kDa, Table 3.1) which is specifically expressed during growth on benzene encodes a gene product similar with 56% sequence identity to benzoate-CoA ligase of *G. metallireducens* (BamY). We name the product of ORF 139 BzlA for benzoate-CoA ligase enzyme of the culture BF(Fig. 3.6a). Noteworthy, the products of ORF 139 could not be identified by N-terminal sequencing of the 60 kDa band due to the predominance of protein products of ORF 138.

Other proteins specifically expressed on benzene were UbiD/UbiX-like carboxylase (3-octaprenyl-4-hydroxybenzoate carboxy-lyase; ORF 140), and a MRP family ATP-binding protein-homologue (multi drug resistance protein; ORFs 127 and 136) (Table 3.1, Fig 3.6a). Moreover, proteins similar to 4-hydroxybenzoyl-CoA reductase subunits (PcmRST; ORFs 34, 35, 197, 199, and 201) were identified from benzene-grown cells (Table 3.1). ORFs 34 and 197 were also expressed in benzoate but not in phenol-grown cells.

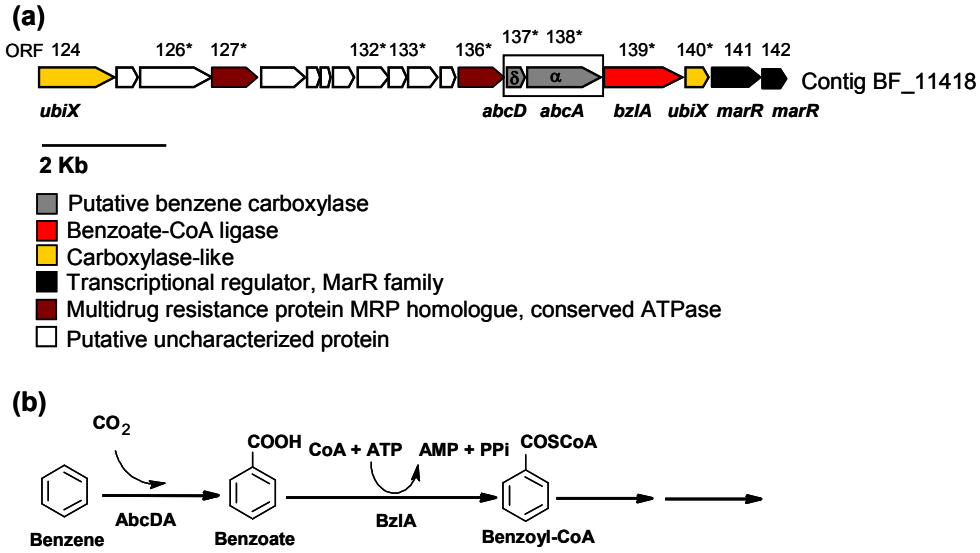

Figure 3.6. Putative benzene degradation cluster of the iron-reducing culture BF. (a) Genes that encode proteins expressed during anaerobic benzene degradation are indicated with stars. Putative genes involved in the initial activation of benzene are shown in a rectangle box. Predicted functions of ORFs are based on sequence similarities to known genes. (b) Proposed roles of predicted genes in the initial activation of anaerobic benzene degradation to benzoate and benzoyl-CoA. The carboxylation step catalyzed by anaerobic benzene carboxylase AbcDA might involve further, not specified gene products, whereas benzoate-ligation step catalyzed by benzoate-CoA ligase BzlA.

3.3.4.3 Proteomic analysis of the sulfate-reducing benzene-grown culture BPL

Metaproteomic analysis was also performed with protein extracts from the sulfate-reducing culture BPL which is able to grow on benzene but not phenol or benzoate as carbon source (Abu Laban*, et al.*, 2009). Peptides of LC/MS-MS analysis were compared against the translated metagenome sequences of the iron-reducing culture BF and the NCBI-blastp data bank. Putative proteins related to phenylphosphate carboxylase (PpcA; ORF 138, and PpcD; ORF 137), benzoate-CoA ligase (BzlA; ORF 87) and 4-hydroxybenzoyl-CoA reductase (PcmT; ORF 186) were expressed in the enrichment culture BPL (Table 3.1). All ORFs mentioned above refer to the annotated metagenome of culture BF. However, no other genes related to aromatic compounds degradation could be identified from the blastp search.

3.4 Discussion

3.4.1 Community structures of the benzene-degrading culture BF with different growth substrates

In the present study, we investigated the anaerobic benzene degradation pathways of the iron- and sulfate-reducing enrichment cultures BF and BPL isolated from soil at a former

manufactured gas plant site. The highly enriched iron-reducing culture BF was dominated by Gram-positive *Peptococcaceae*-related microorganisms. Besides benzene, the microorganisms were able to grow with phenol (1 mM), 4-hyroxybenzoate (1 mM), and benzoate (1 mM), but not with toluene (1 mM), ethylbenzene (1 mM), and xylene isomers (1 mM) as sole source of carbon (Kunapuli*, et al.*, 2007).

With the help of differential expression analysis we aimed at identifying proteins specifically expressed with benzene as growth substrate but not with putative metabolites of benzene degradation such as phenol or benzoate. By comparing the different expression profiles we expected benzoate degradation genes to be induced with all three substrates.

However, culture BF is not pure and a problem for proteome analysis might arise if different community members grew up when the culture is transferred from benzene to phenol or benzoate. Nevertheless, the T-RFLP analysis of cells grown on the respective substrates clearly showed that the community structure is identical during growth with benzene, phenol, and benzoate. This allowed a direct comparison of the expressed proteins of cells grown on the respective substrates.

3.4.2 Putative proteins involved in aromatic compound metabolism

3.4.2.1 Benzoate metabolism

Benzoate is most likely the central intermediate of all three possible options for anaerobic benzene degradation discussed above. In facultative anaerobes, benzoate degradation to benzoyl-CoA involved benzoate activation to benzoyl-CoA catalyzed by benzoate-CoA ligase (Schühle*, et al.*, 2003), and ring reduction of benzoate catalyzed by the key enzyme benzoyl-CoA reductase (Bcr) (Boll & Fuchs, 1995, Boll, 2005). A Bcr enzyme has been isolated and studied in the denitrifying *Thauera aromatica* (Boll & Fuchs, 1995). It is an ATP-dependent oxygen-sensitive enzyme with a γβαδ heterotetramere structure (Boll*, et al.*, 2000). In the strictly anaerobic organism *G. metallireducens*, the membrane protein complex BamBCDEFGI (BamB-I) was proposed to be responsible for benzoyl-CoA reduction. BamY was identified as the benzoate-CoA ligase (Wischgoll*, et al.*, 2005, Heintz*, et al.*, 2009).

In the BF metagenome, ORFs similar to the *bcrCBAD* genes of facultative anaerobes could not be identified. In contrast, gene products similar to BamE and BamF were identified in the proteome of benzoate-grown cells of culture BF. They were part of a large putative benzoate degradation gene cluster (*bamCBDEFHI*) consisting of ORFs 48-58. In addition, gene

products similar to BamY (ORF 194) BamB (ORFs 86 and 155), BamI (ORF 168), and BamF (ORF 178) were identified from both benzene and benzoate grown cells. The identification of bam-like proteins suggests that culture BF utilizes a benzoyl-CoA pathway which is analogous to the one proposed for strictly anaerobic microorganisms such as *G. metallireducens*.

3.4.2.2 Phenol metabolism

Anaerobic phenol catabolism by the denitrifying microorganisms *T. aromatica* and *A. aromaticum* strain EBN1, and the iron-reducing organism *G. metallireducens* has been described in detail (Breinig*, et al.*, 2000, Rabus*, et al.*, 2005, Schleinitz*, et al.*, 2009) and proceeds via phenylphosphate synthase (PpsABC), phenylphosphate carboxylase (PpcABCD), 4-hydroxybenzoate-CoA ligase (HcrL), and 4-hydroxybenzoyl-CoA reductase (HcrCAB/PcmRST) to benzoyl CoA. The mass spectrometric analysis of proteins specifically expressed in phenol-grown cells of culture BF showed two expressed enzymes correlated to the putative ORFs for anaerobic phenol metabolism identified on the metagenome: phenylphosphate carboxylase subunits PpcA (ORFs 29 and 115) and PpcD (ORF 83), and 4-hydroxybenzoyl-CoA reductase subunits PcmS (ORF 77) and HbaD (ORF 184). As ORFs 77, 83, and 115 were constituents of the putative phenol degradation clusters (Fig 3.4c), we conclude that these clusters are responsible for phenol degradation in culture BF. Unfortunately, the other phenol-expressed ORFs 29 and 184 are located on very short contigs and therefore putative information concerning the phenylphosphate synthase (PpsABC) and carboxylase (PpcBC) are not available.

3.4.2.3 Benzene metabolism

Three different pathways have been proposed for anaerobic benzene degradation including a direct methylation to toluene and subsequent degradation via the benzylsuccinate pathway to benzoyl-CoA (Ulrich*, et al.*, 2005), a hydroxylation of benzene to phenol and further degradation by the well known phenol pathway to benzoyl-CoA (Caldwell & Suflita, 2000, Chakraborty & Coates, 2005), and a direct carboxylation to benzoate and activation by CoA to benzoyl-CoA (Caldwell & Suflita, 2000, Phelps*, et al.*, 2001, Kunapuli*, et al.*, 2008, Musat & Widdel, 2008, Abu Laban*, et al.*, 2009). If benzene degradation would proceed via a direct methylation as the first activation reaction of benzene we would expect the benzoyl-CoA pathway enzymes to be expressed, the enzymes of the benzylsuccinate degradation pathway, plus an unknown methylase. If benzene would be activated by a direct hydroxylation to

phenol, one would expect the same phenol degradation enzymes expressed as in phenol grown cells plus some unknown hydroxylase enzyme. Finally, for a carboxylation as the first activation reaction of benzene, we would expect all benzoyl-CoA degradation enzymes to be expressed plus the initial carboxylase but no phenol degradation enzymes.

A) Methylation as a possible initial activation reaction of benzene

The metagenome of culture BF contained ORFs similar to the genes *bssCABD* coding for benzylsuccinate synthase and its activating enzyme in *A. aromaticum* strain EBN1. However, no gene products of *bssCABD* could be identified in benzene-grown cells of cultures BF and BPL. This strongly indicates that benzene is not degraded via toluene as an intermediate which would be the first product of a putative methylation reaction. Moreover, toluene was not utilized as growth substrate by neither of the two cultures BF and BPL (Kunapuli, *et al.*, 2008, Abu Laban, *et al.*, 2009). Recent studies of benzene degradation by other sulfate-reducing enrichment cultures also indicated that toluene could neither be used as a substrate nor as a cosubstrate with benzene (Ulrich, *et al.*, 2005, Musat, *et al.*, 2008, Oka, *et al.*, 2008). Furthermore, benzylsuccinate as the specific metabolite of toluene degradation was also neither detected in the iron-reducing culture BF nor in the sulfate-reducing culture BPL (Kunapuli, *et al.*, 2008, Abu Laban, *et al.*, 2009). Therefore, our proteomic data support the recent metabolite analyses that excluded the formation of toluene during benzene degradation and we therefore exclude methylation as an initial activation mechanism for the anaerobic benzene degradation in our iron- and sulfate-reducing cultures.

B) Hydroxylation as a possible initial activation reaction of benzene

The hydroxylation of benzene to phenol was previously proposed as an initial activation mechanism based on the identification of phenol as metabolite in culture supernatants (Caldwell & Suflita, 2000, Chakraborty & Coates, 2005). Our proteomic data revealed that the phenol degradation genes described above were not expressed in benzene-grown cells. This finding supports recent studies showing that phenol detected in benzene-degrading cultures was formed abiotically during sampling due to contact of reduced ferrous iron in the culture medium with oxygen (Kunapuli, *et al.*, 2008, Abu Laban, *et al.*, 2009).

A theoretical possibility for the hydroxylation of benzene could be by xanthine-oxidase-like proteins (Enroth, *et al.*, 2000) that showed high sequence identity to 4-hydroxybenzoyl-CoA reductase PcmRST subunits of *Geobacter* sp. strain FRC-32 (YP_002535581-YP_002535583) (ORF 34, ORF 35, ORF 197, ORF 199 and ORF 201). PcmRST of *G.*

metallireducens is a special protein belonging to the xanthine-oxidase family of molybdenum cofactor containing enzymes that catalyze the irreversible reductive dehydroxylation of 4-hydroxybenzoyl-CoA yielding benzoyl-CoA and water (Peters*, et al.*, 2007). It is very unlikely that such an enzyme could catalyze the direct hydroxylation of benzene to phenol which would formally be a reversal of the dehydroxylation reaction. Nevertheless, if the gene products of *pcmRST* catalyze the 4-hydroxybenzoyl-CoA reduction, their expression in benzene-grown cells would indicate an active phenol degradation pathway while the initial hydroxylation of benzene to phenol would be performed by other enzymes. However, the *pcmT*-like gene product similar to ORF 186 in culture BF was also induced in the benzene-degrading, sulfate-reducing culture BPL that lacks the ability to grow on phenol or 4-hydroxybenzoate. Furthermore, the *pcmRST*-like ORFs in culture BF were unspecifically expressed with all three growth substrates. This finding was in accordance with the results of other studies showing that *pcmRST* genes were constitutively expressed with all tested aromatic substrates utilized by G. *metallireducens*. On the other hand, 4-hydroxybenzoyl-CoA reductase activity could only be measured with cells grown on *p*-cresol and 4-hydroxybenzoate as substrates, Therefore, the authors suggested a posttranscriptional regulation of the *PcmRST* enzyme activity (Peters*, et al.*, 2007). These observations might indicate that the expressed gene products of ORFs 34, 35, 197, 199, and 201 in benzene-grown cells of culture BF which are similar to PcmRST might not necessarily be active.

Our data provide no direct indications that the expressed *pcmRST*-like genes are involved in phenol degradation in the cultures BF and BPL. At present we can therefore not propose a clear function for the expressed *pcm*-like genes in benzene-grown cells. Together with the lack of proteomic indications that benzene might be hydroxylated and further processed via a phenol degradation pathway we do not support a direct hydroxylation of benzene as the initial activation mechanism.

C) Carboxylation as a possible initial activation reaction of benzene

The most remarkable polypeptide specifically induced in benzene-grown cells was identified by N-terminal sequencing of a very prominent 60 kDa band of the SDS-gels which was expressed with benzene but not with phenol as growth substrate (Fig. 3.5). The corresponding ORF 138 in the metagenome of culture BF showed best similarity to phenylphosphate carboxylase subunit A (PpcA) of *A. aromaticum* strain EbN1, in the NCBI data bank search. As this band was strongly expressed only during growth on benzene and not

on phenol, the protein is probably not involved in phenol metabolism. We rather propose that this enzyme is involved in the direct carboxylation of benzene.

The proteomic study of the SDS-gels revealed a number of other gene products only expressed during growth on benzene which all clustered together on the metagenome of culture BF (Fig. 3.6a). ORF 137 was located adjacent to ORF 138 on the metagenome and is similar to the phenylphosphate carboxylase subunit D (PpcD). Although the two ORFs were similar to phenol carboxylase genes, the gene cluster containing ORFs 137 and 138 did not contain ORFs similar to *ppcB* and *ppcC* needed for phenol metabolism or ORFs similar to *ppsABC* genes encoding for phenylphosphate synthase (PpsABC). It is therefore unlikely that this gene cluster is involved in anaerobic phenol metabolism. As the next ORF in the cluster, ORF 139 is similar to the *bamY* gene of *G. metallireducens* (YP_385097) coding for a benzoate-CoA ligase (56% sequence identity) and is specifically expressed with benzene as growth substrate, we propose that the benzene-induced gene product of ORF 139 constitutes a benzoate-CoA ligase (BzlA) channeling the product of benzene carboxylation, benzoate, into the benzoyl-CoA reduction pathway. ORFs 140 and 133 are similar to genes encoding UbiX (3-octaprenyl-4-hydroxybenzoate carboxy-lyase) and are also expressed with benzene. Furthermore, ATPase-dependent transporter proteins of the multidrug resistance protein family MRP are expressed which might possibly be involved in transport of benzene (ORFs 127 and 136). Further expressed proteins are not similar to proteins with known function (ORFs 126 and 132).

The location in one gene cluster and the simultaneous expression of ORFs 126-140, all related to either carboxylation or transport, together with the specific benzoate-CoA ligase (BzlA) strongly suggests that we have identified the best candidates for genes encoding an enzyme which is directly carboxylating benzene to benzoate.

Based on the genomic and proteomic data discussed above we propose a direct carboxylation of benzene to benzoate as the initial activation mechanism in anaerobic benzene degradation (Fig 3.6b). We name this enzyme anaerobic benzene carboxylase (Abc) which most likely belongs to a carboxylase family including phenylphosphate carboxylase and UbiX (3-octaprenyl-4-hydroxybenzoate carboxy-lyase). Abc probably consists of several subunits two of which are encoded by ORFs 137 and 138 (Fig 3.6b). The direct association of the genes to the different subunits and functions of the enzyme has to be solved by measuring the enzyme reaction and subsequent purification of anaerobic benzene carboxylase. Nevertheless,

the anaerobic carboxylation of benzene constitutes a novel enzyme reaction which is unprecedented in biochemistry. A parallel in chemistry would be a Friedl-Crafts acylation of aromatic compounds but it is out of the scope of such a study to speculate about possible reaction mechanisms.

3.5 References

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4 Two-dimensional stable isotope fractionation analysis to probe for the anaerobic activation mechanism of the unsubstituted hydrocarbon benzene

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4.1 Introduction

Over the last decade the understanding of anaerobic aromatic biodegradation has increased greatly. However, degradation of monoaromatic and polycyclic aromatic hydrocarbons like benzene, naphthalene, anthracene, and phenanthrene is considered to be problematic in nature due to the high stability of the ring structure that makes it difficult to cleave C-H bonds in the absence of oxygen as electron acceptor. In fact, until recently, anaerobic biodegradation of these compounds was thought unlikely due to the lack of activating ring constituents such as the methyl group in toluene (Evans & Fuchs, 1988). However, recent work has shown that mono- and poly-aromatic unsubstituted hydrocarbons can be degraded under nitrate-reducing (Mihelcic & Luthy, 1988, Burland & Edwards, 1999, Rockne*, et al.*, 2000), iron-reducing (Anderson*, et al.*, 1998, Anderson & Lovley, 1999, Caldwell*, et al.*, 1999), sulfate-reducing (Lovley*, et al.*, 1995, Phelps*, et al.*, 1996, Zhang & Young, 1997, Galushko*, et al.*, 1999, Anderson & Lovley, 2000, Meckenstock*, et al.*, 2000, Musat*, et al.*, 2008, Musat & Widdel, 2008), and methanogenic conditions (Grbic-Galic & Vogel, 1987, Weiner & Lovley, 1998). Based on the detection of different signature metabolites in these studies, several mechanisms for the initial activation of unsubstituted aromatic compounds have been proposed. Hydroxylation of compounds like benzene to phenol by nitrate-reducing bacteria (Caldwell & Suflita, 2000, Chakraborty & Coates, 2005) has been reported, a direct carboxylation of naphthalene to 2-naphthoic acid (Zhang & Young, 1997) and benzene to benzoate (Caldwell & Suflita, 2000, Abu Laban*, et al.*, 2009) has been observed with enriched sulfate-reducing cultures. Further experiments indicated that benzene in nitrate-reducing cultures and naphthalene in sulfate-reducing cultures are rather methylated to toluene (Ulrich*, et al.*, 2005, Mancini*, et al.*, 2008) and 2-methylnaphthalene (Safinowski & Meckenstock, 2006), while the intermediates are further degraded by a fumarate-addition reaction analogous to that of anaerobic toluene degradation (Kube*, et al.*, 2004). Despite the evidence of certain

metabolites given by these studies, the initial anaerobic activation mechanism of the unsubstituted aromatic compounds under various electron acceptors remains still unclear.

In recent years Compound Specific Isotope Analysis (CSIA) has become a new innovative approach to assess the natural attenuation of organic pollutants in the environment (Sherwood Lollar*, et al.*, 2001, Abe & Hunkeler, 2006) and to identify degradation pathways of organic contaminants (Elsner*, et al.*, 2005, Zwank*, et al.*, 2005). Compound-specific stable isotope analysis (CSIA) allows determination of the isotope composition of single organic substances in environmental samples.With CSIA, the natural abundance of the heavy (^{h}E) and the light (^{l}E) isotopes of a given element E within a given compound is determined, as expressed by the ratio $R = {}^{h}E/{}^{l}E$. Such bulk isotope ratios are commonly measured by gas chromatography-isotope ratio mass spectrometry (GC-IRMS) and are reported as difference in per mil $\delta^{h}E$ with respect to an international reference standard equation (4.1) (Clark & Fritz, 1997).

$$\delta^{h}E = \left(\frac{R_{Sample} - R_{Ref}}{R_{Ref}} \right) \bullet 1000‰ \qquad (4.1)$$

With this approach transformation reactions can be traced by the isotopic shift in the remaining substrate without the need of metabolite detection. The amount of isotope fractionation can be expressed by the isotope enrichment factor ε, and can be described by the Rayleigh equation (4.2).

$$\ln \frac{R_t}{R_0} = \ln \left(\frac{1 + \delta^{h} E_t}{1 + \delta^{h} E_0} \right) = \varepsilon \cdot \ln f \qquad (4.2)$$

where R_t and R_0 are the compound-specific isotope ratios of heavy versus light isotopes at a given time and at the beginning of the reaction. $\delta^{h}E_t$ and $\delta^{h}E_0$ are the isotopic signatures of the compound for the element E at times t and zero, respectively, while C_t/C_0 is the fraction f of the remaining compound. The bulk isotope enrichment factor ε is a measure for the isotopic enrichment as average over all positions in a molecule. More specifically, the extent of the isotope fractionation depends on the kinetic isotope effect (KIE) of the underlying (bio)chemical reaction. The KIE is caused by a different strength of bonds containing lighter and heavier isotope, respectively (Elsner*, et al.*, 2005). Since different transformation pathways typically involve different bonds with different elements and in different types of chemical reactions, the observable isotope fractionation can be linked to the underlying transformation pathway (Schmidt*, et al.*, 2004, Elsner*, et al.*, 2005)

One way to accomplish this is by consideration of the underlying isotope effects. However, even the same transformation pathways may not always show the same isotope fractionation (Nijenhuis*, et al.*, 2005, Vogt*, et al.*, 2008) One reason is that biotic transformation processes involve preceding steps such as uptake, transport to reactive sites or formation of enzyme-substrate complexes (Northrop, 1981). In particular, if the reverse step of the preceding process is slow, every substrate molecule that reaches the reactive site will be converted, irrespective of its isotopic composition (Elsner*, et al.*, 2005). The observed isotopic fractionation will then not correspond to the intrinsic kinetic isotope effect of the chemical transformation and the KIE is masked. Fortunately, slopes in dual isotope plots (i.e., diagrams plotting changes in isotope ratios of one element relative to another) are much less affected than the isotope effects, since KIE of both elements are masked to the same degree. Recent work therefore showed that two-dimensional isotope plots provide a particularly robust handle to differentiate between different reaction pathways (Elsner*, et al.*, 2007).

In previous studies, dual-isotope analysis for carbon and hydrogen isotopes has been applied to aerobic and anaerobic degradation of benzene using different cultures and electron acceptors (Mancini*, et al.*, 2003, Fischer*, et al.*, 2007, Fischer*, et al.*, 2008, Mancini*, et al.*, 2008). Under aerobic conditions, benzene biodegradation can be initiated by reactions involving monoxygenases and dioxygenases, which break a C-C bond so that a primary carbon isotope effect is expected (Fischer*, et al.*, 2007, Fischer*, et al.*, 2008). In contrast, anaerobic benzene degradation has been observed to lead to pronounced shifts in the hydrogen isotopic composition which are indicative of C-H bond cleavage (Mancini*, et al.*, 2003, Fischer*, et al.*, 2008, Mancini*, et al.*, 2008). Consequently, carbon and hydrogen isotope fractionation investigations hold potential as indicators to probe for the initial activation pathways of benzene. As a general pattern, it was found that methanogenic and sulfate reducers showed a consistently higher slope (Λ = 22-28) compared to nitrate-reducing cultures (Λ = 12-16) (Mancini*, et al.*, 2008). Potentially, these different dual isotope slopes may provide a unique approach to distinguish different initial mechanisms of degradation, even if the actual reaction chemistry and the enzymes involved are not known yet.

In this study, we aimed at using two-dimensional isotope analysis to elucidate differences in the biodegradation pathway of the non-substituted aromatic compound benzene. We therefore used enrichment cultures of the Gram-positive bacteria family *Peptococcaceae* for the determination of hydrogen and carbon isotope fractionation during anaerobic benzene

degradation under sulfate- and - for the first time - also under iron-reducing conditions. The main focus was to link the obtained isotope data with the findings of recent metabolomic and proteomic analysis (Abu Laban*, et al.*, 2009).

4.2 Materials and methods

4.2.1 Enrichment of the benzene-degrading culture and growth conditions

Anaerobic degradation of benzene was carried out by enrichment cultures BF and BPL, which were cultivated as previously described (Kunapuli*, et al.*, 2007, Abu Laban*, et al.*, 2009). For stable isotope analysis cultivation was performed with 10% (v/v) inoculum using 1 L serum bottles, filled with 800 ml fresh mineral medium, 200 mL CO_2/N_2 anoxic gas (20:80 v/v), sealed with Viton rubber stoppers (Mag Technik, Dübendorf, Switzerland) and aluminum crimps. The media of the enrichment cultures BF and BPL were spiked with benzene (200 µM and 500 µM, respectively). Degradation experiments were carried out in three parallel bottles for each of the benzene-degrading active cultures BPL and BF and two parallel bottles containing the autoclaved control cultures. Control bottles with the bacterial inoculum were autoclaved three times on three consecutive days. All cultures bottles were incubated at 30°C in the dark.

4.2.2 Analytical methods

Benzene concentrations were analyzed from 3 mL fresh sample by gas chromatography GC-MS as described by Abu Laban *et al.* (2009). For the autoclaved controls, changes in the concentrations were in the range of the analytical uncertainty.

For benzene carbon and hydrogen isotope analysis, samples of 4-15 mL (depending on the benzene concentration) were periodically taken from active and autoclaved culture bottles, filled into Supelco vials with Teflon coated caps (Sigma-Aldrich) and stored at -20°C prior to analysis by gas chromatograph-isotope ratio mass spectrometry (GC-IRMS). The compound-specific isotope ratios were determined using a TRACE GC Ultra gas chromatograph (GC) (Thermo Fisher Scientific, Milan, Italy), which was coupled to a Finnigan™ MAT 253 isotope ratio mass spectrometer (IRMS) (Thermo Fisher Scientific, Bremen, Germany) via a Finnigan™ GC Combustion III Interface. For carbon isotope measurements the combustion oven was set to 940°C. For hydrogen isotope analysis a pyrolytic oven was operated at 1140°C. The gas chromatograph was equipped with a programmable temperature vaporizer (PTV) injector (Optic 3; ATAS GL International B.V., Veldhoven, Netherlands) with cryofocussing option (with liquid N_2). A purge and trap concentrator Tekmar VelocityXPT™

together with an autosampler Tekmar AQUATek 70 (Tekmar-Dohrmann, Mason, OH, USA) were coupled online to the PTV injector of the GC-IRMS. Operation of the purge & trap system for benzene analysis was done as described elsewhere (Jochmann*, et al.*, 2006).

For carbon isotope analysis the GC was equipped with a Vocol™ capillary column (30m x 0.25, 1.5 µm film thickness, Supelco, Bellefonte, PA, USA) connected to fused-silica pre- and postcolumns (FS-Methyl-Sil, 2 m x 0.32 mm and 1 m x 0.32 mm, respectively; CS Chromatographie Service GmbH, Langerwehe,Germany). The GC-oven was programmed from 40°C (hold: 4 min), ramp 8°C min^{-1} to 100°C , ramp 30°C min^{-1} to 220°C (hold: 5 min).

For hydrogen isotope analysis the GC was equipped with a Supelcowax™ capillary column (30m x 0.25, 0.5 µm film thickness, Supelcoa, Bellefonte, PA, USA). The GC-oven was programmed from 40°C (hold: 0.5 min), ramp 3°C min^{-1} to 45°C (hold: 5min), ramp 4°C min^{-1} to 95°C, ramp 20°C min^{-1} to 200°C (5 min). Helium of grade 5 was used as carrier gas. Split ratio was, depending on concentration of benzene in the sample (404 to 8 µM), in a range of 8 -120. Isotope ratios were expressed in the δ-notation ($\delta^{13}C$ and $\delta^{2}H$) according to equations 4.3 and 4.4:

$$\delta^{13}C\,[‰] = \left(\frac{\left(^{13}C/^{12}C\right)_{Sample} - \left(^{13}C/^{12}C\right)_{Standard}}{\left(^{13}C/^{12}C\right)_{Standard}} \right) \bullet 1000‰ \qquad (4.3)$$

$$\delta^{2}H\,[‰] = \left(\frac{\left(^{2}H/^{1}H\right)_{Sample} - \left(^{2}H/^{1}H\right)_{Standard}}{\left(^{2}H/^{1}H\right)_{Standard}} \right) \bullet 1000‰ \qquad (4.4)$$

During carbon analysis by GC-IRMS, benzene was measured against a laboratory CO_2 calibration gas standard. The laboratory standard had been calibrated to V-PDB by reference CO_2 standards (RM 8562, RM 8563, RM 8564). During hydrogen analysis, benzene was measured against a laboratory H_2 monitoring gas, which has not been calibrated against an international standard. Measured changes in isotope ratios are therefore reported as relative differences $\Delta\delta^{2}H = \delta^{2}H_t - \delta^{2}H_0$ where $\delta^{2}H_0$ is the isotope value of non-reacted benzene. All samples were measured in duplicates. Reproducibility for $\delta^{13}C$ and $\delta^{2}H$ was always better than 0.5‰ and 5‰.

4.2.3 Quantification of isotope fractionation

Isotope fractionation for carbon and hydrogen, as expressed by enrichment factors ε, was obtained by linear regressions according to the Rayleigh equation (equation 4.3). However, the ε values do not distingish between differnt iostopes, therefore measured data only provide bulk isotope fractionation factors. For more detailed mechanistic explanations, fractionation can alternatively be coined in terms of position specific enrichment factors (equation 4.5) according to Elsner *et al.* (2005):

$$\varepsilon_{\text{reactive position}} \approx n/x \bullet \varepsilon_{\text{bulk}} \tag{4.5}$$

where n corresponds to the total positions of the atom and x is the number of reactive positions in the molecule; for carbon and hydrogen in benzene, $n = 6$ and $x = 6$ meaning that $\varepsilon_{\text{bulk}} = \varepsilon_{\text{reactive position}}$). Subsequently, apparent kinetic isotope effects can be calculated from $\varepsilon_{\text{reactive position}}$ to express the isotope fractionation that occurs specifically in the reacting bond. To this end, intramolecular competition between z identical reactive positions needs to be taken into account:

$$\text{AKIE} = \frac{1}{1 + z \bullet \varepsilon_{\text{reactive position}}/1000} \tag{4.6}$$

where for carbon and hydrogen in benzene, $z = 6$. Equation 4.6 refers to apparent kinetic isotope effects (AKIE) rather than intrinsic effects, since it cannot be excluded that the fractionation is masked by the influence of other, non-fractionating steps (Cichocka*, et al.*, 2008) so that smaller kinetic isotope effect values are calculated than would be expected for a certain reaction.

For this reason, dual isotope plots in which $\Delta\delta^2H$ values are plotted versus $\Delta\delta^{13}C$ may alternatively be used to probe for different reaction mechanisms (Elsner*, et al.*, 2007, Fischer*, et al.*, 2008, Vogt*, et al.*, 2008). These plots provide more robust lines of evidence to distinguish different pathways, because isotope fractionation of both elements (H and C) is generally masked to the same extent so that their slope ($\Lambda = \Delta\delta^2H / \Delta\delta^{13}C$) remains the same. A necessary condition for such applications, however, is that additional steps do not show isotope fractionation, since otherwise dual isotope slopes will change depending on the rate of either step (Penning*, et al.*, 2008).

4.3 Results

4.3.1 Carbon and hydrogen isotope fractionation

In this study $^{13}C_6$ stable isotopes in the remaining benzene became significantly enriched during anaerobic benzene degradation using an iron- and a sulfate-reducing cultures (BF and BPL) showing shifts towards more positive $\delta^{13}C$ values from -26‰ to -20‰ in both cultures (Fig 4.1a and b). Evaluation according to equation 4.1 gives carbon enrichment factors ε_C of -3.0 ± 0.5‰ and -2.5 ± 0.2‰, respectively (Fig. 4.1c and d, Table 4.1). No carbon isotope fractionation was observed in control bottles. The corresponding apparent kinetic carbon isotope effects ($AKIE_C$) were 1.018 ± 0.004 and 1.015 ± 0.002 (Table 4.1).

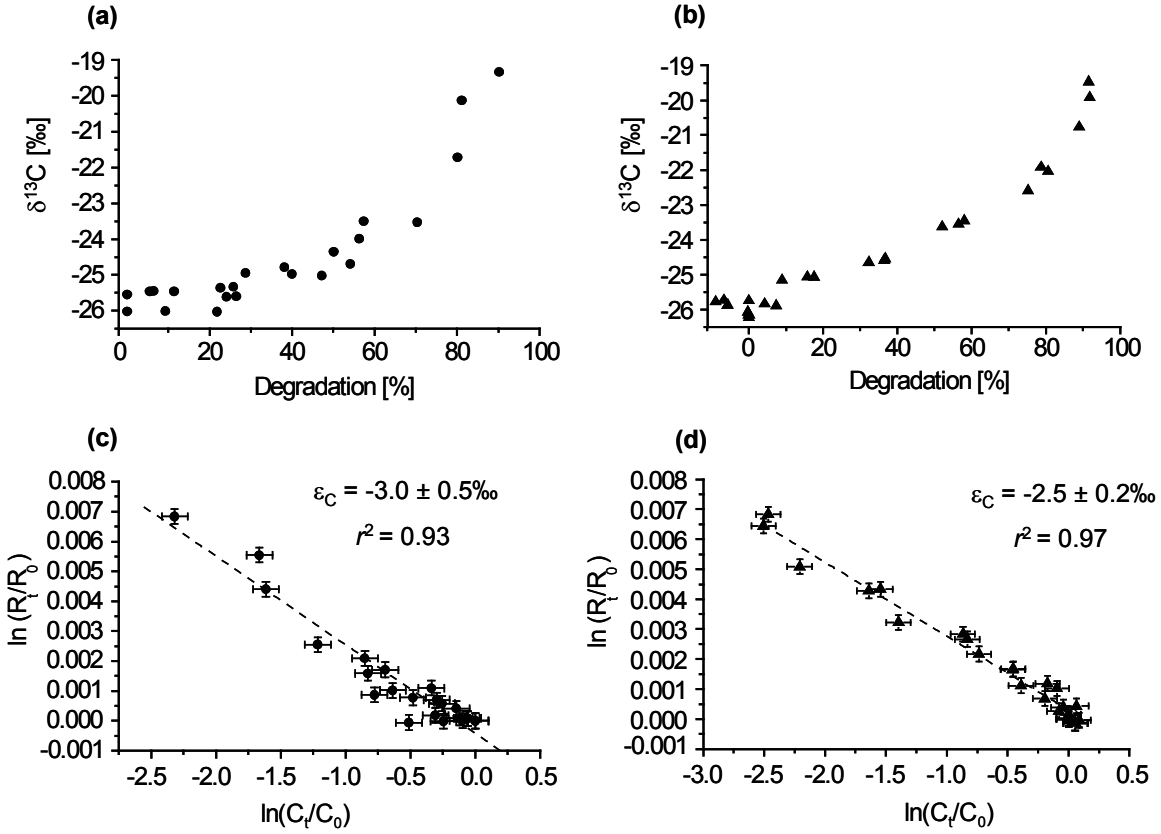

Figure 4.1. Plots of carbon isotope values versus benzene consumption in the iron- and sulfate-reducing cultures. Panels (a) and (b) correlation between benzene concentration and $\Delta\delta^{13}C$ in the iron-reducing (a) and sulfate-reducing (b) cultures where $\Delta\delta^{13}C = \delta^{13}C_t - \delta^{13}C_0$. Panels (c) and (d): logarithmic plot according to the Rayleigh equation (equation 4.3) for changes in $\delta^{13}C$ and benzene concentrations in the iron-reducing (c) and sulfate-reducing (d) cultures. The slope of the linear regression line (dashed line) gives the isotope carbon enrichment factor (ε_C). The reproducibility of carbon isotope measurements was better than 0.5‰ and the uncertainty of the benzene concentration measurements was less than 5% as indicated by the error bars.

Table 4.1. Carbon and hydrogen enrichment factors (ε_C, ε_H), derived apparent Kinetic Isotope Effects, and Λ values of the anaerobic benzene degradation under different electron accepters.

Bacterial culture	Benzene [µM]	$\varepsilon_C \pm$ 95 % CI [‰][a] (r^2)	$AKIE_C$	$\varepsilon_H \pm$ 95 % CI [‰][a] (r^2)	$AKIE_H$	$\Lambda^b \pm$ 95 % CI (r^2)	Reference
Nitrate-reducing mixed	~250	-2.6 ± 0.6 (0.96)	1.015 ± 0.005	-47 ± 11 (0.92)	1.392 ± 0.147	19 ± 3 (0.95)	(Mancini, *et al.*, 2008)
(Gram-negative bacteria)	~250	-2.8 ± 0.6 (0.96)	1.017 ± 0.004	-47 ± 11 (0.99)	1.392 ± 0.147	16 ± 2 (0.98)	(Mancini, *et al.*, 2008)
	~250	-2.2 ± 0.4 (0.91)	1.013 ± 0.003	-35 ± 6 (0.91)	1.265 ± 0.061	8 ± 2 (0.82)	(Mancini, *et al.*, 2003)
	~250	-1.9 ± 0.7 (0.94)	1.011 ± 0.005	-31 ± 7 (0.94)	1.228 ± 0.067	15 ± 4 (0.92)	(Mancini, *et al.*, 2008)
Sulfate-redcuing-mixed	~230	-3.6 ± 0.3 (0.92)	1.020 ± 0.004	-79 ± 4 (0.79)	1.901 ± 0.091	29 ± 3 (0.92)	(Mancini, *et al.*, 2003)
(Gram-negative bacteria)	~150	-1.9 ± 0.3 (0.97)	1.012 ± 0.001	-59 ± 10 (0.99)	1.548 ± 0.158	28 ± 3 (0.92)	(Fischer, *et al.*, 2008)
Sulfate-reducing highly enriched (Gram-positive bacteria)	~450	-2.5 ± 0.2 (0.98)	1.015 ± 0.002	-55 ± 4 (0.97)	1.492 ± 0.056	20 ± 2 (0.98)	This study
Iron-reducing highly enriched (Gram-positive bacteria)	~200	-3.0 ± 0.5 (0.93)	1.018 ± 0.004	-56 ± 8 (0.93)	1.51 ± 0.113	17 ± 1 (0.97)	This study
Methanogenic mixed	~900	-0.8 ± 0.2 (0.93)	1.005 ± 0.001	-34 ± 8 (0.88)	1.204 ± 0.133	39 ± 5 (0.98)	(Mancini, *et al.*, 2008)
(Gram-negative bacteria)	~750	-2.1 ± 0.1 (0.98)	1.013 ± 0.0004	-59 ± 4 (0.86)	1.548 ± 0.059	28 ± 1 (0.95)	(Mancini, *et al.*, 2003)
	~450	-1.1 ± 0.1 (0.88)	1.007 ± 0.0003	-38 ± 6 (0.80)	1.295 ± 0.063	31 ± 4 (0.87)	(Mancini, *et al.*, 2008)

[a]In our study mean enrichment factors calculated from the combined data of triplicate bottles for each culture. [b]Λvalues from our study were derived from the slop of the plot of the linear regression equation of $\Delta\delta^{13}C$ versus $\Delta\delta^{2}H$ benzene values for all triplicate bottles of each growth condition. CI represents 95% confidence intervals determined were determined by linear regression analysis as described elsewhere (Elsner, *et al.*, 2007).

Also, ^2H in benzene became strongly enriched during degradation by the cultures BF and BPL, showing differences of hydrogen isotopic ratios ($\Delta\delta^2H = \delta^2H_t - \delta^2H_0$) of between 0‰ to 116‰ (BF) and 0‰ to 122‰ (BPL) during the time course of benzene degradation (Fig 4.2a and b). In the autoclaved controls, no enrichment of the ^2H was observed in both cultures as indicated by changes of $\Delta\delta^2H$ that were within the analytical uncertainty of the methods (0‰ to -3.5‰). The ε_H values were plotted according to the Rayleigh equation giving correlation factors (r^2) of 0.93 (Fig 4.2c and d). Calculated ε_H and $AKIE_H$ were statistically identical for both cultures (-56 ± 8‰ and -55 ± 4‰; 1.510 ± 0.113 and 1.492 ± 0.056, respectively).

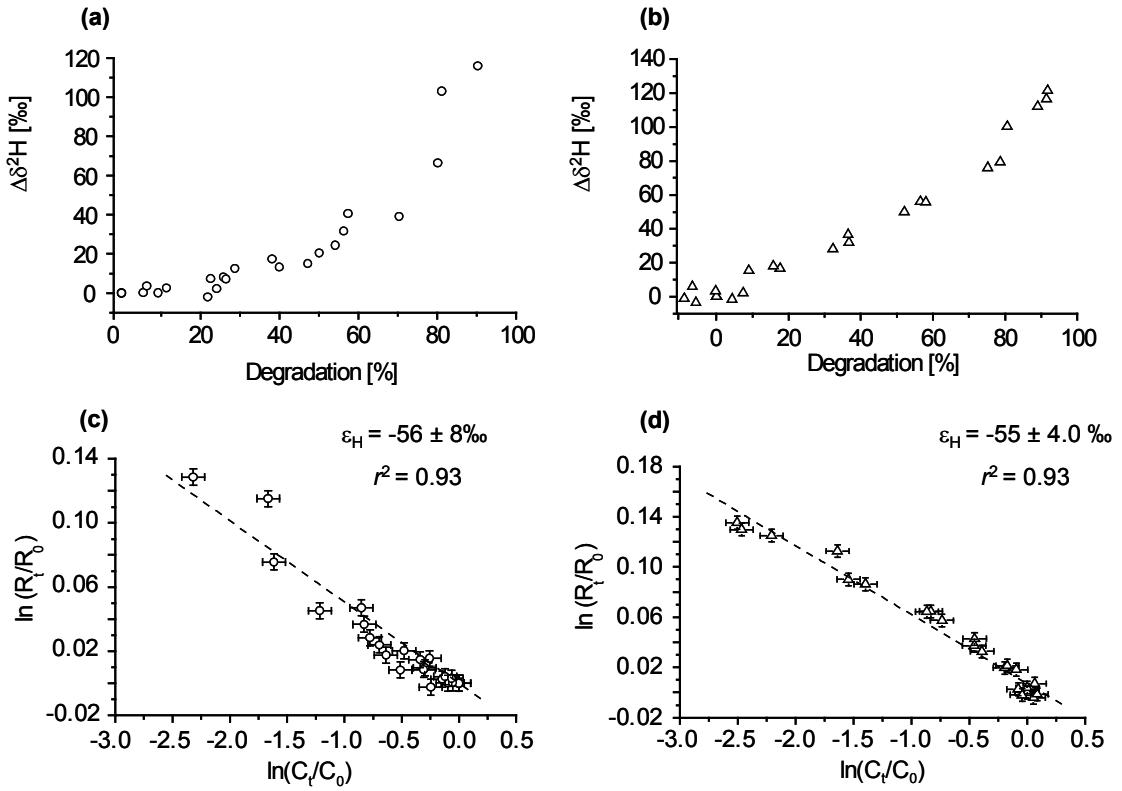

Figure 4.2. Plots of hydrogen isotope composition versus the residual concentration during benzene consumption in the iron-and sulfate-reducing cultures. Panels (a) and (b) benzene concentration and δ^2H in the iron- (a) and (b) sulfate-reducing cultures. Panels (c) and (d): logarithmic plot according to the Rayleigh equation of changes in δ^2H and benzene concentrations in the iron-reducing (c) and sulfate-reducing (d) cultures. The reproducibility of hydrogen isotope measurements was better than 5‰ and the uncertainty of the benzene concentration measurements was less than 5% as indicated by the error bars.

4.3.2 Two-dimensional isotope fractionation investigations

In our study carbon and hydrogen isotope fractionation were analyzed for the first time using benzene-degrading cultures phylogenetically affiliated to Gram-positive bacteria. Since the enrichment factors for a specific transformation reaction can significantly differ due to the influence of preceding non-fractionating steps such as membrane-transport, we plotted the dual isotope fractionation of carbon versus hydrogen ($\Delta\delta^{13}C = \delta_t{}^{13}C - \delta_0{}^{13}C$, $\Delta\delta^{2}H = \delta_t{}^{2}H - \delta_0{}^{2}H$) to decrease the variability (see materials and methods). When the data were plotted for the iron- and sulfate-reducing culture, the observed slopes were statistically not distinguishable (Table 4.1, Fig. 4.3), giving Λ values for the iron- and sulfate-reducing cultures of 17 ± 1 and 20 ± 2, respectively.

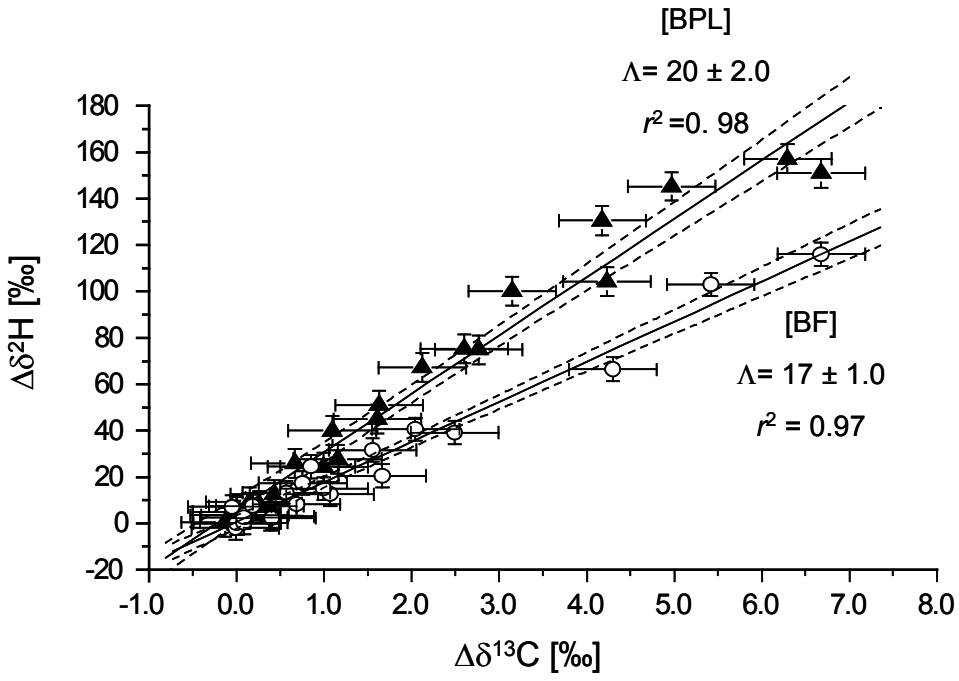

Figure 4.3. Plot of $\Delta\delta^{2}H$ versus $\Delta\delta^{13}C$ for anaerobic benzene biodegradation in the iron-reducing (open circles) and sulfate-reducing (solid triangles) cultures representing triplicate batch experiments of each tested culture. The two solid lines represent linear regressions of the $\delta^{13}C$ and $\delta^{2}H$ values, whereas dashed lines represent 95% confidence intervals. The slope of the regression equation gives the Λ values. Error bars representing the reproducibility of $\delta^{13}C$ and $\delta^{2}H$ were always smaller than 0.5‰ and 5‰, respectively.

4.4 Discussion

4.4.1 Hydrogen and carbon isotope fractionation during anaerobic benzene degradation

Benzene biodegradation has been shown to cause hydrogen and carbon isotopic shifts under aerobic and anaerobic conditions (Hunkeler*, et al.*, 2001, Mancini*, et al.*, 2002, Mancini*, et al.*, 2003, Griebler*, et al.*, 2004, Fischer*, et al.*, 2007, Fischer*, et al.*, 2008, Mancini*, et al.*, 2008). Generally, the isotopic shifts depend on the type of transformation reaction for benzene. Under aerobic conditions, the benzene biodegradation can be initiated by reactions involving monoxygenases or dioxygenases affecting either a C-H bond, and thus a primary carbon isotope fractionation is expected (Fischer*, et al.*, 2007, Fischer*, et al.*, 2008) or, in the case of dioxygenases, by the π-electron system which does not lead to bond breakage and only minor isotope effects. In contrast, anaerobic benzene degradation showed significant shifts in the hydrogen isotopic composition (Mancini*, et al.*, 2003, Fischer*, et al.*, 2008, Mancini*, et al.*, 2008). Therefore, the carbon and hydrogen isotope fractionation investigations might be used as indicator for elucidation of the initial activation pathways of benzene in culture studies but also in the field. In our study, the observed ε_C and $AKIE_C$ (Fig. 4.1 c and d; Table 4.1) for the iron- and sulfate-reducing cultures during anaerobic benzene degradation were in the range of data previously reported from nitrate- and sulfate-reducing cultures, but significantly greater than for methanogenic-mixed cultures (Table 4.1) (Mancini*, et al.*, 2003, Fischer*, et al.*, 2008, Mancini*, et al.*, 2008). On the other hand, the obtained ε_C values were within the range (-2.6 ± 0.8‰ to -3.5 ± 0.3‰) of reported ε_C-values of some aerobic cultures using monoxygenase reactions (e.g., *Alicycliphilus denitrificans* strain BC, *Burkholderia* sp.) (Hunkeler*, et al.*, 2001, Fischer*, et al.*, 2008). Therefore, no clear distinction between aerobic and anaerobic benzene transformation pathways could be obtained from the carbon enrichment investigation.

The hydrogen enrichment factors of benzene-degrading cultures investigated in our study (Fig. 4.2 c and d, Table 4.1) were statistically similar to each others and significantly greater than those reported for aerobic bacteria (Hunkeler*, et al.*, 2001, Fischer*, et al.*, 2008). This suggests the cleavage of a C-H bond in the rate-limiting step of anaerobic benzene degradation as previously indicated by Fischer *et al.* (2008) and Mancini *et al.* (2008). Moreover, the observed ε_H and $AKIE_H$ values were significantly smaller than previous values reported from benzene-degrading, sulfate-reducing, and methanogenic-mixed cultures (Table

4.1) and slightly greater than values observed from nitrate-reducing mixed cultures (Mancini*, et al.*, 2003, Fischer*, et al.*, 2008, Mancini*, et al.*, 2008).The measured ε_H and $AKIE_H$ values for the iron-and sulfate-reducing cultures were statistically within the same range (-55 ± 4 ‰ and -56 ± 8‰). The variability of ε_H and $AKIE_H$ values between our cultures and reported values for sulfate reducers might be related to the bioavailability of substrate associated with nonisotopically sensitive processes, such as transport and uptake of a substrate to the reactive site of the enzyme (Kampara*, et al.*, 2008). Such rate-limiting but not fractionating process could lower the observed isotope fractionation in some cultures such as e.g. the nitrate reducers because they grow very fast compared to the sulfate reducers.

4.4.2 Comparison of dual-isotope fractions of benzene under various electron-accepting conditions

For further mechanistic insight we made a dual parameter plot for $\Delta\delta^{13}C$ and $\Delta\delta^{2}H$. Similar to the ε_H values, also Λ the values derived from our benzene-degrading iron- and sulfate-reducing cultures BF and BPL were statistically non-distinguishable, giving circumstantial evidence that both cultures may be using a similar initial reaction mechanism. Such insight is consistent with our recent detection of benzoate as a metabolite of benzene degradation (Kunapuli*, et al.*, 2008, Abu Laban*, et al.*, 2009), as well as by our recent molecular biology investigation that showed the expression of putative carboxylase-related proteins (AbcA and AbcD, see chapter 3) in the benzene-grown cells of the cultures BF and BPL.

Previous publications report that Λ values ranged between 8 ± 2 and 31 ± 4 for anaerobic benzene degradation with various electron acceptors (Table 4.1) (Mancini*, et al.*, 2003, Fischer*, et al.*, 2008, Mancini*, et al.*, 2008). The lowest Λ values were observed for benzene degradation under nitrate-reducing conditions (8 ± 2 to 19 ± 3). In denitrifying (chlorate-reducing) bacteria, the values were ≤ 10. Since the process of chlorate reduction during benzene degradation produces oxygen, it is assumed that the benzene degradation is initiated by oxygen-dependent enzymatic reactions (Fischer*, et al.*, 2008). However, our Λ values of the iron- and sulfate-reducing culture overlapped significantly (Table 4.1) with those previously obtained for nitrate-reducing (non chlorate-reducing) mixed cultures (Mancini*, et al.*, 2003, Fischer*, et al.*, 2008, Mancini*, et al.*, 2008). In this case, the observed Λ values indicate that the benzene in our tested cultures (BF and BPL) and nitrifying cultures (non chlorate-reducing) is most likely attacked by a similar anaerobic mechanism. These findings are in contrast to other studies that showed significantly higher Λ values (28 ± 3 to 39 ± 5) for

benzene degradation by sulfate-reducing and methanogenic-mixed cultures than nitrate reducer (Mancini*, et al.*, 2003, Fischer*, et al.*, 2008, Mancini*, et al.*, 2008). Our study clearly shows that based on stable isotope fractionation studies it is not possible to distinguish benzene degradation by nitrate-reducing microorganisms from sulfate-reducing microorganisms. Mancini *et al.* (2008) indicated that methylation might be operative for facultative anaerobic nitrifying bacteria, and hydroxylation might be a possible initial activation reaction for strict anaerobes. Our data would rather suggest a common mechanism, but a prediction of a biochemical reaction based on isotope fraction studies is very in accurate and barely possible.

On the other hand, however, in a study of dual isotope fractionation with toluene, significant differences in the Λ values were observed for organisms attacking toluene by the same enzyme, benzylsuccinate synthase, under nitrate- and sulfate-reducing conditions (Vogt*, et al.*, 2008). The authors hypothesized a C-H bond cleavage to be the isoptopically sensitive step of anaerobic toluene degradation in both cases, but pointed out that the values were nonetheless in distinctively different ranges for organisms using nitrate (Λ =11-14) or sulfate (Λ = 28-31) as electron acceptor. Thus, also the observed variations of Λ values among different benzene-degrading ecophysiological groups might not be related to a different enzymatic reaction but could alternatively be caused by the electron accepting conditions leading to e.g. different growth rates in the same way as in the case of toluene.

4.4.3 Relating dual-isotopic data to the initial activation mechanism of benzene

Based on the carbon and hydrogen isotope analysis of the benzene-degrading iron-and sulfate-reducing cultures BF and BPL, we assume that the cultures attacked benzene are using the same reactions mechanism. This is supported by physiological, molecular, and our recent proteomic investigations of the BF and BPL cultures (Kunapuli*, et al.*, 2008, Abu Laban*, et al.*, 2009, chapter 3). In both cultures, the expected intermediate for methylation of benzene, toluene, was not utilized as growth substrate. In addition, genes of benzylsuccinate synthase, the key enzyme of toluene degradation was detected but not expressed in the iron-reducing culture. Furthermore, biochemical evidence for methylation of benzene exists from only traces of toluene detected in the nitrate-reducing cultures (Ulrich*, et al.*, 2005) and has to be interpreted with caution. The measured Λ values for these cultures (15 ± 4 to 19 ± 3) are close to the range of values predicted from our iron-and sulfate-reducing cultures (17 ± 1 to 20 ± 2)

(Mancini, *et al.*, 2008). We therefore propose that anaerobic benzene degradation proceeds via carboxylation also in denitrifying cultures.

In the previous studies, the Λ values for strictly anaerobic methanogenic and sulfate-reducing cultures were significantly higher than for our iron-and sulfate-reducing cultures (Fischer, *et al.*, 2008, Mancini, *et al.*, 2008). The authors indicated that the slopes of the $\Delta\delta^2H/\Delta\delta^{13}C$ are consistent with slopes determined for hydroxylation of benzene by abstraction of oxygen from water and phenol formation, as determined by Ulrich *et al.* (2005) in the same culture. However, anaerobic benzene degradation studies by our sulfate-reducing culture BPL and a marine sulfate-reducing enrichment culture did not show metabolic activity towards phenol (Musat & Widdel, 2008, Abu Laban, *et al.*, 2009). This is consistent with the observation of abiotic hydroxylation of benzene due to contact with oxygen during sampling (Kunapuli, *et al.*, 2008, Abu Laban, *et al.*, 2009). Furthermore, our more recent proteomic analysis identified putative anaerobic benzene carboxylase enzymes (AbcA and AbcD) and other carboxylases as specific benzene-expressed proteins (see chapter 3). These observations rather support a direct carboxylation of benzene. As no evidences indicated hydroxylation could be observed in our cultures, it is most likely that the significantly higher Λ values observed from previously methanogenic and sulfate-reducing cultures have not been related to hydroxylation as a different biochemical reaction mechanism as reported by Mancini *et al.* (2003), Fischer *et al.* (2008), and Mancini *et al.* (2008), but are rather related to phylogenetic and ecophysiological differences among the microorganisms determined our in iron- and sulfate-reducing cultures using the same activation mechanism of benzene, which is most probably a carboxylation. This assumption is supported by the identification of benzoate as metabolite of anaerobic benzene degradation under different electron accepting conditions (Caldwell & Suflita, 2000, Phelps, *et al.*, 2001, Chakraborty & Coates, 2005, Ulrich, *et al.*, 2005, Kunapuli, *et al.*, 2008, Abu Laban, *et al.*, 2009). Such proposed metabolism of benzene via carboxylation has also been proposed as a mechanism for anaerobic activation of naphthalene and phenanthrene (Zhang & Young, 1997, Meckenstock, *et al.*, 2000, Davidova, *et al.*, 2007), which most probably indicated the use of carboxylation as a common mechanism for unsubstituted aromatic compounds.

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5 General conclusions and outlook

The biodegradation of benzene in the absence of molecular oxygen is considered problematic due to the lack of substitute groups (e.g. CH_3, OH^-) being attacked by the microorganisms in the initial activation reaction. Therefore, it is considered one of the most stable and recalcitrant hydrocarbons prevalent in organically polluted subsurface environments. Despite the availability of benzene enrichment cultures, the knowledge about phylogenetic and biochemical characteristics of benzene-degrading microorganisms is still scarce. The work presented in thesis allows a detailed insight into the physiology of the Gram-positive bacteria family *Peptococcaceae*, key-players involved in the anaerobic degradation of benzene, and to emphasize their role in the initial activation reaction of benzene.

The first objective of this study was to perform a detailed molecular and physiological characterization of a benzene-degrading, sulfate-reducing enrichment culture (BPL), derived from a former coal gasification site. Electron and carbon balances with $^{13}C_6$-benzene indicated a total oxidation of the aromatic hydrocarbon to $^{13}CO_2$ coupled to sulfate reduction. Furthermore, even though several aromatic substrates were tested, the enrichment culture BPL could only grown with benzene (up to 2 mM), but not phenol, toluene or benzoate indicating the specificity of the culture for benzene degradation. Based on the analysis of the 16S rRNA gene sequences, the microorganisms phylogenetically related to the genus *Pelotomaculum* were dominant in the culture BPL. Interestingly, members of the genus *Pelotomaculum* are representing the novel subcluster Ih within *Desulfotomaculum* that have lost the ability to reduce sulfate in an evolutionary process and thus are growing syntrophically with hydrogenotrophic methanogens (Imachi*, et al.*, 2002, Imachi*, et al.*, 2006). The quantitative determination of the putative benzene degrader in the culture BPL using *in situ* hybridization with a probe DEM1164r (Stubner & Meuser, 2000) targeting subcluster Ih showed that the enrichment contains (more than 95%) of only one genotype. These findings provided evidence to exclude a syntrophic interaction of *Pelotomaculum*-related microorganisms during benzene degradation. Further analysis proved the absence of methane and methanogenic archaea in the culture BPL and identified a peptide highly affiliated to DsrC (γ-subunit of dissimilatory sulfate reductase) of *Pelotomaculum*, expressed in cultures grown on benzene. Our molecular investigations identified novel bacteria, phylogenetically affiliated to the genus of

Pelotomaculum, but physiologically strongly resembling microorganisms of the genus of *Desulfotomaculum*. Those microorganisms might be the key-players of anaerobic benzene degradation and sulfate reduction in the culture BPL.

The benzene degraders characterized in this study helped to understand the role and importance of Gram-positive bacteria as better competitors in the cultures with high benzene concentrations. This was also observed in a study describing the active benzene degrader of an iron-reducing enrichment culture (BF). Here, using stable isotope probing (SIP), microorganisms belonging to the genus of *Clostridia* and microorganisms related to the *Desulfobulbaceae* have successfully been identified as key-players of benzene degradation (Kunapuli*, et al.*, 2007). Moreover, the studies of microcosms and sand filled columns percolated with groundwater from a benzene-contaminated aquifer showed that high benzene concentrations can cause a major shift from *Beta-proteobacteria* to Gram-positive bacteria related to *Actinobacteria* and *Cryptanaerobacter*/*Pelotomaculum* (Fahy*, et al.*, 2008, Kleinsteuber*, et al.*, 2008). These observations and our cultivation of *Pelotomaculum*-related bacteria further supports the hypothesis that the importance of Gram-positive microorganisms concerning benzene degradation has largely been overlooked, especially their high solvent-tolerance and spore-formation, allowing the microorganisms to survive under various changing environmental conditions. Therefore, Gram-positive bacteria could be important in the natural attenuation of benzene or the remediation of contaminated sites. However, repeated attempts to isolate obligate anaerobic of benzene degrader in pure cultures failed and consequently detailed information about their properties is still needed. Our future goal is to use new approaches for purification of the anaerobic benzene-degrading culture e.g. optical tweezers single cell isolation.

The second objective of this thesis was to elucidate the mechanism of anaerobic benzene degradation using the characterized sulfate-reducing culture (BPL), and the previously enriched ferric iron-reducing culture (BF) (Kunapuli*, et al.*, 2007). The analysis of metabolites in diverse benzene-degrading cultures led to the proposition of three different mechanisms; hydroxylation, forming phenol via a hydroxyl free radical (denitrifying bacteria) or hydroxyl donor like water (methanogenic bacterial) (Vogel & Grbic-Galic, 1986, Grbic-Galic & Vogel, 1987, Caldwell & Suflita, 2000, Chakraborty & Coates, 2005), alkylation forming toluene, followed by fumarate-addition forming benzylsuccinate, and carboxylation forming benzoate (Caldwell & Suflita, 2000, Phelps*, et al.*, 2001). Using LC/MS/MS and HPLC for metabolites

analysis, phenol, 2-hydroxybenzoate, 4-hydroxybenzoate and benzoate were identified during anaerobic benzene degradation by the sulfate-reducing culture. An important finding was the identification of 2-hydroxybenzoate, which is not known to be a specific intermediate compound of anaerobic degradation of monoaromatic hydrocarbons so far. This might indicate that the detected hydroxylated aromatic compounds (phenol, 2- and 4-hydroxybenzoate) were formed abiotically due to the contact of reduced compounds (Fe^{+2} in the culture medium) with oxygen during sampling. This is supported by the detection of phenol in the autoclaved control bottles and the lack of phenol utilization, and the concomitant formation of 4-hydroxybenzoate during benzoate formation by the sulfate-reducing culture. Due to the detection of abiotically formed phenol during benzene degradation by an iron-reducing culture, a similar conclusion was drawn by Kunapuli, *et al.* (2008). Therefore, the data obtained from the metabolite analysis are more indicative for a direct carboxylation of benzene as the initial mechanism.

To resolve the biochemistry of anaerobic benzene degradation, metagenomic, proteomic and peptide identification approaches were performed. So far, only one genome of a benzene-degrading bacterium has been elucidated (*Dechloromonas aromatica* strain RCB; accession number NC_007298). However, no genes coding putative benzene-degrading enzymes were identified neither does the genome contain genes for anaerobic aromatics degradation. In this study, we provide insights into the metagenome of the iron-reducing benzene-grown culture (BF) to describe multiple aromatic compound-degrading genes and putative anaerobic benzene-degrading genes. The microbial community analysis indicated the dominance of *Peptococcaceae*-related bacteria in the iron-reducing benzene-, phenol-, and benzoate-grown cultures. The draft genome contained sequences of 14270 open reading frames (ORFs) distributed in 5832 contigs. About 205 ORFs showed closest sequence similarity to genes encoding enzymes and transcriptional regulators known to be involved in anaerobic aromatic hydrocarbons degradation and 90 genes were related to the transport of xenobiotics, indicating the specificity of these bacteria in aromatic hydrocarbons degradation. Interestingly, gene clusters encoding proteins of anaerobic degradation of toluene, phenol, 4-hydroxybenzoate, and benzoate were identified. Toluene catabolic *bssCABD* and *bbsCABEFD* genes encoding the benzylsuccinate synthase and β-oxidation reactions of benzylsuccinate were found in two different clusters. Furthermore, phenol-degrading *ppsA* and *ppcBCAD* genes encoding putative phenylphosphate synthase and phenylphosphate carboxylase, showing a sequence similarity to the sequences of the respective proteins of *Aromatoleum aromaticum* stain EbN1

(Rabus*, et al.*, 2005), identified in different regions in the genome. A different operon-like organization (~17 Kb) of genes of the putative benzene degradation pathway was discovered with genes encoding of PpcD- and PpcA-like protein subunits clustering together with those for an UbiD/UbiX-like carboxylase (3-octaprenyl-4-hydroxybenzoate carboxy-lyase). An ORF similar to genes encoding the benzoate-CoA ligase/4-hydroxybenzoate-CoA ligase was found to be located nearby the *ppcD*- and *ppcA*-like genes. The later ORF showed 56 % sequence identity to the *bamY* gene encoding benzoate-CoA ligase in *Geobacter metallireducens* (Butler*, et al.*, 2007). These observations indicated the existence of different carboxylase-related ORFs within the genome that could be involved in the carboxylation of unknown aromatic substrates. Moreover, ORFs encoding genes similar of 4-hydroxybenzoyl-CoA reductase (*pcmRST*-homologues) and benzoyl-CoA reductase (*bamB-I*-like), resembling the gene described in *Geobacter* sp. strain FRC-32, were found to be scattered within the genome. The presence of such aromatic-degrading genes with a wide metabolic versatility reflecting the importance of *Peptococcaceae*-related microorganisms can be used as model organism to elucidate the unknown degradation pathways of aromatic compounds.

Another aspect investigated during this study was the identification of putative proteins and enzymes involved in the initial activation of anaerobic benzene degradation in iron- and sulfate-reducing cultures by combining proteomic technologies with genetics. The whole proteomes expressed in benzene-, phenol-, and benzoate-grown cells of the iron-reducing culture BF were compared and correlated to the high-throughput sequenced genome information. Proteomic analysis indicated that the product of genes encoding benzylsuccinate synthase BssCABD and benzylsuccinate β-oxidation BbsCABEFD enzymes could not be indentified from protein extracts of benzene- and benzoate-grown cells. This result agreed with the metabolites analysis of the benzene-degrading cultures (BF and BPL) where benzylsuccinate, the expected downstream intermediate from methylation of benzene, could not be identified (Kunapuli*, et al.*, 2008, Abu Laban*, et al.*, 2009). Here we provided new evidence based on a proteomic approach to exclude methylation as initial reaction of benzene activation under iron- and sulfate-reducing condition. Noteworthy, the only evidence of benzene methylation observed in methanogenic cultures derived from the detection of only traces of toluene; therefore those results have to interpreted carefully (Ulrich*, et al.*, 2005).

Further interesting results obtained by SDS-PAGE analysis of proteins extracts from cells grown on benzene, phenol, and benzoate indicated a specific benzene-expressed peptide band

with a mass of 60 kDa. Peptide identification of this protein, using LC/MS/MS and direct N-terminal sequencing, showed that the expressed protein in the benzene-grown cells was homologue to the phenylphosphate carboxylase PpcA (60 kDa). The gene encoding this protein was part of the putative benzene degradation gene cluster (described above) containing also the other benzene-expressed proteins similar to PpcD (15 kDa) and UbiD/UbiX carboxylase (17 kDa). The PpcD- and PpcA-like protein subunits were not induced in phenol grown-cells and also showed a low sequence similarity (43 % and 37 % identity) to the respective proteins involved in the phenol carboxylation of *Aromatoleum aromatica* strain EbN1 (Rabus*, et al.*, 2005) which might be indicative for another function of these proteins rather than the carboxylation of phenolic compounds. Nearby those genes, another expressed gene can be found, showing 56% sequence similarity to the *bamY* gene encoding the benzoate-CoA ligase in *Geobacter metallireducens* (Butler*, et al.*, 2007). It was previously described to encode a typical aromatic carboxylic acid-CoA ligase (Wischgoll*, et al.*, 2005). Thus, the information obtained from the putative benzene-expressed proteins might provide additional evidence to support a direct carboxylation of benzene, catalyzed by PpcD and PpcA-like protein subunits forming benzoate, followed by a further degradation of benzoate via benzoate-CoA ligation catalyzed by the BamY-like protein. We name PpcD and PpcA-like protein subunits a putative anaerobic benzene carboxylase (Abc) enzyme being encoded by at least *abcD* and *abcA* genes, which most likely belongs to a carboxylase family including phenylphosphate carboxylase and 3-octaprenyl-4-hydroxybenzoate carboxy-lyase enzymes. Noteworthy, the putative AbcD and AbcA protein subunits were also found to be expressed in benzene-grown cells of the sulfate-reducing culture lacking any phenol carboxylation activity, which may be indicative for the benzene degradation specificity of this protein. However, the catalytic mechanism of the putative benzene-expressed proteins still needs to be further investigated, therefore future research aims are to use molecular and biochemical tools to investigate the expression of these genes and to assess their activity under strictly anaerobic conditions.

Other proteins identified from benzene-grown cells of the culture BF were homologues to the 4-hydroxybenzoyl-CoA reductase of the PcmTRS proteins of *Geobacter metallireducens* (Butler*, et al.*, 2007). Interestingly, the large PcmT subunit was found to be expressed in benzene and benzoate, but not in phenol-grown cells. As a member of the xanthine oxidase family, the PcmT protein is expected to catalyze the hydroxylation (dehydrogenation) reaction of benzene using water as substrate and usually NAD^+ as electron acceptor (Lowe, 2002).

However, based on the high similarity (75%) of the PcmT protein to respective proteins of *G. metallireducens*, it seems more likely to catalyze the irreversible reduction of 4-hydroxybenzoate rather than to perform benzene dehydrogenation (Peters*, et al.*, 2007). Thus, the expression of the *pcmT* genes in the benzene-grown cells might not be related to the initial activation reaction. Nevertheless, if the genes product of *pcm* catalysis 4-hydroxybenzoyl-CoA reduction, its expression in benzene-grown cells could possibly indicate an active phenol degradation pathway whereas the initial hydroxylation of benzene to phenol would be performed by other enzymes. However, PcmT was expressed in benzoate-grown cells of the iron-reducing culture BF and in benzene-grown cells of the sulfate-reducing culture BPL, lacking any ability of phenol degradation. This might be a hint that the induced 4-hydroxybenzoyl-CoA reductase is not necessarily active. Taking all of these evidences into account, it is most likely that the hydroxylation of benzene is not operative as initial activation mechanism. As the genome sequence of the iron-reducing culture is not complete, further studies are needed in the future to close the gaps in the genome and to identify the transcriptional regulators of the aromatic-degrading genes e.g. like 4-hydroxybenzoyl-CoA reductase.

Recently, combined carbon ($\delta^{13}C$) and hydrogen ($\delta^{2}H$) stable isotope fractionation "two-dimensional-analysis" have been introduced as powerful tool for the characterization of the transformation pathway of a particular organic compound. The slope (Λ) for the linear regression of hydrogen ($\Delta\delta^{2}H$) and carbon ($\Delta\delta^{13}C$) isotopic fractionation may be used to characterize the reaction mechanism more precisely. The goal of the last part of this study was to compare the benzene stable isotope effects during benzene degradation of the iron-reducing culture BF and sulfate-reducing culture BPL. Similar stable isotope effect would be indicating that both cultures use the same reaction mechanism to attack benzene. The dual-plot for the $\Delta\delta^{13}C/\Delta\delta^{2}H$ indicates that the slopes Λ for the BF and BPL cultures (20 ± 2 and 17 ± 1) were statistically found in the same range, which might indicate that both cultures are using similar mechanisms for the initial attack of benzene. However, previous dual-isotopic analysis suggested different initial reaction mechanisms for anaerobic benzene degradation under methanogenic and sulfate-reducing conditions and nitrate-reducing condition on the other hand (Fischer*, et al.*, 2008, Mancini*, et al.*, 2008). The authors found higher slopes under methanogenic and sulfate-reducing conditions and suggested that hydroxylation of benzene would be proceeded here based on the detection of phenol in the same cultures (Ulrich*, et al.*, 2005). Furthermore, they measured lower slopes in nitrate-reducing cultures and proposed

benzene methylation in these cultures. In the nitrate-reducing cultures traces of toluene were detected, suggesting methylation (Ulrich*, et al.*, 2005). Interestingly, the measured slopes Λ values in nitrate-reducing cultures (15 ± 4 to 19 ± 3) were close to the range of values predicted from our iron- and sulfate-reducing cultures (17 ± 1 to 20 ± 2). In fact, our results of the proteomic and metabolic analysis provided clear evidence to exclude methylation of benzene. Therefore, the interpretation mechanism that benzene is activated by methylation in the nitrate-reducing cultures has to be looked at with caution. On the other hand, our evaluated dual-isotope slopes were not in the range of those cultures proposed to use hydroxylation, as the activation mechanism. Combining the results of physiological, metabolomic, proteomic and dual-isotope analysis of the benzene-degrading cultures, this study strongly supports the direct carboxylation of benzene as common degradation mechanism for anaerobic bacteria.

In summary, the presented data provide comprehensive information to extend our knowledge concerning anaerobic biodegradation of the stable, unsubstituted compound "benzene". The results obtained from several new approaches indicated, that carboxylation is the initial activation mechanism for benzene degradation. Such a mechanism has also been suggested for anaerobic activation of naphthalene and phenanthrene (Zhang & Young, 1997, Meckenstock*, et al.*, 2000, Davidova*, et al.*, 2007). Certainly, more research is required to establish the prevalence of this pathway and the proteins involved in the anaerobic benzene degradation. Metagenomic and proteomic analysis of anaerobic benzene-, naphthalene-, and phenanthrene-degrading cultures using different electron acceptors have to be elucidated in the future.

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Appendix

Data used for figure 2.1a

Concentration of 13C6-benzene (µM), evolution of 13CO2 (mM), and sulfate reduction (mM) monitored during the anaerobic degradation of benzene over three parallel incubations of the active- degrading cultures and two parallel incubations of the controls. (A, D, J) = Standard deviation of respective benzene concentrations (µM); (B, K) = Standard deviation of respective 13CO2 concentrations (mM), and (C, F) = Standard deviation of respective sulfate reduction (mM).

Time	Active-degrading cultures						Autoclaved control cultures					Molybdate inhibited			
Days	Benz (µM)	A	$^{13}CO_2$ (mM)	B	SO_4 (mM)	C	Benz (µM)	D	$^{13}CO_2$ (mM)	SO_4 (mM)	F	Benz (µM)	J	$^{13}CO_2$ (mM)	K
0	354	11	0.0	0.0	1.8	0.1	315	20	0.0	1.9	0.1	535	60	0.0	0.0
14	384	5	0.1	0.1	1.8	0.2	375	24	0.0	1.9	0.4	537	43	0.0	0.0
29	373	65	0.2	0.0	1.7	0.0	368	48	0.0	1.7	0.1	538	59	0.1	0.1
35	282	15	0.5	0.0	1.4	0.3	376	44	0.0	1.7	0.1	533	39	0.1	0.2
42	62	21	1.4	0.1	0.8	0.0	366	30	0.0	1.8	0.1	503	78	0.2	0.3
49	0	0	1.8	0.0	0.6	0.0	383	30	0.0	1.8	0.1	492	85	0.2	0.2
56	0	0	1.8	0.0	0.7	0.0	356	34	0.0	1.7	0.1	486	75	0.3	0.2

Data used for figure 2.2a

Benzene (µM), phenol (mM), and sulfate (mM) concentrations measured from three parallel incubations of the co-substrates experiment and two parallel incubations of the controls.

Time	Co-substrate experiment			Benzene control	
Days	Benzene (µM)	Phenol (µM)	SO_4 (mM)	Benzene (µM)	SO_4 (mM)
0	631	294	2.33	487	2.41
9	637	300	2.28	445	1.7
16	652	308	2.53	253	1.83
23	573	275	2.03	2	1.14
29	535	315	2.02	1	1.1
39	393	314	0.99	-	-
42	350	307	0.84	-	-

Data used for figure 2.2b

Benzene (µM), toluene (mM), and sulfate (mM) concentrations measured from three parallel incubations of the co-substrates experiment and two parallel incubations of the controls.

Time	Co-substrate experiment			Benzene control	
Days	Benzene (µM)	Toluene (µM)	SO_4 (mM)	Benzene (µM)	SO_4 (mM)
0	332.	300	2.87	487	2.41
9	302	396	2.78	445	1.7
16	141	383	2.53	253	1.83
23	26	350	2.14	2	1.14
29	7	329	1.51	1	1.1

Data used for figure 2.6

Cell counts (cells/ml) using DEM1164r-Cy3 labeled probe, EUB338 I-II mix-fluos-labeled probes, and DAPI stain obtained during the time course of anaerobic benzene degradation (A, B, C) = Standard deviation of respective cell counts obtained form duplicate sampling of two different incubations. (D, E) = Standard deviation of benzene and sulfate concentrations obtained from two different incubations.

Time Days	DEM1164r probe Cells/ml	A	EUB338 mix probe Cells/ml	B	DAPI stain Cells/ml	C	Benz (µM)	D	SO_4 (mM)	E
0	3.18E+04	3.9E+03	3.5E+04	7.9E+02	4.0E+04	6.3E+03	424	3	2.8	0.1
7	3.86E+04	8.5E+03	4.4E+04	3.2E+03	5.0E+04	6.9E+03	418	14	2.7	0.0
14	1.17E+05	5.0E+04	1.3E+05	5.4E+04	1.4E+05	4.4E+04	388	3	2.8	0.0
21	1.40E+05	5.3E+03	1.5E+05	5.3E+03	1.6E+05	5.3E+03	321	4	2.5	0.0
28	3.04E+05	7.4E+04	3.2E+05	7.2E+04	3.3E+05	8.2E+04	288	7	2.1	0.1
35	4.21E+05	3.2E+03	4.3E+05	1.3E+04	4.6E+05	2.4E+04	146	13	1.9	0.1
42	9.51E+05	1.2E+05	1.0E+06	1.3E+05	1.0E+06	7.3E+04	89	12	1.4	0.4
49	1.15E+06	8.8E+04	1.2E+06	5.1E+04	1.3E+06	6.2E+04	46	11	1.3	0.1
56	2.16E+06	2.5E+05	2.2E+06	2.9E+05	2.3E+06	2.0E+05	12	7	1.1	0.2

Data used for figure 2.7

Concentration of metabolites (µM) detected and sulfate (mM) reduced during the fulltime course of anaerobic benzene degradation. Data obtained from two parallel incubations of both the active and sterile cultures.

Active benzene-degrading cultures						
Time Days	**Benzene (µM)**	**Phenol (µM)**	**Benzoate (µM)**	**4-OH-benzoate (µM)**	**2-OH-benzoate (µM)**	**SO_4 (mM)**
0	423.824	0.286	0.07568	0.01461	0.00244	4.779
7	418.351	0.428	0.07918	0.01206	0.00381	4.74576
14	387.571	0.47	0.09979	0.01637	0.00474	4.78566
21	321.365	1.267	0.09859	0.01782	0.00613	4.51656
28	287.844	1.794	0.1719	0.02074	0.00631	4.07278
35	146.253	1.694	0.22429	0.06572	0.00957	3.8542
42	89.103	1.489	0.32924	0.0374	0.00931	3.3862
47	46.45	1.302	0.40346	0.10895	0.00933	3.25514
54	12.032	0.464	0.35834	0.14244	0.01441	3.14588
Sterile control cultures						
0	401.872	0.42784	0.06191	0.0129	0.00375	4.8399
7	402.297	0.30688	0.05819	0.01017	0.00189	4.87183
14	397.855	0.16937	0.06623	0.01376	0.00239	4.82512
21	387.859	0.21141	0.04891	0.0056	0.00267	4.56482
28	382.743	0.53289	0.05911	0.00844	0.00279	4.61615
35	376.519	0.92021	0.05871	0.01405	0.00255	4.99608
42	401.446	1.08278	0.0895	0.0255	0.00246	4.71365
47	414.71	1.55682	0.05994	0.02264	0.00248	4.80931
54	392.063	1.32691	0.05258	0.04331	0.00589	4.7875

Data used for figure 3.1

Concentration of Fe(II) (mM) measured during anaerobic benzene, phenol, and benzoate by iron-reducing enrichment culture in triplicate incubations.

Time	**Fe(II) (mM)**		
Days	**Benzene**	**Phenol**	**Benzoate**
0	0.93	0.37	0.91
7	2.24	1.26	0.27
19	3.82	20.22	11.88
23	10.82	27.28	14.72
30	16.55	28.99	31.18
38	25.06	39.57	31.13

Table A1

General features of the benzene-grown, iron-reducing culture metagenome. Data based on the draft of the genome sequence from contigs bzn_200901 PEDANT database.

Genome features		
DNA size (Mb)	**Contigs no.**	***orf* no.**
10.3	5832	14269

Protein encoding genes								
Genes no.	**Protein length (aa)**	**(%)**	**Protein MW (kDa)**	**(%)**	**Isoelectric point (pI)**	**(%)**	**BlastP Identity (%)**	**(%)**
14159	(50-149)	50.6%	<=10	23.6%	4.5 < pI < 5.5	20.3%	> 90%	5.0%
	(150-299)	31.5%	(11_20)	36.8%	5.5 < pI < 6.5	12.7%	> 50%	21.3%
	(300-999)	17.4%	(21-50)	33.0%	6.5 <pI < 7.5	12.3%	> 30%	50.8%
	(1000-3000)	0.4%	(51-100)	5.6%	7.5 < pI < 8.5	10.0%	>0%	59.3%
					8.5 < pI < 9.5	8.6%	0%	40.7%
					9.5 < pI <10.5	14.3%		

Table A2

Aromatic-degrading ORFs in the genome of the iron-reducing culture (BF). For each of the 205 ORFs in the region the following is supplied: ORF identification used in the text, contigs number of the ORF for predicted proteins from bzn-00901-EDANT database (http://pedant.gsf.de:8045/pedant3htmlview/pedant3view?Method=start_method&Db=p3_i5_t1485_Clo_benz_200901_v2); abbreviated gene name, gene product, identity hits (%) obtained from comparison of predicted proteins from the contigs bzn-200901-PEDANT database with the closest homolog in the protein non-redundant BLASTP-NCBI, reference number-NCBI, and species name-NCBI. The sequence of ORF 1-ORF 205 deposited in GenBank under the accession numbers GU357855 to GU358059.

Contigs information		Length (aa)	Size (kDa)	Related gene product (BlastP annotation)					
ORF no.	ORF contig no. (contig size Kb)[a] (Total no. ORFs)[b]			*Gene*	Function	E-value	Identity (%)	Accession no.	Organism
BF_5847	**(50.8)[a] (56)[b]**								
ORF 1	BF_5847_11602	577	66.4		Phosphoenolpyruvate synthase, alpha subunit	2.E-61	29	YP_002524062	*Sphaerobacter thermophilus*
ORF 2	BF_5847_19716	884	100.7	*ppsB*	Phenylphosphate synthase, water dikinase	2.E-76	30	YP_001618497	*Sorangium cellulosum*
ORF 3	BF_5847_26757	634	68.1		Respiratory-chain NADH dehydrogenase domain	0.E+00	55	YP_064419	*Desulfotalea psychrophila*
BF_5874	**(45.6)[a] (56)[b]**								
ORF 4	BF_5874_4459	429	48.1	*orf11*	Transcriptional regulator, Fis family	2.E-119	54	YP_001212988	*Pelotomaculum thermopropionicum*
ORF 5	BF_5874_18992	342	37.6		ABC transporter related	3.E-110	56	YP_001113983	*Desulfotomaculum reducens*
BF_5887	**(107.7)[a] (111)[b]**								
ORF 6	BF_5887_102537	170	18.8	*bamM*	Acyl-CoA dehydrogenase domain protein	3.E-67	72	YP_517949	*Desulfitobacterium hafniense*
ORF 7	BF_5887_16773	264	29.3		Putative FAD-binding subunit of oxidoreductase	3.E-74	56	ZP_02948940	*Clostridium butyricum*
ORF 8	BF_5887_17557	150	16.6		Aldehyde oxidase and xanthine dehydrogenase	8.E-61	70	ZP_02948940	*Clostridium butyricum*
ORF 9	BF_5887_84488	265	29.3		Glycyl-radical enzyme activating protein family	7.E-68	47	YP_001114081	*Desulfotomaculum reducens*
ORF 10	BF_5887_89713	185	21.7		Acetophenone carboxylase, gamma subunit	1.E-38	50	ZP_03494850	*Alicyclobacillus acidocaldarius*
BF_5902	**(23.9)[a] (33)[b]**								
ORF 11	BF_5902_16706	64	7.3	*bssC*	Benzylsuccinate synthase, subunit C	1.E-10	52	YP_158059	*Aromatoleum aromaticum* EbN1
ORF 12	BF_5902_16900	862	9.8	*bssA*	Benzylsuccinate synthase, subunit A	0.E+00	67	YP_158060	*Aromatoleum aromaticum* EbN1
ORF 13	BF_5902_19502	99	11.3	*bssB*	Benzylsuccinate synthase, subunit B	1.E-15	54	YP_158061	*Aromatoleum aromaticum* EbN1
ORF 14	BF_5902_19934	285	32.0	*bssE*	Benzylsuccinate synthase, subunit E	6.E-105	64	AAK50368	*Aromatoleum aromaticum* EbN1

Table A2 (continued):

ORF 15	BF_5092_20784	197	22.9	*bssF*	Putative uncharacterized protein , subunit F	1.E-39	44	CAA05046	*Thauera aromatica*
ORF 16	BF_5092_21374	291	33.4		Von Willebrand factor type A	3.E-46	39	YP_002537889	*Geobacter* sp. FRC-32
ORF 17	BF_5092_22246	74	8.5	*bssF*	Putative uncharacterized protein, subunit F	5.E-15	50	YP_158063	*Aromatoleum aromaticum* EbN1
ORF 18	BF_5902_22489	312	34.9	*bssD*	Benzylsuccinate activating, delta subunit	6.E-80	47	AAK50368	*Azoarcus* sp. T
BF_5926	**(4.8)[a] (5)[b]**								
ORF 19	BF_5926_4087	657	74.2	*bamB*	Aldehyde ferredoxin oxidoreductase	0.E+00	63	YP_002535701	*Geobacter* sp. FRC-32
ORF 20	BF_5926_1476	383	43.2	*bamD*	Protein of unknown function DUF224	3.E-166	70	YP_002535703	*Geobacter* sp. FRC-32
ORF 21	BF_5926_2111	176	19.7	*bamC*	4Fe-4S ferredoxin, iron-sulfur binding protein	3.E-53	64	YP_002535702	*Geobacter* sp. FRC-32
BF_5932	**(21.6)[a] (25)[b]**								
ORF 22	BF_5932_20418	157	16.5	*hcrC*	4-hydroxybenzoyl-CoA reductase, gamma subunit	2.E-47	61	CAI78850	uncultured bacterium
ORF23	BF_5932_21422	334	36.1	*hbaD*	4-hydroxybenzoyl-CoA reductase, beta subunit	3.E-46	30	YP_530898	*Rhodopseudomonas palustris*
BF_5938	**(4.8)[a] (5)[b]**								
ORF 24	BF_5938_5818	465	51.9		Benzoate-CoA ligase	6.E-119	53	ACP50613	*Desulfococcus multivorans*
BF_5966	**(16.9)[a] (16)[b]**								
ORF 25	BF_5966_12885	199	21.8		Probable UbiX-like carboxylase subunit	2.E-72	65	YP_158781	*Aromatoleum aromaticum* EbN1
ORF 26	BF_5966_16788	84	9.9	*ppcD*	Phenylphosphate carboxylase, delta subunit	1.E-13	43	YP_158783	*Aromatoleum aromaticum* EbN1
ORF 27	BF_5966_3804	524	58.0		Benzoate-CoA ligase	7.E-148	48	YP_001212095	*Pelotomaculum thermopropionicum*
BF_5976	**(23.1)[a] (32)[b]**								
ORF 28	BF_5976_700	264	25.9		Putative FAD-binding subunit of oxidoreductase	3.E-74	56	ZP_02948940	*Clostridium butyricum*
BF_5978	**(4.8)[a] (5)[b]**								
ORF 29	BF_5978_1456	479	53.2	*ppcA*	Phenylphosphate carboxylase, alpha subunit	4.E-177	64	YP_158784	*Aromatoleum aromaticum* EbN1
ORF 30	BF_5978_3331	163	19.8	*ppcC*	Phenylphosphate carboxylase, gamma subunit	2.E-36	47	CAC12689	*Aromatoleum aromaticum* EbN1
ORF 31	BF_5978_4461	366	40.7	*ppcB*	Phenylphosphate carboxylase, beta subunit	2.E-150	67	YP_158786	*Aromatoleum aromaticum* EbN1
BF_5983	**(4.5)[a] (4)[b]**								
ORF 32	BF_5983_4123	636	72.4	*bamB*	Aldehyde ferredoxin oxidoreductase	0.E+00	72	YP_384756	*Geobacter metallireducens* GS-15
ORF 33	BF_5983_16635	397	44.3	*bamI*	Formate dehydrogenase, alpha subunit	1.E-130	61	ZP_04353073	*Desulfotomaculum acetoxidans*
BF_6004	**(2.8)[a] (4)[b]**								
ORF 34	BF_6004_1327	289	30.9	*pcmR*	Molybdopterin dehydrogenase, FAD-binding	1.E-84	58	YP_002535583	*Geobacter* sp. FRC-32
ORF 35	BF_6004_346	114	12.2	*pcmS*	4-hydroxybenzoyl-CoA reductase, gamma subunit	7.E-44	70	ZP_03022589	*Geobacter* sp. M21
BF_6099	**(4.7)[a] (9)[b]**								

Table A2 (continued):

ORF 36	BF_6099_3163	199	22.1	*orf3*	Protein stimulating phenylphosphate synthetase	3.E-17	28	YP_158787	*Aromatoleum aromaticum* EbN1
BF_6109	**(3.6)[a] (5)[b]**								
ORF 37	BF_6109_1906	220	24.7	*orf3*	Protein stimulating phenylphosphate synthetase	3.E-11	25	YP_158787	*Aromatoleum aromaticum* EbN1
BF_6226	**(2.6)[a] (6)[b]**								
ORF 38	BF_6226_3375	460	50.6	*orf11*	Transcriptional regulator, Fis family	3.E-129	66	ZP_01287867	*Delta-proteobacterium* MLMS-1
BF_6573	**(2.6)[a] (3)[b]**								
ORF 39	BF_6573_271	89	10.0		PEP-utilising enzyme, mobile region	8.E-25	74	ZP_01291309	*Delta-proteobacterium* MLMS-1
BF_6373	**(2.3)[a] (3)[b]**								
ORF 40	BF_6373_981	237	26.0	*ppcB*	Phosphoenolpyruvate synthase	6.E-85	76	ZP_01291313	*Delta-proteobacterium MLMS-1*
BF_6490	**(2.3)[a] (4)[b]**								
ORF 41	BF_6490_1499	315	33.0		Pyruvate ferredoxin/flavodoxin	1.E-121	75	ZP_01287130	*Delta- proteobacterium* MLMS-1
BF_6642	**(2.1)[a] (5)[b]**								
ORF 42	BF_6642_1587	180	19.8	*ppcC*	Phenylphosphate carboxylase, gamma subunit	2.E-19	48	YP_158785	*Aromatoleum aromaticum* EbN1
BF_6721	**(1.6)[a] (2)[b]**								
ORF 43	BF_6721_545	176	25.4	*ppsC*	Protein stimulating phenylphosphate synthetase	1.E-18	32	YP_158787	*Aromatoleum aromaticum* EbN1
BF_7046	**(1.2)[a] (3)[b]**								
ORF 44	BF_7046_928	225	24.8		Acetyl-CoA carboxylase, beta subunit	4.E-108	82	ZP_01288046	*Delta-proteobacterium* MLMS-1
BF_7915	**(0.7)[a] (3)[b]**								
ORF 45	BF_7915_3	93	10.2		PEP-utilising enzyme, mobile region	4.E-14	54	ZP_01291309	*delta proteobacterium* MLMS-1
BF_5926	**(0.3)[a] (1)[b]**								
ORF 46	BF_9633_2	106	11.4	*bamQ*	Zinc-containing alcohol dehydrogenase	4.E-16	45	ZP_01288429	*delta proteobacterium* MLMS-1
BF_11345	**(537.5)[a] (561)[b]**								
ORF 47	BF_11345_187031	221	24.5		Sigma 54 specific transcriptional regulator	9.E-21	32	YP_284243	*Dechloromonas aromatica*
ORF 48	BF_11345_187653	178	20.1	*bamC*	4Fe-4S ferredoxin, iron-sulfur binding protein	1.E-52	60	YP_002535686	*Geobacter* sp. FRC-32
ORF 49	BF_11345_189593	647	74.1	*bamB*	Aldehyde ferredoxin oxidoreductase	0.E+00	62	YP_002535701	*Geobacter* sp. FRC-32
ORF 50	BF_11345_191335	581	65.0		Molybdopterin oxidoreductase	2.E-149	44	YP_002535624	*Geobacter* sp. FRC-32
ORF 51	BF_11345_191637	80	8.8		Molybdopterin oxidoreductase	3.E-14	46	YP_001381272	*Anaeromyxobacter* sp.
ORF 52	BF_11345_191853	67	7.2	*bamH*	Respiratory-chain NADH dehydrogenase domain	1.E-16	64	YP_002535707	*Geobacter* sp. FRC-32
ORF 53	BF_11345_192359	143	15.9	*bamF*	Methyl-viologen hydrogenase, delta subunit	2.E-45	56	YP_002535705	*Geobacter* sp. FRC-32
ORF 54	BF_11345_194041	535	58.6	*bamE*	4Fe-4S ferredoxin, iron-sulfur binding protein	0.E+00	62	YP_002535704	*Geobacter* sp. FRC-32
ORF 55	BF_11345_195234	373	41.8	*bamE*	4Fe-4S ferredoxin, iron-sulfur binding protein	1.E-148	71	YP_461442	*Syntrophus aciditrophicus*

Table A2 (continued):

ORF 56	BF_11345_195531	76	8.0	*bamE*	4Fe-4S ferredoxin, iron-sulfur binding protein	2.E-26	85	YP_461442	*Syntrophus aciditrophicus*
ORF 57	BF_11345_196736	346	39.3	*bamD*	protein of unknown function DUF224	5.E-134	67	YP_002535687	*Geobacter* sp. FRC-32
ORF 58	BF_11345_197330	179	20.3	*bamI*	4Fe-4S ferredoxin, iron-sulfur binding protein	1.E-54	58	ZP_03022589	*Geobacter* sp. FRC-32
ORF 59	BF_11345_198171	282	32.9		Aldehyde ferredoxin oxidoreductase	3.E-116	69	YP_002535685	*Geobacter* sp. FRC-32
ORF 60	BF_11345_199289	373	41.5		Aldehyde ferredoxin oxidoreductase	6.E-156	70	YP_002535685	*Geobacter* sp. FRC-32
ORF 61	BF_11345_199937	132	15.2		Methylmalonyl CoA epimerase	2.E-24	63	YP_461917	*Syntrophus aciditrophicus*
ORF 62	BF_11345_200420	160	17.1		4-hydroxybenzoyl-CoA reductase, gamma subunit	3.E-49	60	YP_001212129	*Pelotomaculum thermopropionicum*
ORF 63	BF_11345_201456	324	35.2	*hbaD*	4-hydroxybenzoyl-CoA reductase, beta subunit	2.E-41	32	YP_530898	*Rhodopseudomonas palustris*
ORF 64	Bf_11345_203724	437	48.3		4-hydroxybenzoyl-CoA reductase, alpha subunit	1.E-102	44	YP_001212088	*Pelotomaculum thermopropionicum*
ORF 65	BF_11345_265559	595	64.8	*bamH*	Respiratory-chain NADH dehydrogenase domain	0.E+00	65	YP_002506555	*Clostridium cellulolyticum*
ORF 66	BF_11345_266189	178	19.3	*bamG*	NADH dehydrogenase (ubiquinone)	5.E-46	52	YP_001036769	*Clostridium thermocellum*
ORF 67	BF_11345_313117	293	32.2	*hcrB*	Xanthine dehydrogenase	3.E-123	74	YP_001212130	*Pelotomaculum thermopropionicum*
ORF 68	BF_11345_315899	259	27.6	*bamA*	Enoyl-CoA hydratase	1.E-78	56	YP_461962	*Syntrophus aciditrophicus*
ORF 69	BF_11345_318920	360	41.7	*hgdA*	2-hydroxyglutaryl-CoA dehydratase, alpha subunit	9.E-72	38	NP_070782	*Archaeoglobus fulgidus*
ORF 70	BF_11345_455533	326	37.6		2-hydroxyglutaryl-CoA dehydratase	1.E-132	67	YP_001211338	*Pelotomaculum thermopropionicum*
BF_11348	**(61.8)[a] (72)[b]**								
ORF 71	BF_11348_21729	664	74.5	*bamB*	Aldehyde ferredoxin oxidoreductase	0.E+00	83	YP_002535685	*Geobacter* sp. FRC-32
ORF 72	BF_11348_33092	117	12.5		Probable UbiX-like carboxylase subunit,	2.E-36	65	YP_158781	*Aromatoleum aromaticum* EbN1
ORF 73	BF_11348_33442	83	9.5		Probable aromatic acid decarboxylase	4.E-22	68	P57767	*Thauera aromatica*
ORF 74	BF_11348_33695	259	27.6		Short chain enoyl-CoA hydratase	8.E-53	44	YP_001192065	*Metallosphaera sedula*
ORF 75	BF_11348_34496	236	25.5		Acetyl-CoA acetyltransferase	8.E-63	51	YP_001747677	*Pseudomonas putida*
ORF 76	BF_11348_35416	78	86.3		Acetyl-CoA acetyltransferase	1.E-21	72	ZP_01170869	*Bacillus* sp.
ORF 77	BF_11348_35704	95	10.1	*PcmS*	(2Fe-2S)-binding domain protein	6.E-33	73	YP_002535582	*Geobacter* sp. FRC-32
ORF 78	BF_11348_36219	762	83.7	*pcmT*	4-hydroxybenzoyl-CoA reductase, alpha subunit	0.E+00	72	ZP_03023307	*Geobacter* sp. FRC-32
ORF 79	BF_11348_38566	570	66.3	*ppsA*	Phenylphosphate synthase, alpha subunit	9.E-74	30	YP_644349	*Rubrobacter xylanophilus*
ORF 80	BF_11348_40340	472	52.6	*ppcB*	Phenylphosphate carboxylase, beta subunit	6.E-178	61	YP_158786	*Aromatoleum aromaticum* EbN1
ORF 81	BF_11348_41820	179	19.8	*ppcC*	Phenylphosphate carboxylase, gamma subunit	6.E-38	47	YP_158785	*Aromatoleum aromaticum* EbN1
ORF 82	BF_11348_42371	478	52.8	*ppcA*	Phenylphosphate carboxylase, alpha subunit	1.E-174	61	YP_158784	*Aromatoleum aromaticum* EbN1
ORF 83	BF_11348_43821	82	9.4	*ppcD*	Phenylphosphate carboxylase, delta subunit	2.E-14	44	YP_158783	*Aromatoleum aromaticum* EbN1
ORF 84	BF_11348_44136	545	61.1		Acetyl-CoA synthetase	3.E-79	36	ZP_04038819	*Meiothermus ruber*
ORF 85	BF_11348_45786	289	30.9	*pcmR*	Molybdopterin dehydrogenase, FAD-binding domain	8.E-86	59	YP_002535480	*Geobacter* sp. FRC-32

Table A2 (continued):

ORF 86	BF_11348_48177	652	74.2	*bamB*	Aldehyde ferredoxin oxidoreductase	0.E+00	73	YP_002535685	*Geobacter* sp. FRC-32
BF_11352	**(34.6)[a] (31)[b]**								
ORF 87	BF_11352_31718	514	57.0	*bamY*	Benzoate-CoA ligase	2.E-157	54	YP_385097	*Geobacter metallireducens*
BF_11355	**(178.3)[a] (180)[b]**								
ORF 88	BF_11355_137237	456	50.6	*orf11*	Transcriptional regulator, Fis family	5.E-165	63	ZP_02911906	*Geobacillus* sp.
BF_11367	**(59.3)[a] (72)[b]**								
ORF 89	BF_11367_37510	493	55.0	*ppsB*	Phenylphosphate synthase, beta subunit	2.E-147	52	ZP_04354112	*Desulfotomaculum acetoxidans*
BF_11370	**(2.9)[a] (4)[b]**								
ORF 90	BF_11370_11171	587	66.8		UbiD-like carboxylase subunit	8.E-161	50	ZP_01290783	*Delta-proteobacterium*
BF_11386	**(87.3)[a] (95)[b]**								
ORF 91	BF_11386_456	197	22.1	*bamI*	4Fe-4S ferredoxin, iron-sulfur binding protein	1.E-81	74	YP_385033	*Geobacter metallireducens*
BF_11388	**(21.5)[a] (25)[b]**								
ORF 92	BF_11388_14096	399	46.2	*hgdA*	2-hydroxyglutaryl-CoA dehydratase, alpha subunit	7.E-94	42	YP_002605523	*Desulfobacterium autotrophicum*
ORF 93	BF_11388_15452	589	64.3		Indolepyruvate ferredoxin oxidoreductase	1.E-166	55	YP_001230896	*Geobacter uraniireducens*
BF_11393	**(56.2)[a] (63)[b]**								
ORF 94	BF_11393_8876	652	74.4	*bamB*	Aldehyde ferredoxin oxidoreductase	0.E+00	67	YP_002535685	*Geobacter* sp. FRC-32
ORF 95	BF_11393_32034	376	42.4	*bzdY*	6-oxocyclohex-1-ene-1-carbonyl-CoA hydratase	3.E-148	66	AAQ08805	*Azoarcus* sp. CIB
ORF 96	BF_11393_33346	171	18.5	*bzdW*	Putative dienoyl-CoA hydratase	3.E-42	51	CAD21636	*Azoarcus evansii*
ORF 97	BF_11393_33896	70	8.0	*bzdW*	Enoyl-CoA hydratase	7.E-03	35	CAD21628	*Azoarcus evansii*
ORF 98	BF_11393_34473	183	20.1	*bzdX*	6-hydroxycylohex-1-ene-1-carboxyl-CoA dehydrogenase	4.E-51	61	CAI78830	uncultured bacterium
ORF 99	BF_11393_35143	138	15.2		6-hydroxycylohex-1-ene-1-carboxyl-CoA dehydrogenase	4.E-32	45	CAI78830	uncultured bacterium
ORF 100	BF_11393_37778	548	63.9		Carboxyl transferase	0.E+00	75	YP_002535711	*Geobacter* sp. FRC-32
ORF 101	BF_11393_45423	385	42.0	*bamM*	Acyl-CoA dehydrogenase domain protein	1.E-160	69	ZP_03023273	*Geobacter* sp. M21
BF_11401	**(0.4)[a] (1)[b]**								
ORF 102	BF_11401_136	99	10.7		Aldehyde oxidase and xanthine dehydrogenase	5.E-16	46	YP_530898	*Rhodopseudomonas palustris*
BF_11402	**(109.7)[a] (123)[b]**								
ORF 103	BF_11402_616	156	16.4	*hcrC*	4-hydroxybenzoyl-CoA reductase, gamma subunit	1.E-47	62	CAI78850	uncultured bacterium
BF_11406	**(57.4)[a] (60)[b]**								
ORF 104	BF_11406_45912	84	9.8	*ppcD*	Phenylphosphate carboxylase, delta subunit	3.E-14	40	YP_158783	*Aromatoleum aromaticum* EbN1

Table A2 (continued):

ORF 105	BF_11406_47362	475	52.9	*ppcA*	Phenylphosphate carboxylase, alpha subunit	2.E-167	59	YP_158784	*Aromatoleum aromaticum* EbN1
ORF 106	BF_11406_47871	179	18.1	*ppcC*	Phenylphosphate carboxylase, gamma subunit	1.E-34	47	YP_158785	*Aromatoleum aromaticum* EbN1
ORF 107	BF_11406_48783	270	30.2	*ppcB*	Phenylphosphate carboxylase, beta subunit	2.E-93	57	YP_158786	*Aromatoleum aromaticum* EbN1
ORF 108	BF_11406_49148	113	12.9	*ppcB*	Phenylphosphate carboxylase, beta subunit	1.E-31	58	YP_158786	*Aromatoleum aromaticum* EbN1
ORF 109	BF_11406_49356	71	8.0	*ppcB*	Phenylphosphate carboxylase, beta subunit	1.E-05	47	YP_158786	*Aromatoleum aromaticum* EbN1
ORF 110	BF_11406_51227	571	65.9	*ppsA*	Phenylphosphate synthase alpha subunit	7.E-68	30	YP_644349	*Rubrobacter xylanophilus*
ORF 111	BF_11406_53089	558	62.6		2-aminobenzoate-CoA ligase	2.E-92	37	YP_462907	*Syntrophus aciditrophicus* SB
ORF 112	BF_11406_54496	427	48.6		Transcriptional regulator, CdaR	4.E-35	28	YP_919270	*Nocardioides* sp.
ORF 113	BF_11406_55020	165	18.5		Metal dependent phosphohydrolase	1.E-10	35	YP_001230704	*Geobacter uraniireducens*
ORF 114	BF_11406_55472	51	59.6	*ppcD*	Phenylphosphate carboxylase, delta subunit	3.E-04	40	YP_158783	*Aromatoleum aromaticum* EbN1
ORF 115	BF_11406_56921	481	53.2	*ppcA*	Phenylphosphate carboxylase, alpha subunit	4.E-160	59	YP_158784	*Aromatoleum aromaticum* EbN1
ORF 116	BF_11406_57403	154	17.1	*ppcC*	Phenylphosphate carboxylase, gamma subunit	4.E-32	46	YP_158785	*Aromatoleum aromaticum* EbN1
BF_11411	**(42.6)[a] (46)[b]**								
ORF 117	BF_11411_1252	199	21.7		3-polyprenyl-4-hydroxybenzoate decarboxylase	8.E-67	64	YP_160255	*Aromatoleum aromaticum* EbN1
ORF 118	BF_11411_3087	596	66.0	*ppsA*	PEP-utilising enzyme, mobile region	3.E-65	30	YP_644349	*Rubrobacter xylanophilus*
ORF 119	BF_11411_607	196	22.1	*ppcB*	Phenylphosphate carboxylase, beta subunit	1.E-59	59	YP_158786	*Aromatoleum aromaticum* EbN1
BF_11415	**(6.7)[a] (11)[b]**								
ORF 120	BF_11415_4669	302	33.2		Probable UbiD-like carboxylase	6.E-52	41	YP_158782	*Aromatoleum aromaticum* EbN1
BF_11352	**(4.1)[a] (5)[b]**								
ORF 121	BF_11416_3	421	47.4	*ppcA*	Phenylphosphate carboxylase, alpha subunit	1.E-45	31	YP_158784	*Aromatoleum aromaticum* EbN1
ORF 122	BF_11416_1326	206	23.0		3-polyprenyl-4-hydroxybenzoate carboxy-lyase	1.E-19	31	ZP_04376160	*Catenulispora acidiphila*
ORF 123	BF_11416_2012	286	32.5	*ppcA*	Phenylphosphate carboxylase, alpha subunit	7.E-24	30	YP_158784	*Aromatoleum aromaticum* EbN1
BF_11418	**(40.4)[a] (47)[b]**								
ORF 124	BF_11418_237	515	58.3		3-octaprenyl-4-hydroxybenzoate carboxy-lyase	4.E-98	41	YP_138144	*Haloarcula marismortui*
ORF 125	BF_11418_1866	98	11.4		Hypothetical protein	8.E-02	35	XP_757560	*Ustilago maydis*
ORF 126	BF_11418_2188	498	56.1		Hypothetical protein	1.E-44	29	ZP_04375589	*Catenulispora acidiphila*
ORF 127	BF_11418_3776	309	34.2		ATPase-Multidrug resistance protein (MRP)	8.E-57	44	YP_001436025	*Lgnicoccus hospitalis*
ORF 128	BF_11418_4749	307	33.3		Hypothetical protein	2.E-54	41	NP_247256	*Methanocaldococcus jannaschii*
ORF 129	BF_11418_5748	93	10.6		Hypothetical protein	2.E+00	37	YP_002297672	*Rhodospirillum centenum*
ORF 130	BF_11418_6160	67	8.0		Putative regulatory protein, FmdB family	2.E-07	44	ZP_03330044	*Thermotogales bacterium*
ORF 131	BF_11418_6396	145	16.4		Hypothetical protein	3.E-22	40	YP_158780	*Aromatoleum aromaticum* EbN1

Table A2 (continued):

ORF 132	BF_11418_6917	224	25.4		Hypothetical protein	3.E-18	48	NP_143765	*Pyrococcus horikoshii*
ORF 133	BF_11418_7652	131	15.2		Hypothetical protein	3.E+00	28	AAP35074	*Dermatophagoides farinae*
ORF 134	BF_11418_8161	203	21.9		ATPase involved in chromosome partitioning	2.E-31	42	YP_001736462	*Korarchaeum cryptofilum*
ORF 135	BF_11418_8795	103	11.1		ParA family protein	8.E-14	47	NP_952460	*Geobacter sulfurreducens*
ORF 136	BF_11418_9197	317	34.2		ATPase-Multidrug resistance protein (MRP)	3.E-55	42	YP_002352437	*Dictyoglomus turgidum*
ORF 137	BF_11418_10381	126	14.7	*ppcD*	Phenylphosphate carboxylase, delta subunit	2.E-02	37	YP_158783	*Aromatoleum aromaticum* EbN1
ORF 138	BF_11418_10793	508	57.4	*ppcA*	Phenylphosphate carboxylase, alpha subunit	2.E-106	43	YP_158784	*Aromatoleum aromaticum* EbN1
ORF 139	BF_11418_12380	541	60.7	*bamY*	Benzoate-CoA ligase	2.E-168	56	YP_385097	*Geobacter metallireducens*
ORF 140	BF_11418_14073	153	16.8		Probable UbiX-like carboxylase subunit	2.E-52	68	P57767	*Thauera aromatica*
ORF 141	BF_11418_14755	337	38.3		Transcriptional regulator, MarR family	5.E-12	30	YP_001114630	*Desulfotomaculum reducens*
ORF 142	BF_11418_16549	154	19.2		Transcriptional regulator, MarR family	2.E-10	29	YP_001114630	*Desulfotomaculum reducens*
BF_11425	**(80.4)[a] (96)[b]**								
ORF 143	BF_11425_1906	522	58.4		Benzoate-CoA ligase	6.E-169	58	ACP50613	*Desulfococcus multivorans*
BF_11429	**(45.5)[a] (53)[b]**								
ORF 144	BF_11429_1226	383	43.1	*bamD*	Protein of unknown function DUF224	3.E-163	68	YP_460522	*Geobacter* sp. FRC-32
ORF 145	BF_11429_42632	334	36.6		Phosphoenolpyruvate synthase	1.E-94	53	YP_919996	*Thermofilum pendens*
ORF 146*	BF_11429_45028	168	18.9	*ppcB*	Phenylphosphate carboxylase, beta subunit	3.E-58	62	YP_158786	*Aromatoleum aromaticum* EbN1
BF_11433	**(1.7)[a] (3)[b]**								
ORF 147*	BF_11433_1	245	27.4	*ppcB*	Phenylphosphate carboxylase, beta subunit	6.E-97	66	YP_158786	*Aromatoleum aromaticum* EbN1
ORF 148	BF_11433_777	154	19.9	*ppcC*	Phenylphosphate carboxylase, gamma subunit	4.E-35	47	YP_158785	*Aromatoleum aromaticum* EbN1
BF_11434	**(0.9)[a] (1)[b]**								
ORF 149	BF_11434_2	278	30.7	*ppcA*	Phenylphosphate carboxylase, alpha subunit	2.E-99	60	YP_158784	*Aromatoleum aromaticum* EbN1
BF_11437	**(3.6)[a] (4)[b]**								
ORF 150	BF_11437_246	86	10.2	*ppcD*	Phenylphosphate carboxylase, delta subunit	2.E-11	38	YP_158783	*Aromatoleum aromaticum* EbN1
ORF 151	BF_11437_631	445	48.9		Probable UbiD-like carboxylase subunit	1.E-101	45	YP_158782	*Aromatoleum aromaticum* EbN1
BF_11447	**(3.6)[a] (4)[b]**								
ORF 152	BF_11447_30914	102	11.5	*bamF*	Methyl-viologen-reducing hydrogenase, delta subunit	5.E-09	50	YP_002535689	*Geobacter* sp. FRC-32
BF_11449	**(3.8)[a] (5)[b]**								
ORF 153	BF_11449_3102	281	41.1	*bamE*	4Fe-4S ferredoxin, iron-sulfur binding protein	1.E-150	66	YP_002535688	*Geobacter* sp. FRC-32
ORF 154	BF_11449_577	191	21.5	*bamF*	Methyl-viologen- hydrogenase, delta subunit	2.E-53	52	YP_002535705	*Geobacter* sp. FRC-32

Table A2 (continued):

BF_11452	**(64.8)[a] (63)[b]**								
ORF 155	BF_11452_2886	654	74.3	*bamB*	Aldehyde ferredoxin oxidoreductase	0.E+00	73	YP_002535685	*Geobacter* sp. FRC-32
ORF 156	BF_11452_29919	355	39.6	*ppsB*	phenylphosphate synthase, beta subunit	5.E-72	42	ABN80333	*Desulfobacterium* sp.
ORF 157	BF_11452_935	184	20.7	*bamC*	4Fe-4S ferredoxin, iron-sulfur binding protein	1.E-54	57	YP_384757	*Geobacter metallireducens*
BF_11460	**(114.2)[a] (1121)[b]**								
ORF 158	BF_11460_64269	251	28.0		4-hydroxybenzoate-CoA ligase / benzoate-CoA ligase	7.E-82	64	YP_460853	*Syntrophus aciditrophicus*
ORF 159	BF_11460_73605	637	72.6	*bamB*	Aldehyde ferredoxin oxidoreductase	0.E+00	70	YP_002535685	*Geobacter* sp. FRC-32
ORF 160	BF_11460_75531	180	20.1	*bamC*	4Fe-4S ferredoxin, iron-sulfur binding protein	5.E-50	63	ZP_03022589	*Geobacter* sp. FRC-32
ORF 161	BF_11460_88951	289	30.9	*pcmR*	Molybdopterin dehydrogenase, FAD-binding	1.E-54	57	YP_384757	*Geobacter metallireducens*
ORF 162	BF_11460_89839	165	17.5	*pcmS*	Ferredoxin:(2Fe-2S)-binding	6.E-68	75	YP_385089	*Geobacter metallireducens*
ORF 163	BF_11460_92723	405	45.2	*bamM*	Acyl-CoA dehydrogenase domain protein	2.E-116	51	YP_146298	*Geobacillus kaustophilus*
BF_11464	**(63.1)[a] (73)[b]**								
ORF 164	BF_11465_14747	178	20.1	*bamC*	4Fe-4S ferredoxin, iron-sulfur binding protein	3.E-52	64	YP_002535685	*Geobacter* sp. FRC-32
BF_11473	**(10.7)[a] (14)[b]**								
ORF 165	BF_11473_3811	205	23.9		Glycyl-radical enzyme activating protein family	3.E-75	62	YP_001921250	*Clostridium botulinum* E3
BF_11479	**(102.6)[a] (120)[b]**								
ORF 166	BF_11479_64833	603	64.7		Respiratory-chain NADH dehydrogenase domain	0.E+00	67	ZP_03729943	*Dethiobacter alkaliphilus* AHT 1
ORF 167	BF_11479_65215	129	13.8	*bamG*	NADH dehydrogenase (ubiquinone)	2.E-39	67	ZP_03729874	*Dethiobacter alkaliphilus* AHT 1
BF_11492	**(155.7)[a] (194)[b]**								
ORF 168	BF_11492_155188	365	40.5	*bamI*	4Fe-4S ferredoxin, iron-sulfur binding protein	8.E-38	29	YP_002430370	*Geobacter metallireducens*
BF_11494	**(107)[a] (123)[b]**								
ORF 169	BF_11494_7273	573	63.4	*orfII*	Transcriptional regulator, Fis family	0.E+00	61	YP_001211905	*Pelotomaculum thermopropionicum*
ORF 170	BF_11494_9862	52	5.9	*bbsA*	SubName: Full=*bbsA*	3.E-03	43	AAF89836	*Thauera aromatica*
ORF 171	BF_11494_10048	381	40.4	*bbsB*	SubName: Full=Putative thiolase	3.E-99	51	ACL11821	*Mycobacterium brisbanense*
ORF 172	BF_11494_111231	251	26.7	*bbsD*	2-[hydroxy(Phenyl)methyl]-succinyl-CoA dehydro	1.E-74	51	YP_158076	*Aromatoleum aromaticum* EbN1
ORF 173	BF_11494_12795	77	8.7	*bbsE*	SubName: Full=*bbsE*	2.E-11	49	AAF89840	*Thauera aromatica*
ORF 174	BF_11494_15272	399	45.4	*bbsG*	Full=Acyl-CoA dehydrogenase domain	2.E-128	55	YP_002537876	*Geobacter* sp. FRC-32
ORF 175	BF_11494_16498	255	27.6	*bbsF*	Full=Putative E-phenylitaconyl-CoA hydratase	2.E-83	61	YP_158071	*Aromatoleum aromaticum* EbN1
BF_11500	**(72.1)[a] (77)[b]**								
ORF 176	BF_11500_28122	360	38.5		Benzyl alcohol dehydrogenase	3.E-161	74	ZP_01169860	*Bacillus* sp.

Table A2 (continued):

BF_11515	**(71.7)[a] (85)[b]**								
ORF 177	BF_11515_17560	351	37.4		Probable zinc-containing alcohol dehydrogenase	1.E-122	62	ZP_03697404	*Lutiella nitroferrum*
ORF 178	BF_11515_54022	732	82.0	*bamF*	Acetyl-CoA decarbonylase, beta subunit	0.E+00	74	YP_360060	*Carboxydothermus hydrogenoformans*
BF_11517	**(6.4)[a] (9)[b]**								
ORF 179	BF_11517_3906	129	14.6		3-octaprenyl-4-hydroxybenzoate carboxy-lyase	2.E-08	33	YP_002829391	*Sulfolobus islandicus*
BF_11522	**(88.2)[a] (90)[b]**								
ORF 180	BF_11522_61196	659	72.9		Acetophenone carboxylase	3.E-155	42	YP_158348	*Aromatoleum aromaticum* EbN1
ORF 181	BF_11522_6293	447	48.5		Benzoate transporter	3.E-86	45	YP_430616	*Moorella thermoacetica*
BF_11546	**(46.8)[a] (48)[b]**								
ORF 182	BF_11546_950	315	35.1		UbiD-like carboxylase subunit	2.E-47	38	gb_ACC80490	*Nostoc punctiforme*
BF_11550	**(27.4)[a] (30)[b]**								
ORF 183	BF_11550_8887	161	17.5		(2Fe-2S)-binding protein	2.E-45	52	ZP_03650477	*Clostridiales* bacterium
ORF 184	BF_11550_9899	339	36.6	*hbaD*	4-hydroxybenzoyl-CoA reductase, beta subunit	1.E-47	37	NP_946025	*Rhodopseudomonas palustris*
ORF 185	BF_11550_10462	185	19.5	*hbaC*	4-hydroxybenzoyl-CoA reductase, alpha subunit	3.E-28	40	ABJ04548	*Rhodopseudomonas palustris*
ORF 186	BF_11550_12177	543	58.7	*pcmT*	4-hydroxybenzoyl-CoA reductase, alpha subunit	3.E-109	40	YP_002535581	*Geobacter* sp. FRC-32
ORF 187	BF_11550_13758	516	57.6	*bamY*	Benzoate-CoA ligase family	7.E-151	49	YP_385097	*Geobacter* sp. FRC-32
BF_11554	**(34.8)[a] (44)[b]**								
ORF 188	BF_11554_7745	289	32.1		UbiD-like carboxylase subunit	2.E-111	66	CAI10592	*Azoarcus* sp.
ORF 189	BF_11554_15475	477	52.7		Carboxylyase-related protein	5.E-65	34	YP_002822423	*Rhizobium* sp.
ORF 190	BF_11554_18813	334	37.2		UbiD-, 3-octaprenyl-4-hydroxybenzoate carboxy-lyase	5.E-30	30	YP_297275	*Ralstonia eutropha*
ORF 191	BF_11554_19805	135	15.0		3-octaprenyl-4-hydroxybenzoate carboxy-lyase	2.E-09	34	YP_002499955	*Methylobacterium nodulans*
ORF 192	BF_11554_20212	54	6.3		Hypothetical protein	2.E+00	39	XP_002155012	*Hydra magnipapillata*
ORF 193	BF_11554_20474	657	73.1		Sigma 54 specific transcriptional regulator	2.E-124	40	YP_360088	*Carboxydothermus hydrogenoformans*
ORF 194	BF_11554_22461	521	57.7	*bamY*	Benzoate-CoA ligase family	7.E-168	55	YP_002535674	*Geobacter* sp. FRC-32
ORF 195	BF_11554_31496	117	13.3		Transcriptional regulator, IclR family	7.E-13	37	YP_001232370	*Geobacter uraniireducens*
ORF 196	BF_11554_31924	131	14.3		Transcriptional regulator, IclR family	8.E-15	34	YP_360107	*Carboxydothermus hydrogenoformans*
ORF 197	BF_11554_34403	774	85.0	*pcmT*	4-hydroxybenzoyl-CoA reductase, alpha subunit	0.E+00	77	YP_002535581	*Geobacter* sp. FRC-32
ORF 198	BF_11554_34808	129	13.8	*pcmS*	Ferredoxin:(2Fe-2S)-binding protein	2.E-48	74	YP_002535582	*Geobacter* sp. FRC-32
BF_11555	**(0.5)[a] (0)[b]**								

Table A2 (continued):

ORF 199	BF_11555_509	167	17.8	*pcmS*	Ferredoxin:(2Fe-2S)-binding protein	2.E-67	70	ZP_03023308	*Geobacter* sp. M21
BF_11556	**(5.1)[a] (5)[b]**								
ORF 200	BF_11556_1106	218	23.0	*pcmR*	Molybdopterin dehydrogenase, FAD-binding	1.E-59	60	YP_002535583	*Geobacter sp.* FRC-32
ORF 201	BF_11556_4755	773	84.9	*pcmT*	4-hydroxybenzoyl-CoA reductase, alpha subunit	0.E+00	76	YP_002535581	*Geobacter sp.* FRC-32
BF_11563	**(59.4)[a] (77)[b]**								
ORF 202	BF_11563_26098	156	16.6	*pcmS*	Ferredoxin:(2Fe-2S)-binding protein	4.E-46	56	YP_385089	*Geobacter metallireducens*
ORF 203	BF_11563_26571	187	20.6	*pcmT*	4-hydroxybenzoyl-CoA reductase, alpha subunit	4.E-38	50	YP_728905	*Ralstonia eutropha*
BF_11571	**(52.4)[a] (60)[b]**								
ORF 204	BF_11571_28857	161	17.8	*bamG*	NADH dehydrogenase (ubiquinone)	2.E-56	65	YP_001213199	*Pelotomaculum thermopropionicum*
ORF 205	BF_11571_29773	597	64.3	*bamH*	Respiratory-chain NADH dehydrogenase domain	0.E+00	72	ZP_01665951	*Thermosinus carboxydivorans*

Data used for figure 4.1a and b

Carbon isotope ratio $\delta^{13}C/^{12}C$ measured and anaerobic benzene degradation (%) for two and three parallel incubations of the iron- and sulfate-reducing cultures, respectively.

Iron-reducing culture (BF)			Sulfate-reducing culture (BPL)		
	Benzene degradation (%)	$\delta^{13}C/^{12}C$ (‰)		Benzene degradation (%)	$\delta^{13}C/^{12}C$ (‰)
S1	0.0	-26.02	**S1**	0.0	-25.739
	21.8	-26.03		-5.7	-25.872
	9.3	-26.01		-8.9	-25.768
	24.1	-25.62		17.6	-25.073
	25.8	-25.33		32.3	-24.646
	28.7	-24.95		52.1	-23.62
	47.2	-25.02		75.2	-22.593
	50.1	-24.35		89.0	-20.77
	70.3	-23.53	**S2**	0.0	-26.147
	80.1	-21.72		-6.7	-25.719
	90.2	-19.34		7.4	-25.884
S2	0.0	-25.54		9.0	-25.151
	5.5	-25.46		36.7	-24.541
	6.4	-25.45		56.5	-23.55
	11.4	-25.46		78.7	-21.916
	22.6	-25.36		91.5	-19.47
	26.5	-25.60	**S3**	0.0	-26.214
	40.0	-24.98		4.3	-25.831
	38.1	-24.79		-0.2	-26.064
	54.1	-24.69		15.8	-25.06
	56.3	-23.99		36.5	-24.584
	57.4	-23.50		58.0	-23.45
	81.1	-20.13		80.6	-22.036
				91.8	-19.92

Data used for figure 4.1c and d

Data used for predicting of the carbon enrichment factors (ε_C) during anaerobic benzene degradation by two and three parallel incubations of the iron- and sulfate-reducing cultures, respectively. (A, C) = The uncertainty (5%) of the benzene concentration measurements. (B, D) = The reproducibility (0.25‰) of carbon isotope measurements.

Iron-reducing culture (BF)				Sulfate-reducing culture (BPL)			
$\ln(C_t/C_o)$	A	$\ln(R_t/R_0)$	B	$\ln(C_t/C_o)$	C	$\ln(R_t/R_0)$	D
S1 0.0	0.1	0	2.50E-04	**S1** 0.0	0.1	0	2.50E-04
-0.24631	0.1	-1.23E-05	2.50E-04	0.05551	0.1	-1.37E-04	2.50E-04
-0.09788	0.1	7.49E-06	2.50E-04	0.0851	0.1	-2.98E-05	2.50E-04
-0.14637	0.1	4.13E-04	2.50E-04	-0.19355	0.1	6.83E-04	2.50E-04

-0.29788	0.1	7.02E-04	2.50E-04	-0.39052	0.1	0.00112	2.50E-04
-0.33821	0.1	0.0011	2.50E-04	-0.73612	0.1	0.00217	2.50E-04
-0.63777	0.1	0.00102	2.50E-04	-1.39624	0.1	0.00322	2.50E-04
-0.69489	0.1	0.00171	2.50E-04	-2.208	0.1	0.00509	2.50E-04
-1.21507	0.1	0.00255	2.50E-04	**S2** 0.0	0.1	0	2.50E-04
-1.61486	0.1	0.0044	2.50E-04	0.06496	0.1	4.39E-04	2.50E-04
-2.31938	0.1	0.00683	2.50E-04	-0.07724	0.1	2.70E-04	2.50E-04
S2 0.0	0.1	0	2.50E-04	-0.0947	0.1	0.00102	2.50E-04
-0.05618	0.1	8.52E-05	2.50E-04	-0.45778	0.1	0.00165	2.50E-04
-0.1215	0.1	9.65E-05	2.50E-04	-0.83231	0.1	0.00266	2.50E-04
-0.06585	0.1	8.52E-05	2.50E-04	-1.54429	0.1	0.00434	2.50E-04
-0.30761	0.1	1.87E-04	2.50E-04	-2.46637	0.1	0.00683	2.50E-04
-0.51163	0.1	-5.54E-05	2.50E-04	**S3** 0.0	0.1	0	2.50E-04
-0.2567	0.1	5.83E-04	2.50E-04	-0.04409	0.1	3.93E-04	2.50E-04
-0.48043	0.1	7.74E-04	2.50E-04	0.00243	0.1	1.54E-04	2.50E-04
-0.77823	0.1	8.73E-04	2.50E-04	-0.17169	0.1	0.00118	2.50E-04
-0.82762	0.1	0.00159	2.50E-04	-0.45443	0.1	0.00167	2.50E-04
-0.8525	0.1	0.00209	2.50E-04	-0.86742	0.1	0.00283	2.50E-04
-1.6653	0.1	0.00554	2.50E-04	-1.6395	0.1	0.00428	2.50E-04
				-2.50468	0.1	0.00644	2.50E-04

Data used for figure 4.2a and b

Hydrogen isotope ratio $\delta^2H/^1H$ measured and anaerobic benzene degradation (%) for two and three parallel incubations of the iron- and sulfate -reducing cultures, respectively.

Iron-reducing culture (BF)			**Sulfate-reducing culture (BPL)**		
	Benzene degradation (%)	**$\delta^2H/^1H$ (‰)**		**Benzene degradation (%)**	**$\delta^2H/^1H$ (‰)**
S1	0.0	-155.00	**S1**	0.0	-156.10
	21.8	-156.97		-5.7	-159.70
	9.3	-154.84		-8.9	-157.30
	24.1	-152.81		17.6	-139.40
	25.8	-146.81		32.3	-128.10
	28.7	-142.37		52.1	-106.30
	47.2	-140.00		75.2	-80.10
	50.1	-134.47		89.0	-43.90
	70.3	-115.83	**S2**	0.0	-157.70
	80.1	-88.54		-6.7	-151.70
	90.2	-38.97		7.4	-155.60
S2	0.0	-156.29		9.0	-142.30
	5.5	-156.01		36.7	-125.80
	6.4	-152.73		56.5	-101.50
	11.4	-153.68		78.7	-78.35
	22.6	-148.93		91.5	-40.96
	26.5	-149.15	**S3**	0.0	-158.50
	40.0	-143.09		4.3	-160.10
	38.1	-138.88		-0.2	-155.20
	54.1	-131.88		15.8	-140.40
	56.3	-124.67		36.5	-121.80

57.4 81.1	-115.66 -53.28	58.0 80.6 91.8	-102.60 -58.10 -36.75

Data used for figure 4.2c and d

Data used for predicting of the hydrogen enrichment factors (ε_H) during anaerobic benzene degradation by two and three parallel incubations of the iron- and sulfate-reducing cultures, respectively. (A, C) = The uncertainty (5%) of the benzene concentration measurements. (B, D) = The reproducibility (5‰) of hydrogen isotope measurements.

Iron-reducing culture (BF)				**Sulfate-reducing culture (BPL)**			
ln(C_t/C_o)	**A**	**ln(R_t/R_0)**	**B**	**ln(C_t/C_o)**	**C**	**ln(R_t/R_0)**	**D**
S1 0.0	0.1	0.0	0.005	**S1** 0.0	0.1	0.0	0.005
-0.24631	0.1	-0.00233	0.005	0.05551	0.1	-0.00428	0.005
-0.09788	0.1	1.92E-04	0.005	0.0851	0.1	-0.00142	0.005
-0.14637	0.1	0.00258	0.005	-0.19355	0.1	0.0196	0.005
-0.29788	0.1	0.00965	0.005	-0.39052	0.1	0.03264	0.005
-0.33821	0.1	0.01484	0.005	-0.73612	0.1	0.05734	0.005
-0.63777	0.1	0.0176	0.005	-1.39624	0.1	0.08623	0.005
-0.69489	0.1	0.024	0.005	-2.208	0.1	0.12483	0.005
-1.21507	0.1	0.04531	0.005	**S2** 0.0	0.1	0.0	0.005
-1.61486	0.1	0.07571	0.005	0.06496	0.1	0.0071	0.005
-2.31938	0.1	0.12867	0.005	-0.07724	0.1	0.00249	0.005
S2 0.0	0.1	0.0	0.005	-0.0947	0.1	0.01812	0.005
-0.05618	0.1	3.28E-04	0.005	-0.45778	0.1	0.03717	0.005
-0.1215	0.1	0.0042	0.005	-0.83231	0.1	0.06459	0.005
-0.06585	0.1	0.00308	0.005	-1.54429	0.1	0.09003	0.005
-0.30761	0.1	0.00868	0.005	-2.46637	0.1	0.1298	0.005
-0.51163	0.1	0.00842	0.005	**S3** 0.0	0.1	0.0	0.005
-0.2567	0.1	0.01552	0.005	-0.04409	0.1	-0.0019	0.005
-0.48043	0.1	0.02042	0.005	0.00243	0.1	0.00391	0.005
-0.77823	0.1	0.02851	0.005	-0.17169	0.1	0.02128	0.005
-0.82762	0.1	0.03679	0.005	-0.45443	0.1	0.04269	0.005
-0.8525	0.1	0.04703	0.005	-0.86742	0.1	0.06432	0.005
-1.6653	0.1	0.11519	0.005	-1.6395	0.1	0.11271	0.005
				-2.50468	0.1	0.13513	0.005

Data used for figure 4.3

Data used for plotting of the dual-isotope fractionation for carbon versus hydrogen during anaerobic benzene degradation by two and three parallel incubations of the iron- and sulfate-reducing cultures, respectively.(A, C) = The reproducibility (0.5 ‰) of the $\delta^{13}C$. (B, D) = The reproducibility (5‰) of the $\delta^{2}H$.

Iron-reducing culture (BF)				Sulfate-reducing culture (BPL)			
$\Delta\delta^{13}C$ (‰)	A	$\Delta\delta^{2}H$ (‰)	B	$\Delta\delta^{13}C$ (‰)	C	$\Delta\delta^{2}H$ (‰)	D
S1 0.0	0.5	0.00	5	**S1** 0.0	0.5	0.00	0
-0.012	0.5	-1.97	5	-0.133	0.5	-3.60	5
0.007	0.5	0.16	5	-0.029	0.5	-1.20	5
0.402	0.5	2.19	5	0.666	0.5	16.70	5
0.684	0.5	8.19	5	1.093	0.5	28.00	5
1.068	0.5	12.63	5	2.119	0.5	49.80	5
0.995	0.5	15.01	5	3.146	0.5	76.00	5
1.665	0.5	20.53	5	4.969	0.5	112.20	5
2.489	0.5	39.17	5	**S2** 0.0	0.5	0.00	5
4.296	0.5	66.46	5	0.428	0.5	6.00	5
6.678	0.5	116.03	5	0.263	0.5	2.10	5
S2 0.0	0.5	0.00	5	0.996	0.5	15.40	5
0.083	0.5	0.28	5	1.606	0.5	31.90	5
0.094	0.5	3.55	5	2.597	0.5	56.20	5
0.083	0.5	2.61	5	4.231	0.5	79.35	5
0.182	0.5	7.36	5	6.677	0.5	116.74	5
-0.054	0.5	7.14	5	**S3** 0.0	0.5	0.00	5
0.568	0.5	13.20	5	0.383	0.5	-1.60	5
0.755	0.5	17.40	5	0.150	0.5	3.30	5
0.851	0.5	24.40	5	1.154	0.5	18.10	5
1.552	0.5	31.61	5	1.630	0.5	36.70	5
2.041	0.5	40.63	5	2.764	0.5	55.90	5
5.418	0.5	103.01	5	4.178	0.5	100.40	5
				6.294	0.5	121.75	5

Curriculum Vitae

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Abu Laban N, Selesi D, Jobelius C & Meckenstock RU (2009) Anaerobic benzene degradation by Gram-positive sulfate-reducing bacteria. *FEMS Microbiology Ecology* **68**: 300-311.

Abu Laban N, Selesi D, Rattei T, Tischler P & Meckenstock RU (2010) Identification of enzymes involved in anaerobic benzene degradation by two strictly anaerobic enrichment cultures (a revision has been submitted to *Enviromental Microbiology*).

Abu Laban N, Bergmann F, Meyer A, Elsner M & Meckenstock RU (2010) Two-dimensional stable isotope fractionation analysis to probe for the anaerobic activation mechanism of the unsubstituted hydrocarbon (in preparation).

Selected contributions to national and international scientific meetings:

Abu Laban N, Selesi D & Meckenstock RU (2007) Physiological and molecular characterization of anaerobic benzene-degrading culture with sulfate as electron acceptor. Annual Conference of the Association for General and Applied Microbiology (VAAM) 01-04.04.2007, Osnabrück, Germany, (Poster presentation).

Abu Laban N, Selesi D & Meckenstock RU (2008). Initial activation mechanism of anaerobic benzene degradation in a sulfate-reducing enrichment culture. Annual Conference of the Association for General and Applied Microbiology (VAAM) 09-11.03.2008, Frankfurt, Germany, (Poster presentation).

Abu Laban N, Selesi D & Meckenstock RU (2009) Combining proteomic and stable isotope fractionation analyses to elucidate anaerobic benzene degradation pathway by iron- and sulfate-reducing microorganisms. Annual conference of the Association for General and Applied Microbiology (VAAM) 08-11.03.2009, Bochum, Germany (Poster presentation).

Abu Laban N, Selesi D & Meckenstock RU (2009) Metabolic and proteomic analyses to elucidate the mechanism of anaerobic benzene degradation. The 3rd Congress of European Microbiologists (FEMS) 28.06-02.07.2009, Gothenburg, Sweden, (Poster presentation).

Clarification of contributions of other scientists

Chapter 2 "Anaerobic benzene degradation by Gram-positive sulfate-reducing bacteria" has been published in *FEMS Microbiology Ecology* **68** (2009) 300-311. The following coauthors contributed to this work:

1) Prof. Dr. Rainer Meckenstock: contributed to the experimental design (mass balance, substrates and co-substrates utilization) and proof reading of the manuscript.

2) Dr. Draženka Selesi: contributed to the experimental design (molecular analysis) and proof-reading of the manuscript.

3) Carsten Jobelius (University of Karlsruhe): performed LC-ESI-MS-MS metabolites analysis.

Chapter 3 "Identification of enzymes involved in anaerobic benzene degradation by two strictly anaerobic enrichment cultures" has been submitted to *Environmental Microbiology* in 13.10.2009. The following coauthors contributed to this work:

1) Prof. Dr. Rainer Meckenstock: contributed to the experimental design (proteomic and Edman sequencing) and proof-reading the manuscript.

2) Dr. Draženka Selesi: contributed to the experimental design (proteomic) and reviewing the result part.

3) Dr. Thomas Rattei and Dr. Patrick Tischler (Technical University Munich): performed annotation of the genome of the iron-reducing culture BF by bioinformatics algorithms.

The genome was sequenced by Eurofins MWG Operon, Ebersberg, Germany. ESI/LC-MS/MS protein identification was provided by the Core Facility Proteomics, Helmholtz Zentrum München, Germany. Edman sequencing was performed at the Max Planck Institute of Neurobiology, Martinsried, Germany.

Chapter 4 "Two-dimensional stable isotope fractionation analysis to probe for the anaerobic activation mechanism of the unsubstituted hydrocarbon benzene" is prepared for publication. The following coauthors contributed to this work:

1) Prof. Dr. Rainer Meckenstock and Dr. Martin Elsner: contributed to the experimental design (carbon and hydrogen stable isotope analysis) and proof-reading of the chapter.

2) Armin Meyer: Developed analytical methods and measured stable isotope ratios with GC-IRMS (carbon and hydrogen isotopes for benzene).

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