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Research Article

RESEARCH ARTICLE

Reconstructing metabolic pathways of a member of the genus *Pelotomaculum* suggesting its potential to oxidize benzene to carbon dioxide with direct reduction of sulfate

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One sentence summary: *Pelotomaculum* candidate BPL not only degraded benzene without molecular oxygen but also performed sulfate reduction, making it the first representative of the genus *Pelotomaculum* which grows by dissimilatory sulfate reduction.

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ABSTRACT

The enrichment culture BPL is able to degrade benzene with sulfate as electron acceptor and is dominated by an organism of the genus *Pelotomaculum*. Members of *Pelotomaculum* are usually known to be fermenters, undergoing syntrophy with anaerobic respiring microorganisms or methanogens. By using a metagenomic approach, we reconstructed a high-quality genome (~2.97 Mbp, 99% completeness) for *Pelotomaculum* candidate BPL. The proteogenomic data suggested that (1) anaerobic benzene degradation was activated by a yet unknown mechanism for conversion of benzene to benzoyl-CoA; (2) the central benzoyl-CoA degradation pathway involved reductive dearomatization by a class II benzoyl-CoA reductase followed by hydrolytic ring cleavage and modified β-oxidation; (3) the oxidative acetyl-CoA pathway was utilized for complete oxidation to CO_2. Interestingly, the genome of *Pelotomaculum* candidate BPL has all the genes for a complete sulfate reduction pathway including a similar electron transfer mechanism for dissimilatory sulfate reduction as in other Gram-positive sulfate-reducing bacteria. The proteome analysis revealed that the essential enzymes for sulfate reduction were all formed during growth with benzene. Thus, our data indicated that, besides its potential to anaerobically degrade benzene, *Pelotomaculum* candidate BPL is the first member of the genus that can perform sulfate reduction.

Keywords: benzene; sulfate reduction; *Pelotomaculum*; anaerobic degradation; genome; proteome

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INTRODUCTION

Benzene is a widely distributed pollutant in groundwater posing a hazardous risk to drinking water resources (Manoli and Samara 1999). Several recent microcosm studies and lab cultures have shown that benzene and other aromatic compounds can be mineralized to CO_2 under nitrate-reducing, iron-reducing, sulfate-reducing and methanogenic conditions (Ulrich and Edwards 2003; Kunapuli, Lueders and Meckenstock 2007; Musat and Widdel 2008; van der Zaan *et al.* 2012; Luo *et al.* 2014).

Three types of initial activation reactions for anaerobic benzene degradation have been proposed: methylation to toluene, hydroxylation to phenol and direct carboxylation to benzoate. Methylation of benzene to toluene was mostly based on the recovery of labeled toluene when labeled benzene was added to cultures (Meckenstock and Mouttaki 2011; Aburto-Medina and Ball 2014). However, the key product of anaerobic toluene activation, benzylsuccinate, has never been detected in benzene-degrading cultures, and so far no other evidence has been reported to support ring methylation as the initial activation reaction. There is also indication for hydroxylation of benzene to phenol as initial activation reaction by *Geobacter metallireducens* which metabolized benzene with Fe(III) citrate as the electron acceptor (Caldwell and Suflita 2000; Zhang *et al.* 2012, 2013). Several groups suggested a direct carboxylation of benzene to benzoate as the first step for anaerobic benzene metabolism (Abu Laban *et al.* 2010; Holmes *et al.* 2011; Luo, Devine and Edwards 2016). In the Fe(III)-reducing enrichment culture BF, combined genomic and proteomic studies revealed genes encoding for two subunits of a putative anaerobic benzene carboxylase (AbcD and AbcA) (Abu Laban *et al.* 2010).

The enrichment culture BPL used in this study is able to degrade benzene with sulfate as electron acceptor (Abu Laban *et al.* 2009). Previous community analysis based on 16S rRNA gene sequencing and FISH revealed that the dominant bacterial member (~95%) belongs to the phylum Firmicutes genus *Pelotomaculum* (Abu Laban *et al.* 2009). Members of the genus *Pelotomaculum* are mostly obligate syntrophs that need methanogens as syntrophic partners to oxidize propionate, alcohols, or aromatic compounds (Imachi *et al.* 2002; Stams and Plugge 2009). Recent studies propose that syntrophic metabolism during biodegradation of benzene and other hydrocarbons might occur in the presence of electron acceptors, such as sulfate or nitrate (Gieg, Fowler and Berdugo-Clavijo 2014). For example, stable isotope probing (SIP) with the Fe(III)-reducing enrichment culture BF revealed that members of the *Peptococcaceae* were the primary benzene degraders and shared electrons with members of the family *Desulfobulbaceae* (Kunapuli, Lueders and Meckenstock 2007). Similar results were obtained for two denitrifying cultures based on either batch or chemostat studies (van der Zaan *et al.* 2012; Luo *et al.* 2014).

Pelotomaculum thermopropionicum is the only member whose genome has been sequenced in the genus *Pelotomaculum*. Similar to other known representatives in the genus, it lacks the capability to reduce sulfate, even though the related genes for sulfate reduction have been identified in the genome (Plugge *et al.* 2011). Previously, a draft genome for an uncultured *Pelotomaculum* spp. was retrieved from a terephthalate-degrading culture (Lykidis *et al.* 2011). Combined with the subsequent proteomic studies, *Pelotomaculum* spp. was confirmed to degrade terephthalate via syntrophic interactions with methanogens (Wu *et al.* 2013). The given examples provide more and more evidence that members of the genus *Pelotomaculum* carry out syntrophic metabolism with anaerobic respiring microorganisms. However, we hypothesize here that the anaerobic benzene degradation in the enrichment culture BPL is performed as a single cell process by a novel member of the genus *Pelotomaculum* which is able to reduce sulfate as electron acceptor.

MATERIALS AND METHODS

Cultivation of the enrichment culture BPL

The anaerobic sulfate-reducing culture BPL was enriched from soil at a former coal gasification site in Gliwice, Poland, and was cultivated in bicarbonate-buffered (30 mM) freshwater medium as described earlier (Abu Laban *et al.* 2009). Benzene (0.5 mM) (Sigma-Aldrich, Steinheim, Germany) was added as sole electron donor and sulfate (10 mM) as electron acceptor. The enrichment culture was transferred in 1:10 (vol/vol) dilutions and the metabolic activity was monitored by consumption of sulfate (Abu Laban *et al.* 2009).

DNA sequencing, assembly and annotation

Genomic DNA was extracted from a 1200-ml BPL culture when ~2 mM sulfate was reduced using the modified CTAB (hexadecyltrimethylammonium bromide) method (Zhou, Bruns and Tiedje 1996). Whole metagenome sequencing was performed with a 454 GS FLX Titanium system (Roche, Penzberg, Germany) in two different runs. The resulting reads were co-assembled into contigs with the Newbler v2.7 assembly software. The automated assembly of the sequences was checked manually by mapping the raw 454 reads against the contigs with Mosaik v2.2.3 (Lee *et al.* 2014) followed by visual inspection with Tablet v1.14 (Milne *et al.* 2013). The assembled contigs were submitted to IMG-M 4.530 (Project ID: Gp0111374) (Markowitz *et al.* 2014) for automated gene calling and annotation. Automatic annotations were manually curated for genes described in this study by bioinformatics tools in the IMG platform (e.g. KEGG and BLAST) (Markowitz *et al.* 2014).

Binning draft genomes from BPL metagenome

All contigs were annotated taxonomically without length restriction with taxator-tk v1.3.0e using default settings and the refpack (microbial_20150430) provided by Dröge, Gregor and McHardy (2015). Additionally, we screened all contigs for (partial) marker genes (5S, 16S, 18S and 31 conserved genes) (Wu and Scott 2012) and classified them taxonomically, both using PhyloPythiaS+ v1.4 (Gregor *et al.* 2016). The two assignment methods provided similar results. Then, we validated the results by checking (i) their consistency with the taxonomic profiles obtained from both independent 16S rRNA gene amplicon sequencing data and the contigs-extracted SSU (16S) sequences data for the same community, (ii) the read coverage and (iii) the GC content of contigs assigned to the same clades.

Finally, we identified the major taxa based on the taxonomic annotations and used them to extract sample-specific training data for the training stage of the composition-based taxonomic classifier PhyloPythiaS+ using default settings. By considering the taxonomic annotation, GC content and average read coverage, we divided the metagenome contigs into four genome bins.

Phylogenetic analysis

We picked the near-complete *Pelotomaculum* candidate BPL 16S rRNA gene, 16S RefSeq sequences for the closely related genera *Pelotomaculum* and *Desulfotomaculum*, every *Desulfotomaculum*

sequence that was deposited in SILVA SSU r123 as well as an outgroup sequence *Thermincola potens* JR, which we used later to root the tree. We aligned those sequences to the SILVA reference alignment using SINA v1.2.12 and trimmed the resulting multiple sequence alignment to SILVA positions 1007 to 43293. Finally, a maximum-likelihood tree was calculated with RAxML v.8.2.8 using the combined rapid bootstrapping (1000 replicates) and maximum-likelihood search algorithm with the GTRCAT approximation model and with random seed 29582.

Protein extraction

For proteomics, two independent experiments were performed in a similar way as follows. Extraction of proteins from bacterial cultures was performed as previously described (Abu Laban *et al.* 2010; Selesi *et al.* 2010). Comparative proteomic analysis is impossible here, because the culture cannot grow with any of the possible downstream metabolites like toluene, benzoate or phenol. Furthermore, it is an enrichment culture, and easily degradable substrates like butyrate and acetate will change the compositions of the microbial community. Thus, we performed a qualitative proteomics experiment with benzene as substrate. Cells were harvested from a total of 1200 ml culture by centrifugation (30 min at 3739 × g and 4°C). The cell pellet was washed three times with 1× phosphate buffered saline (PBS buffer), and resuspended in a mixture of 400 μl lysis buffer, including 9 M urea, 2 M thiourea, 4% 3-[(3-cholamidopropyl)-dimethylammonio]-1-propanesulfonate (CHAPS) and 1% dithiothreitol (DTT), and 66.8 μl of a 7× stock solution containing a complete EDTA-free mini-protease inhibitor cocktail tablet (Roche Diagnostics GmbH, Penzberg, Germany). After 30 min of incubation at room temperature, the cell-buffer mixture was subjected to sonication in an ice-water bath for 2 min (amplitude: 0.3, cycle: 60%; UP50H, Hielscher GmbH, Germany). The homogenized solution was centrifuged for 10 min at 20 000 × g and 4°C. The supernatant was treated for 30 min at room temperature with 4 μl nuclease mix (GE Healthcare, Piscataway, NJ, USA) and centrifuged for 30 min at 15 000 × g and 4°C. Estimation of protein concentrations in the supernatant was performed with the two-dimensional Quant kit according to the protocol of the manufacturer (GE Healthcare, Piscataway, NJ, USA). However, quantification of protein was affected by interference with ferrous sulfide, prohibiting a proper estimation of protein concentrations.

Proteome analysis

For sample preparation, a modified filter-aided sample preparation approach was performed (Wisniewski *et al.* 2009). After tryptic digestion, samples were stored at –20°C until further use.

LC-MS/MS analysis was performed as described previously on a LTQ-Orbitrap XL (Thermo Fisher) (von Toerne *et al.* 2013). Briefly, the pre-fractionated samples were automatically injected and loaded onto the trap column (Acclaim PepMap100, C18, 5 μm, 100 Å pore size, 300 μm ID × 5 mm μ-Precolumn - No 160454; Thermo Scientific). After 5 min, the peptides were eluted and passed to the analytical column (Acclaim PepMap100, C18, 3 μm, 100 Å pore size, 75 μm ID × 15 cm, nanoViper-No 164568; Thermo Scientific) by reversed phase chromatography which was operated on a nano-HPLC (Ultimate 3000, Dionex). A non-linear 170-min gradient was used for elution with a mobile phase of 35% acetonitrile in 0.1% formic acid in water (A) and 0.1% formic acid in 98% acetonitrile (B) at a flow rate of 300 nL min^{-1}. The gradient settings were: 5–140 min: 14.5%–90% A, 140–145 min: 90% A–95% B, 145–150 min: 95% B followed by equilibration for 15 min to starting conditions. The 10 most abundant peptide ions were selected from the MS pre-scan for fragmentation in the linear ion trap if they exceeded an intensity of at least 200 counts and were at least doubly charged. A high-resolution (60 000 full-width half maximum) MS spectrum was acquired in the Orbitrap with a mass range from 200 to 1500 Da during fragment analysis.

MS-MS spectra were searched against the BPL metagenome database (20 979 entries) via the MASCOT search engine (version 2.5.1; Matrix Science). A mass tolerance of 10 ppm for peptide precursors and 0.6 Da for MS-MS peptide fragments was applied allowing no more than one missed cleavage. Fixed modifications were set to carbamidomethylation of cysteine and variable modifications to oxidation of methionine and deamidation of asparagine and glutamine.

The Scaffold software (version 4.4.1.1., Proteome Software Inc.) was used to validate peptide identifications and visualize results (Keller *et al.* 2002). Peptide threshold was set to a false discovery rate (FDR) of 1%. A decoy protein FDR of 2.2% was observed. Protein identifications were accepted if two or more unique peptides were identified.

RESULTS AND DISCUSSION

Phylogenetic binning and proteome

Metagenomic sequencing of enrichment culture BPL yielded an assembly of 16.9 million base pairs into 5842 contigs with a N50 of 12.3 thousand base pairs. Gene prediction with the IMG/M pipeline produced 20 979 open reading frames with 12 736 (60.03%) being assigned to a putative functional name.

The metagenome contigs were manually divided into four genome bins: (1) *Pelotomaculum* operational taxonomic unit (OTU) (described as *Pelotomaculum* candidate BPL in this article) with a relative abundance of 54.27%; (2) *Gracilibacter* OTU with 32.40%; (3) *Desulfomonile* OTU with 7.41%; and (4) *Ignavibacterium* OTU with 5.92%. The discrepancy for population abundance estimates for *Pelotomaculum* candidate BPL between 454 read coverage here and PCR-based 16S rRNA gene frequency reported previously (Abu Laban *et al.* 2009) can be explained by experimental bias and varying 16S rRNA gene operon copy numbers. For instance, rrnDB v.4.4.4 (Nov 2015) (Stoddard *et al.* 2015) reports two operons for *Pelotomaculum*, only one for *Desulfomonile* and *Ignavibacterium*, and an unknown number for *Gracilibacter*. The program CheckM v1.0.3 (Parks *et al.* 2015) was used to determine the completeness and contamination of the four genome bins. Based on the lineage-specific single-copy marker gene analysis by CheckM, the constructed genomes for *Pelotomaculum* and *Gracilibacter* OTUs are 99% and 97% complete, respectively (Fig. S1, Supporting Information). The genomes for *Desulfomonile* and *Ignavibacterium* OTUs could not be recovered in full length (Fig. S1).

The shotgun proteomic analysis of the enrichment culture BPL community from two independent experiments produced a total of 545 proteins encoded by the genes located on the contigs of the metagenome. Almost 97% of the proteins were assigned to both the *Pelotomaculum* candidate BPL (354 proteins, 64%, Table S1, Supporting Information) and *Gracilibacter* OTU bin (166 proteins, 30%). We did not analyze the *Desulfomonile* and *Ignavibacterium* OTUs because of their minor abundances in the metagenome and their low coverage in the proteome (only 3% of the total proteins).

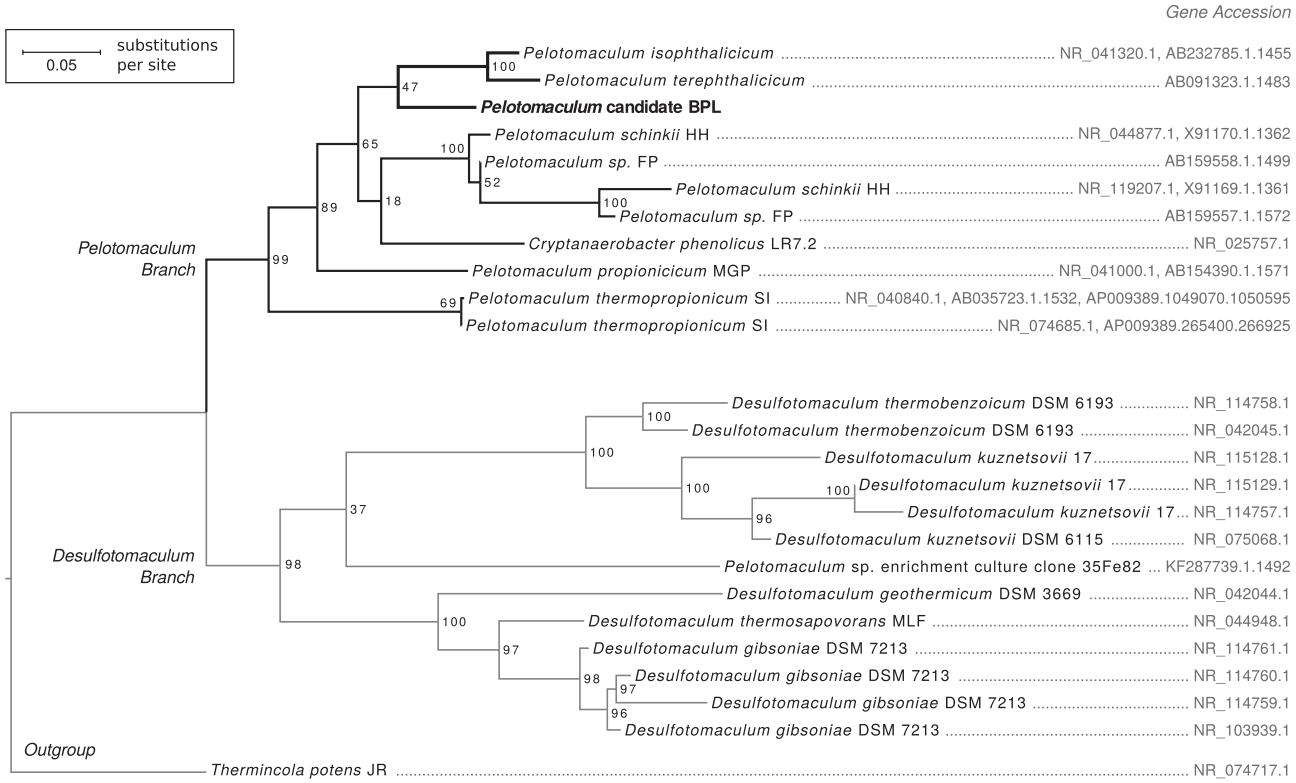

Figure 1. Maximum-likelihood tree of the near-complete 16S rRNA genes with bootstrap support values (%) showing the phylogenetic affiliation of *Pelotomaculum* candidate BPL. Strain names are followed by the corresponding RefSeq or SILVA sequence identifiers. Thick branch lines indicate a closer phylogenetic neighborhood of *Pelotomaculum* candidate BPL. The sequence of the 16S rRNA gene of *Pelotomaculum* candidate BPL can be found in Text S1.

In the genome of *Gracilibacter* OTU (IMG ID: *Clostridium* Ga0073690), no genes for sulfate reduction could be detected. If *Gracilibacter* activated benzene, *Gracilibacter* should have the capability to perform the corresponding downstream degradation. However, among the proteins classified to the *Gracilibacter* OTU, only a few matched enzymes that could be related to benzoate degradation. Possibly, the *Gracilibacter* OTU grew by fermentation of metabolites produced by *Pelotomaculum* candidate BPL. For example, an indication could be the identification of pyruvate ferredoxin oxidoreductase in the proteome.

A novel member of the genus *Pelotomaculum*

The reconstructed *Pelotomaculum* candidate BPL genome (IMG ID: pelotomaculum Ga0073689) consisted of 99 high-quality contigs with a total length of 2.97 Mbp and an average GC content of 53.71%. They were assembled from 454 reads (mean > 500 bp) according to standard procedures in isolate genome sequencing, using a 72-fold average positional coverage. Their length ranges from 1 to 234 kb with a mean of 30 kb. *Pelotomaculum thermopropionicum* SI is the only strain in the genus *Pelotomaculum* for which a full genome is available in the public resources. The genome has a total length of 3.03 Mbp with an average G+C content of 53.0%. It is reported to be a fermenting syntrophic organism whose metabolism depends on coupling with other microbes. Thus, we performed genomic comparisons between the *Pelotomaculum* candidate BPL and the *P. thermopropionicum* SI genomes (NCBI Reference Sequence: NC_009454.1). Comparative analyses of the average nucleotide identity (ANI) based on the IMG/M platform indicated that the two genomes represent different *Pelotomaculum* species, with an ANI value of 75.98%, far below an ANI threshold range for species delineation (95%–96%) (Kim *et al.* 2014). This was in agreement with their low 16S rRNA gene sequence identity (<95%). The corresponding identity scatter plot showed that the contigs from *Pelotomaculum* candidate BPL covered the entire genome of *P. thermopropionicum* SI (Fig. S2, Supporting Information). However, the nucleotide sequence similarity was limited leaving many gaps (Fig. S2), which emphasized the differences between the reconstructed *Pelotomaculum* candidate BPL genome and the RefSeq isolate genome sequence. The gene distributions of the genomes were compared for Clusters of Orthologous Groups (COG). We found significant differences in the COG categories distributions at the level of $P < 0.05$ by the chi-squared test, with several differences in the reference genome of *P. thermopropionicum* SI (Fig. S3, Supporting Information), indicating different metabolic capacities. Employing a phylogenetic analysis of the near-complete 16S rRNA gene (position 113–1661, Text S1, Supporting Information), we found that among known members of the *Peptococcaceae* family, the BPL candidate is clearly separate from *P. thermopropionicum* SI (Fig. 1). Furthermore, it has the closest phylogenetic relationship (with 95% 16S rRNA gene sequence similarities) to *P. isophthalicicum* and *P. terephthalicicum* that both can utilize benzoate and other low-molecular-weight aromatic compounds when grown in co-culture with hydrogenotrophic methanogens (Qiu *et al.* 2006) (Fig. 1).

Initial activation of benzene

In order to find evidence for methylation as the initial activation reaction, we tried to identify genes related to anaerobic degradation of toluene in the genome. Benzylsuccinate synthase (Bss)

is a key enzyme in the anaerobic toluene degradation pathway. However, we could neither identify bss alpha-subunit (*bssA*)-like genes in the genome nor genes for beta-oxidation of benzylsuccinate to benzoyl-CoA. Thus, we exclude a degradation of benzene via methylation to toluene. Anaerobic phenol degradation to benzoyl-CoA was known to proceed with the participation of phenylphosphate synthase, phenylphosphate carboxylase, 4-hydroxybenzoate-CoA ligase and 4-hydroxybenzoyl-CoA reductase (Abu Laban *et al*. 2010). Only a few hits could be identified for genes showing homology to individual subunits of these enzymes. However, when looking at the genetic context, genes encoding for the other subunits of these proteins which would be needed to build catalytically active enzymes are lacking. The absence of relevant key genes for anaerobic phenol degradation excludes activation of benzene via hydroxylation in this culture. The earlier reported inability of culture BPL to grow on toluene or phenol confirmed this genomic analysis (Abu Laban *et al*. 2009).

Previous metabolite analyses (e.g. benzoate as an intermediate) and substrate tests indicated a direct carboxylation of benzene as the initial activation mechanism for *Pelotomaculum* candidate BPL (Abu Laban *et al*. 2009). Thus, we searched for genes encoding for the proposed anaerobic benzene carboxylase (AbcA and AbcD) responsible for direct carboxylation to benzoate in the iron-reducing culture BF (Abu Laban *et al*. 2010). In the genome of *Pelotomaculum* candidate BPL, we identified one gene copy (Ga0073689_2617920776) assigned as 4-hydroxy-3-polyprenylbenzoate decarboxylase which shared 33% amino acid sequence identity to the anaerobic benzene carboxylase gene *abcA*. However, no *abcD* homolog could been detected in the proximity of gene Ga0073689_2617920776 and no gene product of gene Ga0073689_2617920776 could be identified in the proteome. We thus conclude that *Pelotomaculum* candidate BPL does not utilize an anaerobic benzene carboxylase similar to culture BF.

Furthermore, in culture BF, a gene for a putative benzoate-CoA ligase was located next to the carboxylase genes. This ligase is intended to convert the carboxylation product benzoate to benzoyl-CoA which can be further degraded through the benzoyl-CoA degradation pathway. If benzene carboxylation occurred, we would expect genes encoding benzoate-CoA ligase which further activates benzoate to benzoyl-CoA in the genome. In *Geobacter metallireducens* GS-15, there are two different enzymes for benzoyl-CoA production: the ATP-dependent benzoate-CoA ligase (BamY) and succinyl-CoA: benzoate CoA transferase (Bct) (Oberender *et al*. 2012). Three hits (Ga0073689_2617922070, 2617921892 and 2617920988) were obtained in our genome showing only 27%–29% amino acid sequence identities to the *bamY* gene of *G. metallireducens* GS-15. Additionally, we could not identify genes homologous to *bct* in *G. metallireducens* GS-15 or the putative benzoate-CoA ligase *bzlA* in the iron-reducing enrichment culture BF (Abu Laban *et al*. 2010).

Based on the current data, we propose that *Pelotomaculum* candidate BPL might not employ any of the three abovementioned initial activation reactions proposed for benzene degradation. *Pelotomaculum* candidate BPL is not the only case of lacking genomic evidence for any of the activation reactions proposed so far. In a benzene-degrading methanogenic culture, benzene degradation was affiliated to a *Deltaproteobacterium* bacterium ORM2 (Devine 2013; Luo, Devine and Edwards 2016). There, the authors could not identify genes involved in toluene or phenol activation, either. Additionally, they failed to identify genes encoding for UbiD-like carboxylases and benzoate-CoA ligases. They therefore proposed an alternative carbonylation pathway for transformation of benzene to benzoyl-CoA via either a Wood–Ljungdhal-type reaction or a Gatterman–Koch-type mechanism (Devine 2013). Similarly, in a sulfate-reducing, benzene-degrading consortium enriched by Phelps, Zhang and Young (2001), benzoate was detected in culture supernatants but it could not support cell growth. Thus, the authors proposed that benzene was directly transformed to benzoyl-CoA (Phelps, Zhang and Young 2001). In another study performed by Taubert *et al*. (2012), a similar benzene-degrading, sulfate-reducing enrichment culture was analyzed using protein-based SIP. Although this might be due to the poor detection limit of the method, they could not find proteins of known or proposed pathways for benzene activation.

Taking together, the genomic information on anaerobic benzene degradation from literature and the data presented here indicate a common observation for benzene activation. The iron- and nitrate-reducing bacteria known so far seem to activate benzene via carboxylation (Abu Laban *et al*. 2010; Luo *et al*. 2014). Fermenting- and sulfate-reducing bacteria including candidate *Pelotomaculum* BPL studied here might have developed so far unknown, oxygen-independent strategies for anaerobic benzene activation.

Central benzoyl-CoA degradation pathway

According to present knowledge, anaerobic degradation of benzene has to proceed via the central intermediate, benzoyl-CoA, regardless of the initial activation mechanisms. Two distinct classes of benzoyl-CoA reductases (BCRs) are presently known for the reduction of benzoyl-CoA to cyclohexa-1,5-diene-1-carboxyl-CoA (dienoyl-CoA). The ATP-dependent class I BCR (BcrABCD) is predominantly found in facultative anaerobes; the ATP-independent and tungsten cofactor-containing class II BCR (BamBCDEFGHI) occurs in obligate anaerobic bacteria (Kung *et al*. 2009; Fuchs, Boll and Heider 2011; Loffler *et al*. 2011). As expected for an obligate anaerobic sulfate reducer degrading aromatic compounds, a protein similar to the active site subunit of the class II BCR in *G. metallireducens* GS-15 (BamB, Ga0073689_2617921884) was detected in the proteome (Fig. 2, Table S1, Supporting Information). The gene is located adjacent to a *bamC* paralog (Ga0073689_2617921883) with 66% amino acid sequence identity to the *bamC* gene in *G. metallireducens*. Candidate genes for *bamDEFGHI* putatively encoding BCR were identified in at least one copy in the genome of *Pelotomaculum* candidate BPL. They shared amino acid sequence identities ranging from 33% to 71% to the respective proteins described in *G. metallireducens* GS-15 (Wischgoll *et al*. 2005; Loffler *et al*. 2011), indicating that *Pelotomaculum* candidate BPL degraded benzoate-CoA via the class II BCR (Bam type).

Products of a gene cluster (Ga0073689_2617919856–8) were detected in the proteome putatively encoding 6-oxo-cyclohex-1-ene-carbonyl-CoA hydrolase (Oah), cyclohex-1,5-diencarbonyl-CoA hydratase (Dch) and 6-hydroxycyclohex-1-ene-1-carbonyl-CoA dehydrogenases (Had), respectively. These genes and gene products suggest further transformation of dienoyl-CoA to 3-hydroxypimelyl-CoA (Fig. 2, Table S1). The hydratase (Dch) could catalyze water addition to a carbon–carbon double bond, followed by dehydrogenation (Had), and hydrolytic ring cleavage (Oah) (Wischgoll *et al*. 2005; Holmes *et al*. 2012). Proteins for further β-oxidation of 3-hydroxypimelyl-CoA to acetyl-CoA and CO_2 via glutaryl-CoA were also identified in the proteome, i.e. glutaryl-CoA dehydrogenase (Ga0073689_2617919859), enoyl-CoA hydratase (Ga0073689_2617921893) and 3-hydroxyacyl-CoA dehydrogenase (Ga0073689_2617921894) (Table S1).

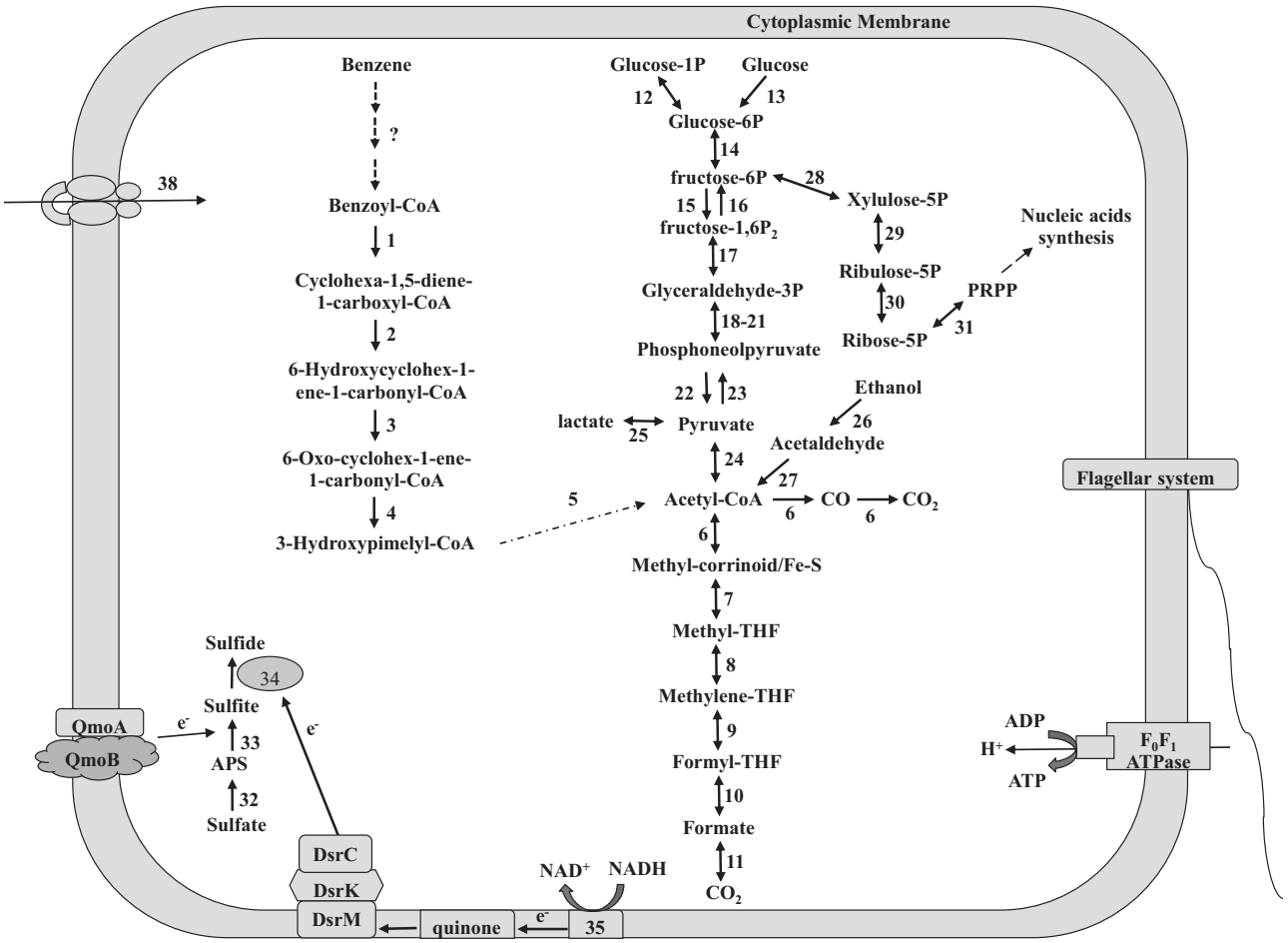

Figure 2. Schematic view of a *Pelotomaculum* candidate BPL cell showing metabolic reconstruction of the pathways of benzene degradation, sulfate reduction, glycolysis/gluconeogenesis, pentose phosphate pathway and ABC transporters deduced from the *Pelotomaculum* candidate BPL genome. Benzene degradation (enzymes 1–11) and sulfate reduction pathways (enzymes 32–35) are evidenced by proteome analysis. Numbers correspond to the following enzymes: 1, class II benzoyl-CoA reductase (Bam type); 2, cyclohex-1,5-diencarbonyl-CoA hydratase; 3, 6-hydroxycyclohex-1-ene-1-carbonyl-CoA dehydrogenases; 4, 6-oxo-cyclohex-1-ene-carbonyl-CoA hydrolase; 5, β oxidation pathway; 6, carbon monoxide dehydrogenase/acetyl-CoA synthetase; 7, 5-methyltetrahydrofolate methyltransferase; 8, methylenetetrahydrofolate reductase; 9, methylene-tetrahydrofolate dehydrogenase; 10, formate-tetrahydrofolate synthetase; 11, formate dehydrogenase; 12, phosphoglucomutase; 13, glucokinase; 14, glucose-6-phosphate isomerase; 15, 6-phosphofructokinase; 16, fructose-1,6-bisphosphatase; 17, fructose 1,6-bisphosphate aldolase, class I; 18, glyceraldehyde-3-phosphate dehydrogenase (NAD(P)); 19, phosphoglycerate kinase; 20, 2,3-bisphosphoglycerate-independent phosphoglycerate mutase; 21, enolase; 22, pyruvate kinase; 23, phosphoenolpyruvate synthetase; 24, pyruvate ferredoxin oxidoreductase; 25, lactate dehydrogenase; 26, alcohol dehydrogenase; 27, aldehyde dehydrogenase; 28, transketolase; 29, ribulose-phosphate 3-epimerase; 30, ribose 5-phosphate isomerase; 31, ribose-phosphate pyrophosphokinase; 32, ATP sulfurylase, 33, adenosine-5′-phosphosulfate reductase; 34, dissimilatory sulfite reductase; 35, NADH-quinone oxidoreductase; 36, ABC transporters for molybdate/tungstate, phosphate, phosphonate, iron complex, zinc, cobalt, nickel, biotin and lipopolysaccharide. APS, adenosine-5′-phosphosulfate; QmoAB, quinone-interacting membrane-bound oxidoreductase; DsrABC, dissimilatory sulfite reductase cytoplasmic subunits; DsrMK, transmembrane dissimilatory sulfite reductase complex; PRPP, phosphoribosyl pyrophosphate; e^-, electron.

Complete oxidation via the Wood–Ljungdahl pathway

For complete oxidation of acetyl-CoA to CO_2, many sulfate reducers run the Wood–Ljungdahl pathway in reverse, also termed as oxidative carbon monoxide dehydrogenase/acetyl-CoA pathway. These are e.g. *Desulfatibacillum alkenivorans* AK-01, *Desulfobacula toluolica* Tol2, *Desulfobacterium autotrophicum* HRM2 or *Desulfotomaculum acetoxidans* DSM 771 (Spormann and Thauer 1988; Strittmatter *et al.* 2009; Callaghan *et al.* 2012; Wohlbrand *et al.* 2013). Likewise, all necessary enzymes involved in the Wood–Ljungdahl pathway were detected in the proteome and encoded in the *Pelotomaculum* candidate BPL genome (Fig. 2, Table S1). Namely, they contained the key enzyme carbon monoxide dehydrogenase/acetyl coenzyme A (CoA) synthetase CODH/ACS (Ga0073689_2617920888 and 2617921376–80), and the enzymes for the methyl branch of the pathway including 5-methyltetrahydrofolate methyltransferase AcsE (Ga0073689_2617921126), methylenetetrahydrofolate reductase MetF (Ga0073689_2617921113), methylene-tetrahydrofolate dehydrogenase FolD (Ga0073689_2617920380), formate-tetrahydrofolate synthetase Fhs (Ga0073689_2617921323) and formate dehydrogenase FhdA (Ga0073689_2617922225).

An interesting feature is the detection of genes encoding heterodisulfide reductase (Hdr)-related proteins which co-localize with methylenetetrahydrofolate reductase MetF (Ga0073689_2617921113) (Fig. S4, Supporting Information). The methylenetetrahydrofolate reductase gene *metF* is tightly associated with a gene (Ga0073689_2617921112) designated as the

N-terminus of MetF (*metV*) and they are located next to *mvhD* encoding the delta subunit of methyl-viologen-reducing hydrogenase and *hdrA* encoding heterodisulfide reductase iron-sulfur subunit A (Fig. S4). The function of the delta subunit MvhD and HdrA is described as transferring reducing equivalents to methylenetetrahydrofolate reductase MetF in an electron bifurcation process (Buckel and Thauer 2013; Wohlbrand *et al*. 2013; Mock *et al*. 2014). Similar gene clusters of *metVF* genes together with the *hdrA-mvhD* genes are also present in other acetate-oxidizing sulfate reducers, i.e. *Desulfatibacillum alkenivorans* AK-01, *Desulfobacula toluolica* Tol2, *Desulfobacterium autotrophicum* HRM2 (Wohlbrand *et al*. 2013). We conclude that *Pelotomaculum* candidate BPL has a similar way of energy conservation from acetate oxidation and sulfate reduction as described previously for sulfate-reducing bacteria.

Among possible genes of the tricarboxylic acid (TCA) cycle, we have found malate dehydrogenase (NAD) (Ga0073689_2617921286) from the oxidative branch and isocitrate dehydrogenase (NADP) (Ga0073689_2617922024) from the reductive branch indicating that this part of the genome might be incomplete. Absence of the key enzyme α-ketoglutarate dehydrogenase at least indicates that *Pelotomaculum* candidate BPL cannot operate a TCA cycle for oxidation of acetyl-CoA to CO_2 which agrees with the presence of a complete Wood–Ljungdahl pathway for oxidation of acetate.

Sulfate reduction in the genus *Pelotomaculum*

So far, members of the genus *Pelotomaculum* are supposed to be unable to grow by sulfate reduction (Imachi *et al*. 2006). However, the closely related genus *Desulfotomaculum* is well known for sulfate reduction. The key enzymes of dissimilatory sulfate reduction ATP sulfurylase (Sat) (Ga0073689_2617919560), adenosine -5′-phosphosulfate reductase (Apr) (Ga0073689_2617919558-9) and dissimilatory sulfite reductase (Dsr) (Ga0073689_2617920264-5) have been found in the proteome of our *Pelotomaculum* candidate BPL (Fig. 2, Table S1). ATP sulfurylase catalyzes the initial activation of sulfate to adenosine-5′-phosphosulfate (APS) releasing pyrophosphatase. Pyrophosphate can be used to create a proton motive force by an annotated, putative proton-translocating membrane pyrophosphatase (Ga0073689_2617919937). Genome analysis revealed the genes *aprA* and *aprB* encoding the sulfite-forming APS reductase are located adjacent to the *sat* gene. In close proximity to the *sat* and *apr* genes are *qmoAB* genes (Ga0073689_2617919556-7) probably encoding for an adenylylsulfate reductase-associated electron transfer protein which is homologous to heterodisulfide reductases (Ramos *et al*. 2015). The QmoAB redox complex is proposed to be the electron donor of APS reductase in Gram-positive sulfate-reducing bacteria (Junier *et al*. 2010). Interestingly, gene Ga0073689_2617919555 was automatically annotated by the IMG platform as the membrane subunit of the QmoABC complex (*qmoC*), which has been observed only in Gram-negative sulfate-reducing bacteria so far (Pereira *et al*. 2011). Our further search found that the product of the gene lacked transmembrane helices and should therefore encode a different function (Junier *et al*. 2010). Further reduction of sulfite to sulfide could be catalyzed by dissimilatory sulfite reductase encoded by *dsrAB* genes (Ga0073689_2617920264-5). The electrons for DsrAB reductase are probably transferred from the quinone pool by the DsrMK complex and DsrC (Junier *et al*. 2010). According to the Gene Ortholog Neighborhoods tool in the IMG platform, *Pelotomaculum* candidate BPL exhibited the same gene organization for the *dsr* operon as other Gram-positive sulfate-reducing bacteria, e.g. *D. reducens* MI-1 or *D. acetoxidans* DSM 771 (Junier *et al*. 2010). Similar to *D. reducens* MI-1, the identified *dsrM* (Ga0073689_2617920256) encoding a membrane protein functioning as a conduit to transfer electrons from quinones in *D. reducens* MI-1 was also annotated as the nitrate reductase gamma subunit in the IMG platform. The electrons for the quinone pool might come from NADH oxidation coupled with proton translocation catalyzed by the energy-conserving NADH-quinone oxidoreductase (complex I, Ga0073689_2617921751-61). Another essential protein, dissimilatory sulfite reductase subunit C (DsrC, Ga0073689_2617920254), was also identified in the proteome. The proteogenomic data presented here clearly indicate that *Pelotomaculum* candidate BPL has a complete sulfate reduction pathway which is active during anaerobic benzene degradation.

In another study, *Pelotomaculum* members were also proposed to degrade benzene in a similar sulfate-reducing enrichment culture (Herrmann *et al*. 2010). The authors hypothesized that benzene degradation by *Pelotomaculum* occurred by fermentation coupled to sulfate reduction performed by another organism in the enrichment culture (Herrmann *et al*. 2010; Rakoczy *et al*. 2011; Taubert *et al*. 2012). Interspecies hydrogen transfer was suggested to play an important role for benzene mineralization (Rakoczy *et al*. 2011). However, our data for *Pelotomaculum* candidate BPL indicate that benzene degradation and sulfate reduction have been rather performed by the same organism.

Hydrogenases and formate dehydrogenases

No genes encoding [FeFe] and [NiFe] hydrogenases could be identified in the genome. Their absence suggests that *Pelotomaculum* candidate BPL neither has the capability for H_2 oxidation nor for fermentation with H_2 production. This was unexpected as members of the genus *Pelotomaculum* are typical fermenting organisms and usually possess hydrogenases. Three genes putatively encoding formate dehydrogenases (Ga0073689_2617920664, 2617920666 and 2617922225) were identified in the genome but neither of them could be identified in the proteome. This suggests that formate transfer rather than hydrogen transfer may be important if *Pelotomaculum* candidate BPL would possibly grow in syntrophic fermentation when sulfate is not available.

CONCLUSIONS

Our proteogenomic study of *Pelotomaculum* candidate BPL sheds new light on anaerobic benzene degradation by sulfate-reducing microorganisms and in particular by the genus *Pelotomaculum*. The member of the genus *Pelotomaculum* identified here, candidate strain BPL, did not only degrade benzene in the absence of molecular oxygen but also performed sulfate reduction, making it the first representative of the genus *Pelotomaculum* which has the capacity to grow by dissimilatory sulfate reduction. Comparison with other benzene-degrading cultures indicates that such co-cultures might be widespread. Detection of *Pelotomaculum* species in cultures or natural samples therefore does not literally indicate fermentation, especially in the case of the anaerobic hydrocarbon degradation.

SUPPLEMENTARY DATA

Supplementary data are available at *FEMSEC* online.

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Conflict of interest. None declared.

REFERENCES

Abu Laban N, Selesi D, Jobelius C *et al*. Anaerobic benzene degradation by Gram-positive sulfate-reducing bacteria. *FEMS Microbiol Ecol* 2009;**68**:300–11.

Abu Laban N, Selesi D, Rattei T *et al*. Identification of enzymes involved in anaerobic benzene degradation by a strictly anaerobic iron-reducing enrichment culture. *Environ Microbiol* 2010;**12**:2783–96.

Aburto-Medina A, Ball AS. Microorganisms involved in anaerobic benzene degradation. *Ann Microbiol* 2014;**65**:1201–13.

Buckel W, Thauer RK. Energy conservation via electron bifurcating ferredoxin reduction and proton/Na+ translocating ferredoxin oxidation. *Biochim Biophys Acta* 2013;**1827**:94–113.

Caldwell ME, Suflita JM. Detection of phenol and benzoate as intermediates of anaerobic benzene biodegradation under different terminal electron-accepting conditions. *Environ Sci Technol* 2000;**34**:1216–20.

Callaghan AV, Morris BE, Pereira IA *et al*. The genome sequence of *Desulfatibacillum alkenivorans* AK-01: a blueprint for anaerobic alkane oxidation. *Environ Microbiol* 2012;**14**:101–13.

Devine CE. Identification of key organisms, genes and pathways in benzene-degrading methanogenic cultures volume. *Ph.D. Thesis*. University of Toronto, 2013.

Dröge J, Gregor I, McHardy AC. Taxator-tk: precise taxonomic assignment of metagenomes by fast approximation of evolutionary neighborhoods. *Bioinformatics* 2015;**31**:817–24.

Fuchs G, Boll M, Heider J. Microbial degradation of aromatic compounds - from one strategy to four. *Nat Rev Microbiol* 2011;**9**:803–16.

Gieg LM, Fowler SJ, Berdugo-Clavijo C. Syntrophic biodegradation of hydrocarbon contaminants. *Curr Opin Biotechnol* 2014;**27**:21–9.

Gregor I, Dröge J, Schirmer M *et al*. PhyloPythiaS+: a self-training method for the rapid reconstruction of low-ranking taxonomic bins from metagenomes. *PeerJ* 2016;**4**:e1603.

Herrmann S, Kleinsteuber S, Chatzinotas A *et al*. Functional characterization of an anaerobic benzene-degrading enrichment culture by DNA stable isotope probing. *Environ Microbiol* 2010;**12**:401–11.

Holmes DE, Risso C, Smith JA *et al*. Anaerobic oxidation of benzene by the hyperthermophilic archaeon *Ferroglobus placidus*. *Appl Environ Microb* 2011;**77**:5926–33.

Holmes DE, Risso C, Smith JA *et al*. Genome-scale analysis of anaerobic benzoate and phenol metabolism in the hyperthermophilic archaeon *Ferroglobus placidus*. *ISME J* 2012;**6**:146–57.

Imachi H, Sekiguchi Y, Kamagata Y *et al*. *Pelotomaculum thermopropionicum* gen. nov., sp. nov., an anaerobic, thermophilic, syntrophic propionate-oxidizing bacterium. *Int J Syst Evol Micr* 2002;**52**:1729–35.

Imachi H, Sekiguchi Y, Kamagata Y *et al*. Non-sulfate-reducing, syntrophic bacteria affiliated with *Desulfotomaculum* Cluster I are widely distributed in methanogenic environments. *Appl Environ Microb* 2006;**72**:2080–91.

Junier P, Junier T, Podell S *et al*. The genome of the Gram-positive metal- and sulfate-reducing bacterium *Desulfotomaculum reducens* strain MI-1. *Environ Microbiol* 2010;**12**:2738–54.

Keller A, Nesvizhskii AI, Kolker E *et al*. Empirical statistical model to estimate the accuracy of peptide identifications made by MS/MS and database search. *Anal Chem* 2002;**74**:5383–92.

Kim M, Oh HS, Park SC *et al*. Towards a taxonomic coherence between average nucleotide identity and 16S rRNA gene sequence similarity for species demarcation of prokaryotes. *Int J Syst Evol* **Micr** 2014;**64**:346–51.

Kunapuli U, Lueders T, Meckenstock RU. The use of stable isotope probing to identify key iron-reducing microorganisms involved in anaerobic benzene degradation. *ISME J* 2007;**1**:643–53.

Kung JW, Loffler C, Dorner K *et al*. Identification and characterization of the tungsten-containing class of benzoyl-coenzyme A reductases. *P Natl Acad Sci USA* 2009;**106**:17687–92.

Lee WP, Stromberg MP, Ward A *et al*. MOSAIK: a hash-based algorithm for accurate next-generation sequencing short-read mapping. *PLoS One* 2014;**9**:e90581.

Loffler C, Kuntze K, Vazquez JR *et al*. Occurrence, genes and expression of the W/Se-containing class II benzoyl-Coenzyme A reductases in anaerobic bacteria. *Environ Microbiol* 2011;**13**:696–709.

Luo F, Devine CE, Edwards EA. Cultivating microbial dark matter in benzene-degrading methanogenic consortia. *Environ Microbiol* 2016;**18**:2923–36.

Luo F, Gitiafroz R, Devine CE *et al*. Metatranscriptome of an anaerobic benzene-degrading, nitrate-reducing enrichment culture reveals involvement of carboxylation in benzene ring activation. *Appl Environ Microb* 2014;**80**:4095–107.

Lykidis A, Chen CL, Tringe SG *et al*. Multiple syntrophic interactions in a terephthalate-degrading methanogenic consortium. *ISME J* 2011;**5**:122–30.

Manoli E, Samara C. Polycyclic aromatic hydrocarbons in natural waters: sources, occurrence and analysis. *Trends Analyt Chem* 1999;**18**:417–28.

Markowitz VM, Chen IM, Chu K *et al*. IMG/M 4 version of the integrated metagenome comparative analysis system. *Nucleic Acids Res* 2014;**42**:D568–73.

Meckenstock RU, Mouttaki H. Anaerobic degradation of non-substituted aromatic hydrocarbons. *Curr Opin Biotechnol* 2011;**22**:406–14.

Milne I, Stephen G, Bayer M *et al*. Using Tablet for visual exploration of second-generation sequencing data. *Brief Bioinform* 2013;**14**:193–202.

Mock J, Wang S, Huang H *et al*. Evidence for a hexaheteromeric methylenetetrahydrofolate reductase in *Moorella thermoacetica*. *J Bacteriol* 2014;**196**:3303–14.

Musat F, Widdel F. Anaerobic degradation of benzene by a marine sulfate-reducing enrichment culture, and cell hybridization of the dominant phylotype. *Environ Microbiol* 2008;**10**:10–9.

Oberender J, Kung JW, Seifert J *et al*. Identification and characterization of a succinyl-Coenzyme A (CoA):benzoate CoA transferase in *Geobacter metallireducens*. *J Bacteriol* 2012;**194**:2501–8.

Parks DH, Imelfort M, Skennerton CT *et al*. CheckM: assessing the quality of microbial genomes recovered from isolates, single cells, and metagenomes. *Genome Res* 2015;**25**:1043–55.

Pereira IA, Ramos AR, Grein F *et al*. A comparative genomic analysis of energy metabolism in sulfate reducing bacteria and archaea. *Front Microbiol* 2011;**2**:69.

Phelps CD, Zhang ZM, Young LY. Use of stable isotopes to identify benzoate as a metabolite of benzene degradation in a sulphidogenic consortium. *Environ Microbiol* 2001;**3**:600–3.

Plugge CM, Zhang W, Scholten JC *et al*. Metabolic flexibility of sulfate-reducing bacteria. *Front Microbiol* 2011;**2**:81.

Qiu YL, Sekiguchi Y, Hanada S *et al*. *Pelotomaculum terephthalicum* sp. nov. and *Pelotomaculum isophthalicum* sp. nov.: two anaerobic bacteria that degrade phthalate isomers in syntrophic association with hydrogenotrophic methanogens. *Arch Microbiol* 2006;**185**:172–82.

Rakoczy J, Schleinitz KM, Muller N *et al*. Effects of hydrogen and acetate on benzene mineralisation under sulphate-reducing conditions. *FEMS Microbiol Ecol* 2011;**77**:238–47.

Ramos AR, Grein F, Oliveira GP *et al*. The FlxABCD-HdrABC proteins correspond to a novel NADH dehydrogenase/heterodisulfide reductase widespread in anaerobic bacteria and involved in ethanol metabolism in *Desulfovibrio vulgaris* Hildenborough. *Environ Microbiol* 2015;**17**:2288–305.

Selesi D, Jehmlich N, von Bergen M *et al*. Combined genomic and proteomic approaches identify gene clusters involved in anaerobic 2-methylnaphthalene degradation in the sulfate-reducing enrichment culture N47. *J Bacteriol* 2010;**192**:295–306.

Spormann AM, Thauer RK. Anaerobic acetate oxidation to CO_2 by *Desulfotomaculum acetoxidans*. *Arch Microbiol* 1988;**150**:374–80.

Stams AJ, Plugge CM. Electron transfer in syntrophic communities of anaerobic bacteria and archaea. *Nat Rev Microbiol* 2009;**7**:568–77.

Stoddard SF, Smith BJ, Hein R *et al*. rrnDB: improved tools for interpreting rRNA gene abundance in bacteria and archaea and a new foundation for future development. *Nucleic Acids Res* 2015;**43**:D593–8.

Strittmatter AW, Liesegang H, Rabus R *et al*. Genome sequence of *Desulfobacterium autotrophicum* HRM2, a marine sulfate reducer oxidizing organic carbon completely to carbon dioxide. *Environ Microbiol* 2009;**11**:1038–55.

Taubert M, Vogt C, Wubet T *et al*. Protein-SIP enables time-resolved analysis of the carbon flux in a sulfate-reducing, benzene-degrading microbial consortium. *ISME J* 2012;**6**:2291–301.

Ulrich AC, Edwards EA. Physiological and molecular characterization of anaerobic benzene-degrading mixed cultures. *Environ Microbiol* 2003;**5**:92–102.

van der Zaan BM, Saia FT, Stams AJ *et al*. Anaerobic benzene degradation under denitrifying conditions: *Peptococcaceae* as dominant benzene degraders and evidence for a syntrophic process. *Environ Microbiol* 2012;**14**:1171–81.

von Toerne C, Kahle M, Schäfer A *et al*. Apoe, Mbl2, and Psp plasma protein levels correlate with diabetic phenotype in NZO mice-an optimized rapid workflow for SRM-based quantification. *J Proteome Res* 2013;**12**:1331–43.

Wischgoll S, Heintz D, Peters F *et al*. Gene clusters involved in anaerobic benzoate degradation of *Geobacter metallireducens*. *Mol Microbiol* 2005;**58**:1238–52.

Wisniewski JR, Zougman A, Nagaraj N *et al*. Universal sample preparation method for proteome analysis. *Nat Meth* 2009;**6**:359–62.

Wohlbrand L, Jacob JH, Kube M *et al*. Complete genome, catabolic sub-proteomes and key-metabolites of *Desulfobacula toluolica* Tol2, a marine, aromatic compound-degrading, sulfate-reducing bacterium. *Environ Microbiol* 2013;**15**:1334–55.

Wu JH, Wu FY, Chuang HP *et al*. Community and proteomic analysis of methanogenic consortia degrading terephthalate. *Appl Environ Microb* 2013;**79**:105–12.

Wu M, Scott AJ. Phylogenomic analysis of bacterial and archaeal sequences with AMPHORA2. *Bioinformatics* 2012;**28**:1033–4.

Zhang T, Bain TS, Nevin KP *et al*. Anaerobic benzene oxidation by *Geobacter* species. *Appl Environ Microb* 2012;**78**:8304–10.

Zhang T, Tremblay PL, Chaurasia AK *et al*. Anaerobic benzene oxidation via phenol in *Geobacter metallireducens*. *Appl Environ Microb* 2013;**79**:7800–6.

Zhou J, Bruns MA, Tiedje JM. DNA recovery from soils of diverse composition. *Appl Environ Microb* 1996;**62**:316–22.