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Enzymes involved in phthalate degradation in sulphate-reducing bacteria

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Summary

The complete degradation of the xenobiotic and environmentally harmful phthalate esters is initiated by hydrolysis to alcohols and *o*-phthalate (phthalate) by esterases. While further catabolism of phthalate has been studied in aerobic and denitrifying microorganisms, the degradation in obligately anaerobic bacteria has remained obscure. Here, we demonstrate a previously overseen growth of the δ-proteobacterium *Desulfosarcina cetonica* with phthalate/sulphate as only carbon and energy sources. Differential proteome and CoA ester pool analyses together with in vitro enzyme assays identified the genes, enzymes and metabolites involved in phthalate uptake and degradation in *D. cetonica.* Phthalate is initially activated to the short-lived phthaloyl-CoA by an ATP-dependent phthalate CoA ligase (PCL) followed by decarboxylation to the central intermediate benzoyl-CoA by an UbiD-like phthaloyl-CoA decarboxylase (PCD) containing a prenylated flavin cofactor. Genome/metagenome analyses predicted phthalate degradation capacity also in the sulphate-reducing *Desulfobacula toluolica*, strain NaphS2, and other δ-proteobacteria. Our results suggest that phthalate degradation proceeds in all anaerobic bacteria via the labile phthaloyl-CoA that is captured and decarboxylated by highly abundant PCDs. In contrast, two alternative strategies have been established for the formation of phthaloyl-CoA, the possibly most unstable CoA ester in biology.

Introduction

Phthalic acid esters (PAEs), esters of various alcohols with *o*-phthalic acid (1,2-dicarboxybenzene, referred to as phthalate), are annually produced at the million ton scale and are mainly used as plasticizers that are non-covalently incorporated into high-molecular weight polymers such as polyvinyl chloride. They are classified as xenobiotics that have been produced industrially only since the 1960s (Giam *et al*., 1984; Caldwell, 2012; Benjamin *et al*., 2015; Net *et al*., 2015; Gao and Wen, 2016). Among many adverse effects, in particular the endocrine disrupting activity of PAEs threatens wildlife and human health. Due to their global utilization and facile migration into the environment during the production, transport and disposal of plastics, PAEs are categorized as industrially important hazardous pollutants (Caldwell, 2012; Benjamin *et al*., 2015; Gao and Wen, 2016).

Biodegradation of PAEs by microorganisms is considered as the most effective process to eliminate them from the environment (Cousins *et al*., 2003; Huang *et al*., 2013; Gao and Wen, 2016). Bacterial PAE degradation is initiated by hydrolysis of the two ester bonds to the corresponding alcohols and phthalate (Liang *et al*., 2008; Gao and Wen, 2016). In aerobic bacteria, dioxygenases and decarboxylases convert the latter then into protocatechuate, a central intermediate of the aerobic degradation of aromatic compounds (Eaton and Ribbons, 1982; Nomura *et al*., 1992).

At anoxic habitats, complete oxidation of phthalate to CO_2 is considered a rate-limiting step of PAE degradation (Gao and Wen, 2016). The decarboxylation of phthalate to benzoate, either directly or after activation to a CoA thioester, has been proposed more than three decades ago (Taylor and Ribbons, 1983; Nozawa and Maruyama, 1988; Kleerebezem, 1999). However, the genes and enzymes involved in oxygen-independent degradation of phthalate

were only recently elucidated in studies with denitrifying, phthalate-degrading *Thauera, Azoarcus* and *Aromatoleum* strains (Junghare *et al*., 2016; Ebenau-Jehle *et al*., 2017). Note that formerly phthalate/aromatic compounds degrading *Azoarcus* sp. have recently been reclassified to the novel genus *Aromatoleum* (Rabus *et al*., 2019). The genomes of denitrifying phthalate degraders contain gene clusters comprising phthalate-induced genes putatively encoding a tripartite ATP-independent periplasmic (TRAP) transporter, a class III CoA-transferase, an UbiD-like decarboxylase, and an UbiX-like prenyltransferase involved in UbiD flavin cofactor maturation. In accordance, extracts of phthalate-grown *Thauera chlorobenzoica* CB-1, *Aromatoleum aromaticum EbN1* and *Azoarcus* sp. PA01 activated phthalate to phthaloyl-CoA by a succinyl-CoA-dependent CoA transferase, followed by decarboxylation to benzoyl-CoA (Fig. 1) (Junghare *et al*., 2016; Ebenau-Jehle *et al*., 2017). In denitrifying bacteria, benzoyl-CoA is dearomatized by ATP-dependent class I benzoyl-CoA reductases (Kung *et al*., 2010; Boll *et al*., 2014; Buckel *et al*., 2014; Tiedt *et al*., 2018). The cyclic dienoyl-CoA product is then converted to three acetyl-CoA and CO_2 by modified β-oxidation reactions of the benzoyl-CoA degradation pathway (Rabus *et al*., 2005; Fuchs *et al*., 2011; Schmid *et al*., 2015; Rabus *et al*., 2016).

A highly specific, heterodimeric class III succinyl-CoA: phthalate CoA transferase (SptAB) was characterized from *A. aromatoleum* after heterologous expression in *Escherichia coli* (Mergelsberg *et al*., 2018*).* The product phthaloyl-CoA is extremely labile due to intramolecular substitution yielding CoA and phthalic anhydride (half-life = 7 min). To overcome the general problem of phthaloyl-CoA instability, the next enzyme of the pathway, phthaloyl-CoA decarboxylase (PCD) is produced at high amounts in denitrifying phthalate degraders (>100 μM, 5%–15% of soluble protein). Thus, phthaloyl-CoA formed by SptAB will be immediately complexed and decarboxylated by PCD, which explains the very low cellular phthaloyl-CoA concentrations far below 1 μM. No evidence of a direct interaction of SptAB and PCD was observed (Mergelsberg *et al*., 2018).

A phthaloyl-CoA decarboxylase has so far been isolated only from extracts of *T. chlorobenzoica* cells grown with phthalate and nitrate (Mergelsberg *et al*., 2017). It belongs to the UbiD-family of (de)carboxylases containing a prenylated FMN (prFMN) and Fe^{2+} as cofactors. Upon incubation in air, the Fe^{2+} is oxidized to the Fe^{3+}-state with a concomitant release of the active site prFMN resulting in a complete loss of activity (half-life in air 13.3 ± 3 min). As shown for other UbiD-like decarboxylases, PCD maturation is expected to depend on an UbiX-like prenyltransferase that modifies the active site FMN using dimethylallyl-monophosphate as co-substrate (Payne *et al*., 2015; White *et al*., 2015). Among UbiD-like decarboxylases, PCD represents the only member catalysing the difficult C—C bond cleavage of a non-phenolic arylcarboxyl-CoA thioester. As a consequence, the mechanism is considered to substantially differ from those of recently characterized UbiD-like decarboxylases (Mergelsberg *et al*., 2017) indicating a functional diversity of UbiD-family members (Leys and Scrutton, 2016; Leys, 2018).

Only very little is known about anaerobic phthalate degradation in strictly anaerobic bacteria. Phthalate degradation coupled to sulphate reduction and methanogenesis was observed in sediment slurries or enrichment cultures (Kleerebezem *et al*., 1999; Liu and Chi, 2003; Chang *et al*., 2005; Liu *et al*., 2005a; Liu *et al*., 2005b), and *Pelotomaculum* species were reported to degrade phthalate in syntrophic association with hydrogenotrophic methanogens (Qiu *et al*., 2006). However, the genes and enzymes involved in phthalate degradation have not yet been studied in an obligately anaerobic organism. Here, we report on the previously unknown capacity of the sulphate-reducing δ-proteobacterium *Desulfosarcina cetonica* to fully degrade phthalate under sulphate-reducing conditions. This bacterium probably uses the class II benzoyl-CoA reductase for degradation of aromatic compounds and oxidizes the resulting acetyl-CoA through the Wood-Ljungdahl pathway (Janssen and Schink 1995a, b). We identified the genes, enzymes and metabolites involved in the phthalate degradation.

Results and discussion

Growth of Desulfosarcina cetonica *with phthalate and sulfate*

The genome of *D. cetonica* contains a gene encoding an UbiD-like product (locus tag JCM12296_RS19395) with

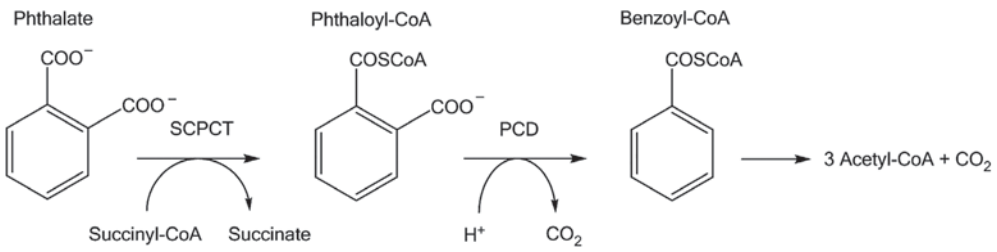

Fig. 1. Enzymatic steps involved in phthalate degradation in denitrifying bacteria.
SCPCT = succinyl-CoA:phthalate CoA transferase; PCD = phthaloyl-CoA decarboxylase.

marked similarities to the experimentally verified PCD from *T. chlorobenzoica* (72% amino acid sequence identity) (Mergelsberg *et al*., 2017). This finding motivated us to test the phthalate degradation potential of this sulphate-reducing δ-proteobacterium that was originally isolated from a flooded oil stratum (Galushko and Rozanova, 1991). Indeed, during anoxic cultivation in gas-tight sealed 100-ml bottles, the complete degradation of 5 mM phthalate coupled to sulphide production was observed at a doubling time of 36 ± 3.5 h with an OD_{578} reached of around 0.4. The doubling time was slightly higher than during growth with benzoate (29 ± 2.5 h), or butyrate (25 ± 1.5 h). For large-scale cultivation, *D. cetonica* was grown in a 200 l fermenter (for growth curve see Supporting Information Fig. S1). To remove H_2S and to adjust the pH to 7.4, this fermenter was frequently gassed with N_2 and/or CO_2. Under these condition, 1.3 mol of phthalate were consumed with a yield of 128 g (wet mass) of *D. cetonica* cells giving a yield coefficient of Y = 0.18 g (dry mass) per mol phthalate consumed.

In vitro *assays for enzymes involved in phthalate degradation*

Due to the instability of phthaloyl-CoA (Mergelsberg *et al*., 2018), *in vitro* enzyme assays were routinely carried out in a coupled, discontinuous assay following benzoyl-CoA formation from phthalate by ultra-performance liquid chromatography (UPLC). In the presence of 5 mM MgATP and 0.5 mM CoA, the protein- and time-dependent conversion of phthalate to benzoyl-CoA was observed at a rate of 4.5 nmol min^{-1} mg^{-1} (Fig. 2A and B); virtually no phthaloyl-CoA intermediate was detected (<0.1 μM). When ATP or CoA was omitted, no formation of benzoyl-CoA was observed. Neither succinyl-CoA nor acetyl-CoA (0.2 mM each) could substitute for CoA + ATP. These results suggest that phthalate is converted to benzoyl-CoA by an ATP-dependent phthalate CoA ligase (PCL) and a PCD, whereas no evidence of the involvement of a succinyl-CoA:phthalate CoA transferase, that was identified in all denitrifying, phthalate-degrading bacteria investigated so far, was obtained.

To test the oxygen sensitivity of benzoyl-CoA formation from phthalate, CoA and ATP, extracts of *D. cetonica* cells grown with phthalate/sulphate were incubated in air for different time points on ice after which the activity was measured under anoxic conditions (N_2:H_2, 95:5, by vol.). Upon exposure to air, a clear decrease of activity was observed with a half life of around 7 min (Fig. 2C). In contrast the anaerobically incubated control was stable for almost 1 h. While the activation of phthalate to its CoA ester was stable in air (see below), the oxygen sensitivity is assigned to PCD with a half-life being even shorter than that of extracts from *T. chlorobenzoica* cells grown with phthalate/nitrate (20 min) (Mergelsberg *et al*., 2017).

Formation of benzoyl-CoA from phthalate, CoA and MgATP was negligible in cells grown with benzoate (<0.1 nmol min^{-1} mg^{-1}), whereas benzoyl-CoA formation from benzoate in the presence of MgATP and CoA was observed in both, cells grown with phthalate or benzoate (4.8 nmol min^{-1} mg^{-1}). This finding suggests a clear induction of specific PCL/PCD activities during growth with phthalate. To further substantiate the induction of PCD during growth with phthalate, we analysed extracts of exponentially grown cells with phthalate/sulphate and benzoate/sulphate for the presence of prFMN, the modified flavin-cofactor of PCD from *T. chlorobenzoica* (Mergelsberg *et al*., 2017) and other UbiD-like enzymes. Using ESI-QTOF-MS analyses, a compound with m/z = 525.1746 ± 0.0008 Da was identified at high abundance in cells grown with phthalate/sulphate that fitted to the catalytically active azomethine ylid (iminium form) of prFMN of UbiD-like enzymes (Payne *et al*., 2015) including PCD from *T. chlorobenzoica* (Mergelsberg *et al*., 2017). In contrast, virtually no compound fitting to this mass was identified in cells grown with benzoate (abundance in phthalate vs benzoate grown cells ≥500), which is in line with the differential abundance and activity of PCD in cells grown with phthalate or benzoate (see below).

The dependence on K^+ ions is a common property of UbiD-like enzymes and was recently also reported also for PCD from *T. chlorobenzoica* (Mergelsberg *et al*., 2017). To test whether this applies to PCD from *D. cetonica*, the PCD activity was determined at varying KCl concentrations. A clear up to 2-fold stimulation of PCD activity was observed in comparison to a control without KCl supplement with a maximum around 100 mM KCl (Fig. 2D). In summary, results from cell extracts assays indicate that PCD from *D. cetonica* appears share similar properties with the enzyme from *T. chlorobenzoica* in terms of cofactor content, oxygen-sensitivity, and KCl dependence.

Rate limiting step during conversion of phthalate to benzoyl-CoA in cell-free extracts

To determine the individual PCL and PCD activities and thus the rate-limiting step during the conversion of phthalate to benzoyl-CoA, crude cell extract assays were supplemented with fractions of enriched PCL (obtained in this work, see below) or purified PCD from *T. chlorobenzoica*, respectively. As these fractions contained exclusively either PCL or PCD activity, an increase of the benzoyl-CoA formation rate should be unambiguously assigned to one of the two enzymes. Addition of enriched PCL (up to 50 nmol min^{-1} mg^{-1} crude extract) had virtually no effect on the observed rate of benzoyl-CoA formation from phthalate in the presence of MgATP and CoA. Thus, the observed specific activity of 4.5 nmol min^{-1} mg^{-1} can be assigned to PCD, catalysing the rate-limiting step during

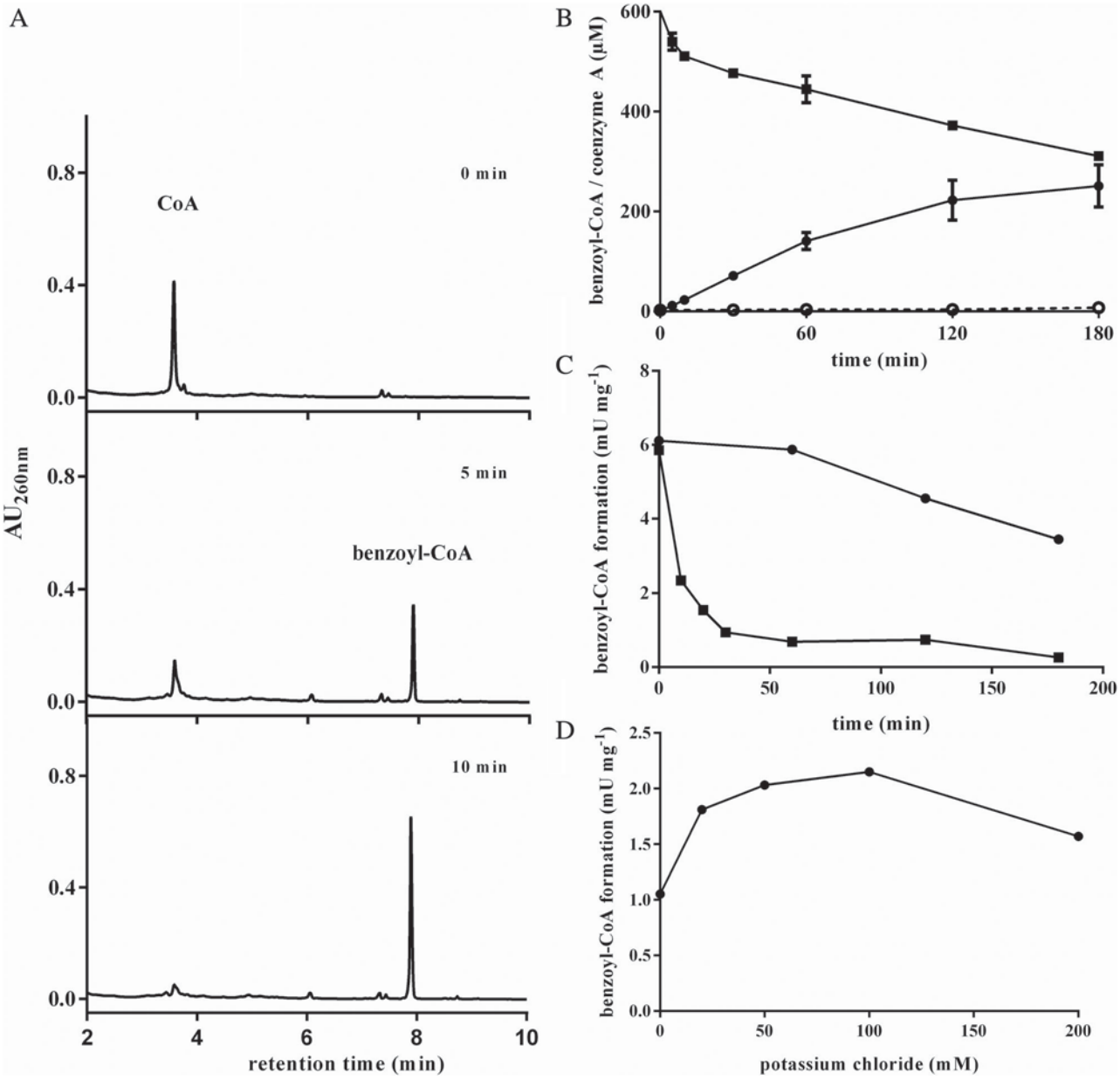

Fig. 2. *In vitro* conversion of phthalate to benzoyl-CoA and properties of PCD.
A. UPLC-analysis of the time-, phthalate-, and ATP-dependent conversion of CoA to phthaloyl-CoA by extracts of *D. cetonica* cells grown with phthalate/sulphate. Virtually to transient accumulation of the proposed phthaloyl-CoA intermediate was observed.
B. Course of CoA (■) consumption, and benzoyl-CoA (●) formation in the presence of phthalate; (○) benzoyl-CoA formation in the absence of phthalate.
C. Oxygen sensitivity of benzoyl-CoA formation from phthalate and CoA; crude extract (■) incubated in air at 4 °C, (●) incubated under anoxic conditions at 4 °C.
D. KCl dependence of the benzoyl-CoA formation from phthalate and CoA.

the conversion of phthalate to benzoyl-CoA. In contrast, the addition of increasing amounts of PCD gradually stimulated the rate of benzoyl-CoA formation reaching a maximal activity of 29.5 nmol min^{-1} mg^{-1}. In conclusion, this activity is assigned to PCL activity in cell extracts of *D. cetonica.*

The phthaloyl-CoA forming and decarboxylating activities in *D. cetonica* crude extracts were in the range of the corresponding activities reported for extracts of denitrifying phthalate degraders (18–31 nmol min^{-1} mg^{-1} for succinyl-CoA:phthalate CoA transferase and 6–13 nmol min^{-1} mg^{-1} for phthaloyl-CoA decarboxylase activities) (Ebenau-Jehle *et al*., 2017).

A phthalate-induced gene cluster in D. cetonica *and similar clusters in other sulphate-reducing bacteria.* A

whole genome shotgun sequence of *D. cetonica* is available (accession Nr. NZ_BBCC00000000). To identify genes and proteins specifically involved in phthalate degradation, extracts of *D. cetonica* cells grown with phthalate/sulphate were subjected to mass spectrometry (MS)-based proteome analyses and compared with those from cells grown with benzoate/sulphate and butyrate/sulphate (for proteome data see Supporting Information Tables S1 and S2). By analysing six biological replicates, the PCD-like gene product with the locus tag JCM12296_RS19395 was identified at a 740-fold higher abundance in cells grown with phthalate than in cells grown with benzoate as carbon and electron source. Proteins that were only identified in *D. cetonica* cells grown with phthalate but not in cells grown with benzoate or butyrate comprise putative gene products with amino acid sequence similarities to the respective phthalate-induced gene products from *T. chlorobenzoica* including a UbiX-like prenyltransferase (83% identity), a TRAP transporter (85% identity) and a periplasmic binding protein (96% identity). A further gene product uniquely identified in cells grown with phthalate is assigned to a putative AMP-forming, long chain fatty acid CoA ligase (JCM12296_RS19390) fitting perfectly to the observed ATP-dependent benzoyl-CoA formation from phthalate and CoA in crude extracts of *D. cetonica.* Neither in cells grown with phthalate nor with benzoate, a gene product for a SptAB-like class III CoA transferase was identified.

The phthalate-induced genes of *D. cetonica* are organized in a single cluster (Fig. 3A). Next to the phthalate-induced gene products identified by proteome analyses, additional putative gene products of this cluster are annotated as kinase inhibitor, stress protein and flavin-dependent dehydrogenase/isomerase. Their role is less clear but they may be involved in regulatory processes and/or cofactor maturation.

We searched in genomes from other known strictly anaerobic sulphate reducing, metal oxide reducing or fermenting bacteria for similar gene clusters using the UbiD-like putative phthaloyl-CoA decarboxylase from *D. cetonica* (JCM12296_RS19395) as query sequence. In the completed genome of the sulphate-reducing *Desulfobacula toluolica* Tol2 (Wöhlbrand *et al*., 2013, accession Nr. NC_018645.1), a highly similar gene cluster was identified, that, possibly as a result of an artefact during genome assembly was present five identical copies (Fig. 3B). The cluster contains all genes present in the phthalate-induced gene cluster of *D. cetonica* including the encoding gene of a putative UbiD-like decarboxylase (TOL2_RS14825) with 83% amino acid sequence identity to the corresponding gene product from *D. cetonica* (JCM12296_RS19395). Based on theses finding, *D. toluolica* is proposed to have the capacity to degrade phthalate employing similar gene products that are hitherto similar to those of *D. cetonica.* Using BLAST a putative PCD-like decarboxylase was identified also in the genome of the naphthalene degrading, sulphate-reducing strain NaphS2 (WP_006419763.1, 84% identity) (Musat *et al*., 2009; DiDonato Jr. *et al*., 2010). However, no other gene typical of anaerobic phthalate degradation was identified in the near genomic environment. Notably, naphthalene degradation is initiated by naphthalene carboxylation, catalysed by a further UbiD-like enzyme present in sulphate-reducing bacteria during growth with aromatics (Koelschbach *et al*., 2019). No other clearly significant homologue of PCD (amino acid sequence identity ≥65%) was identified in the genomes of other strictly anaerobic bacteria that are known to have the enzyme inventory of the benzoyl-CoA degradation pathway. However, a number of δ-proteobacterial PCD homologues (amino acid sequence identity ≥66%) were identified in the metagenomes of a shallow sediment-hosted perennially suboxic/oxic aquifer (Anantharaman *et al*., 2016), (Supporting Information Table S3), suggesting that next to *D. cetonica*, *D. toluolica* and NaphS2, many so far unknown strictly anaerobic δ-proteobacteria have acquired the capability of phthalate degradation.

Enrichment and some properties of enriched phthalate CoA ligase

The results of *in vitro* enzyme assays and of proteome analyses suggest that in sulphate-reducing bacteria phthalate is activated by a phthalate CoA ligase (PCL) instead of a succinyl-CoA dependent CoA transferase present in denitrifying phthalate degraders. To separate PCL from PCD and other potential acyl-CoA synthetases 100,000 × *g* supernatants of extracts of *D. cetonica* cells grown with phthalate/sulphate in a 200 l fermenter were subjected to DEAE Sepharose anionic exchange chromatography under anoxic conditions. PCL activity assays were routinely supplemented with PCD from *T. chlorobenzoica* (130 nmol min^{-1} ml^{-1}) and the MgATP and CoA-dependent formation of benzoyl-CoA was followed by UPLC. If necessary, the amount of PCD added in the assays was adjusted to guarantee that PCL but not PCD activity was rate limiting in the coupled assay. After a wash step with 35 mM KCl in morpholino propanesulfonic acid (Mops) buffer at pH 7.8, PCL activity was eluted in buffer containing 85 mM KCl in with a 3.2-fold enrichment and a yield of 4.5% (Table 1). SDS PAGE analysis of the PCL activity fraction did not reveal an enriched band migrating at the deduced mass (52 kDa) of the putative phthalate-induced carboxylic acid CoA ligase (JCM12296_RS19390, not shown) suggesting that PCL represents a rather low abundant enzyme with a high specific activity. All attempts to further enrich PCL by other chromatographic materials including Q-Sepharose anionic

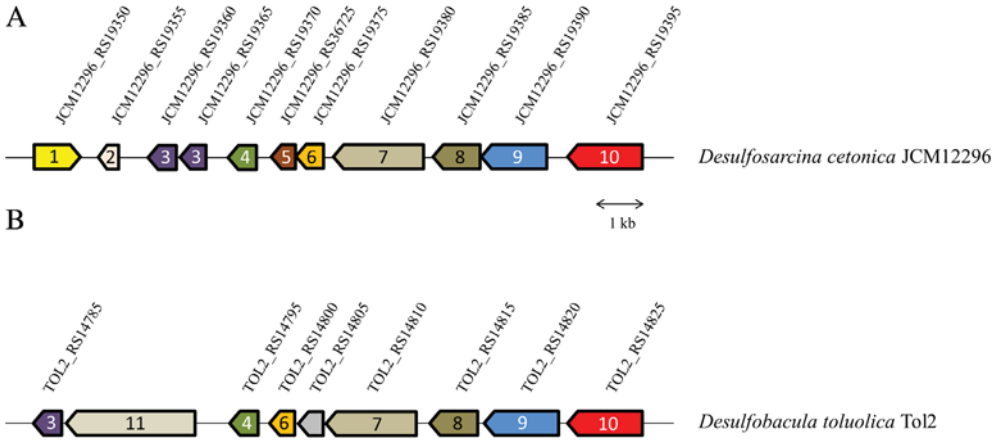

Fig. 3. Phthalate-induced gene cluster in *D. cetonica* as identified by differential proteome analyses and similar genes in *Desulfobacula toluolica*. A. Phthalate-induced genes in D. cetonica. 1, Transcriptional regulator; 2, flavin reductase-like protein; 3, FMN-dependent dehydrogenase/isomerase; 4, UbiX-like enzyme; 5, putative kinase inhibitor; 6, putative nucleotide-binding protein; 7, TRAP transporter; 8, periplasmic binding protein; 9, long-chain fatty acid CoA ligase; 10, UbiD-like (de)carboxylase. The gene products 4, 7–10 were identified as phthalate induced during proteome analyses.
B. Similar gene cluster in D. toluolica. Similar colour/numbers indicate high amino acid sequence similarities and predicted similar functions. [Color figure can be viewed at wileyonlinelibrary.com]

exchanger, a number of reactive dye resins for affinity chromatography, or gel filtration were always accompanied with a major or complete loss of PCL activity without any further enrichment.

Using the PCL activity containing fractions obtained after chromatography on DEAE Sepharose, phthalate + CoA conversion to benzoyl-CoA followed Michaelis–Menten kinetics with an apparent K_m of 22.5 ± 2.8 μM for phthalate (Supporting Information Fig. S2). Notably this fraction contained virtually no benzoate CoA ligase activity indicating that a specific PCL but no promiscuous benzoate CoA ligase catalysed phthalate activation. Enriched PCL was stable in air; virtually no loss of activity was observed compared to an anaerobically incubated control for several hours at 4 °C or for weeks in liquid nitrogen.

Enrichment and properties of phthaloyl-CoA decarboxylase

Enrichment of PCD activity from crude extracts from *D. cetonica* grown with phthalate/sulphate was carried out by DEAE anion exchange chromatography under anoxic conditions. Using the same buffer as for PCL enrichment plus 10% glycerol, PCD activity eluted at 75 mM KCl with an almost five-fold enrichment and a yield of 4.4% (Table 1). Similar as for PCL, all attempts for further enrichment by the chromatographic methods did not result in a further enrichment of PCD. Thus, PCL and PCD appear to be far less stable than the phthaloyl-CoA forming/decarboxylating enzymes from denitrifying bacteria (Mergelsberg *et al*., 2017; Mergelsberg *et al*., 2018).

SDS-PAGE analysis of the protein fractions obtained after DEAE chromatography during PCD enrichment revealed a dominant band migrating at around 60 kDa (Fig. 4), fitting to the theoretical mass of the putative PCD (JCM12296_RS19395). UPLC/electrospray-ionization-quadrupole-time-of-flight mass spectrometry (ESI-QTOF-MS) analyses of tryptic digests of the excised protein band unambiguously identified it as JCM12296_RS19395 (95.7% sequence coverage).

Differential intracellular CoA ester pool analyses

To identify accumulating metabolites during phthalate degradation in *D. cetonica*, CoA esters were extracted from cells grown exponentially with phthalate or benzoate

Table 1. Enrichment of phthalate CoA ligase (PCL) and phthaloyl-CoA decarboxylase (PCD) from extracts of *D. cetonica* cells grown with phthalate and sulphate on DEAE anion exchange chromatography. PCL was purified from 1.6 g, PCD from 3.5 g (wet mass, respectively).

Enzyme/Enrichment step	Total protein (mg)	Total activity (nmol min^{-1})	Specific activity (nmol min^{-1} mg^{-1})	Enrichment (–fold)	Recovery (%)
PCL					
100.000 × *g* supernatant	98	2892	29.5	1	100
DEAE-Sepharose	1.4	133	95	3.2	4.5
PCD					
100.000 × *g* supernatant	350	1190	3.4	1	100
DEAE-Sepharose	3.1	52.7	17	5	4.4

(OD_{578} between 0.1 and 0.2); 2-chlorobenzoyl-CoA was added at defined amounts as an external reference standard. LC/ESI-Q-TOF-MS analyses revealed that benzoyl-CoA was clearly the most abundant CoA-ester in extracts of cells grown with phthalate or benzoate, followed by acetyl-CoA (other typical CoA esters of the benzoyl-CoA degradation pathway identified at lower concentrations are not listed here) (Fig. 5). In contrast, not even traces of phthaloyl-CoA were detected (cellular concentration < 0.1 μM). These findings are in agreement with results from previous studies of phthalate-degrading denitrifying bacteria where phthaloyl-CoA was also identified only in trace amounts (Mergelsberg *et al*., 2018). Remarkably, the abundance of succinyl-CoA was below the detection limit (<0.1 μM) under both growth conditions. This finding is in accordance with the absence of a gene encoding a putative succinyl-CoA:phthalate CoA transferase in the phthalate induced gene cluster in sulphate-reducing bacteria. In contrast, succinyl-CoA represents a highly abundant CoA ester in phthalate-degrading denitrifying bacteria and serves as CoA donor for a phthalate induced CoA transferase (Junghare *et al*., 2016; Ebenau-Jehle *et al*., 2017; Mergelsberg *et al*., 2018). The inability to detect phthaloyl-CoA is assigned to its reported instability which after its formation affords an immediate complexation and decarboxylation by the highly abundant PCD, which prevents its accumulation and subsequent decay (Mergelsberg *et al*., 2018).

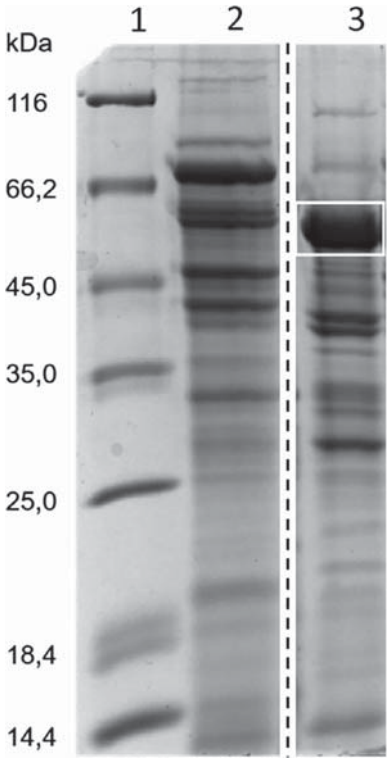

Fig. 4. SDS gel demonstrating enrichment of PCD from extracts of *D. cetonica* cells grown with phthalate and sulphate. Lane 1, molecular weight standard; lane 2, 100,000 × *g* supernatant; lane 3, protein fraction eluting at 75 mM KCl during DEAE anion exchange chromatography. The framed protein band at around 60 kDa was identified by mass spectrometry as JCM12296_RS19395 with 72% amino acid sequence identity to PCD from *T. chlorobenzoica.*

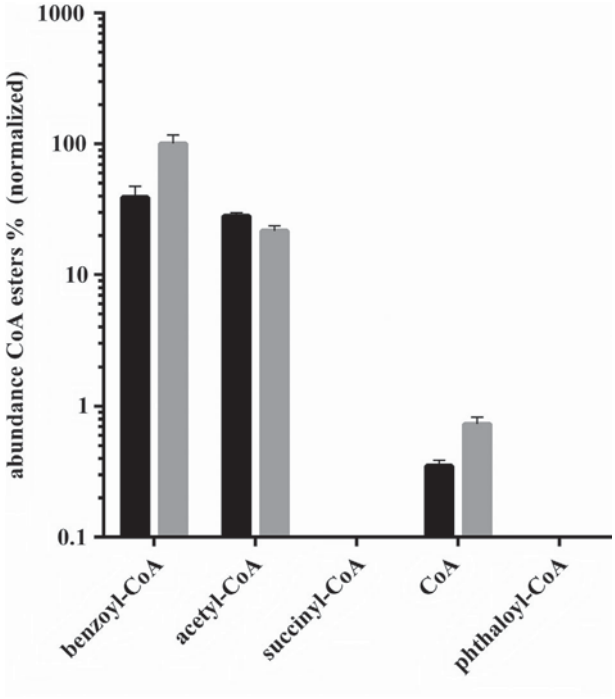

Fig. 5. Cellular abundance of CoA esters. CoA esters were identified by UPLC/ESI-Q-TOF MS analysis. Black bars: cells grown with phthalate; grey bars: cells grown with benzoate. Values were normalized to the highest abundant CoA ester benzoyl-CoA (100%), abundance of succinyl-CoA and phthaloyl-CoA was ≤0.1% of benzoyl-CoA.

Conclusions

Until recently, the pathway of oxygen-independent phthalate degradation has been studied exclusively in a number of denitrifying bacteria of the genera *Thauera* and *Aromatoleum.* The previously overseen capacity of *D. cetonica* to use phthalate as carbon/energy source opened the door to identify the genes and study the enzymatic reactions involved in phthalate degradation in an obligately anaerobic model organism. The results obtained suggest that anaerobic phthalate degradation follows similar strategies in denitrifying and sulphate-reducing bacteria: phthalate is initially activated to the extremely labile phthaloyl-CoA that serves as a substrate for a highly abundant PCD, which immediately captures and decarboxylates it to benzoyl-CoA, thereby preventing futile decay to phthalate and CoA. Next to the encoding genes for catabolic enzymes, also those involved in phthalate uptake and PCD maturation appear to be highly similar in nitrate- and sulphate-reducing bacteria.

The only but remarkable exception is the phthalate activation step: while all denitrifying bacteria investigated so far use a class III succinyl-CoA:phthalate CoA transferase, *D. cetonica* and most probably *D. toluolica* use an ATP-dependent phthalate CoA ligase. At the first view, it appears surprising that sulphate-reducing bacteria that gain far less energy from complete degradation of aromatic substrates than denitrifyers do (Rabus *et al*., 1993; Peters *et al*., 2004) activate phthalate by an AMP-forming CoA ligase instead of a less energy-consuming succinyl-CoA-dependent CoA transferase. However, the inability to detect succinyl-CoA in *D. cetonica* during growth with phthalate clearly indicates that this organism oxidizes the three acetyl-CoA formed from phthalate in the benzoyl-CoA degradation pathway completely to six CO_2 not via the citric acid cycle but via the Wood-Ljungdahl pathway as reported for most alcohols/fatty acid oxidizing sulphate-reducing bacteria (Schauder *et al*., 1986; Janssen and Schink, 1995b). Thus, the use of a succinyl-CoA-dependent CoA transferase is no option for sulphate-reducing bacteria. It is remarkable that despite the relatively short period since PAEs have been introduced at the industrial and thus environmentally relevant scale, obviously two non-related enzymatic strategies have evolved for the initiation of phthalate degradation.

The capacity to use phthalate as growth substrate appears to be less abundant under sulphate- than under nitrate-reducing conditions, though putative PCD encoding genes were identified in unknown δ-proteobacteria during metagenomic aquifer analyses (Anantharaman *et al*., 2016). A rational explanation for this observation may be that xenobiotic PAEs have predominantly been released to the environment at terrestric sites as a result of urbanization and industrialization, and less in marine sediments where sulphate respiration represents the major form of energy metabolism (Gao and Wen, 2016). It can be speculated whether the ongoing pollution of marine environments by plasticizer-containing plastics (Bergmann *et al*., 2017; Haward, 2018) will probably result in a higher frequency of the capability to fully degrade phthalate among marine sulphate-reducing bacteria. Surprisingly, no phthalate catabolic gene cluster was found in the genomes of obligately anaerobic Fe(III)-respiring or fermenting bacteria with sequenced genomes (e.g. *Geobacter* or syntrophic fermenting species). Within the latter physiological group, *Pelotomaculum isophthalicum* is the only reported phthalate-degrading pure culture (Qiu *et al*., 2006). Recently, isophthalate degradation was shown to follow principles in syntrophically fermenting *Syntrophorhabdus aromaticivorans* that are similar to phthalate degradation in sulphate-reducing bacteria involving an ATP-dependent isophthaloyl-CoA synthetase and a UbiD-like isophthaloyl-CoA decarboxylase (Junghare *et al*., 2019).

Experimental procedures

Cultivation of D. cetonica *and preparation of cell-free extracts*

Desulfosarcina cetonica strain 480 (JCM12996, DSM-7267) (Galushko and Rozanova, 1991) was grown anaerobically in bicarbonate-buffered mineral salt medium at 30 °C in a 200 l bioreactor (GEA Diessel GmbH, Hildesheim) containing an initial concentration of 5 mM phthalate and 20 mM Na_2SO_4. The bioreactor was operated in a continuous fed-batch mode using a 0.25 M phthalate and 1 M Na_2SO4 stock solution. Cells were harvested in the exponential growth phase at OD_{578} = 0.7. Harvested cells were kept frozen in liquid nitrogen until use.

Cells used for proteome analysis were grown anaerobically in 80-ml medium containing 10 mM butyrate, 5 mM benzoate or 5 mM phthalate as carbon and energy source in addition to 20 mM Na_2SO_4 as electron acceptor. Cells were harvested in the exponential growth phase at an OD_{578} of 0.14–0.2, centrifuged (10 min, 20.800 × *g*, 4°C) and washed twice with 10 mM Tris/HCl (pH 7). The pellets obtained were frozen in liquid nitrogen until proteome analysis. Extracts were prepared under anaerobic conditions. Frozen cells were suspended in a three- to four-fold volume of 20 mM potassium phosphate buffer (pH 8) for *in vitro* assays. In case of PCD enrichment, cell suspension was in buffer A (50 mM MOPS, 50 mM KCl, glycerol 10% v/v, DTE 1 mM, pH 7.5). Buffers were supplemented with 0.1 mg mL^{-1} DNase I, 0.1 mg mL^{-1} RNase A, and 0.1 mg mL^{-1} lysozyme. Cell suspensions were passed through a chilled French pressure cell at 9 MPa. The cell lysate was ultra-centrifuged (1 h, 200.000 × *g*, 4° C) and the supernatant was used immediately. In case of PCL enrichment, cells were suspended in buffer A (50 mM MOPS, 10 mM KCl, pH 7.8) containing 0.1 mg mL^{-1} DNase I and 0.1 mg mL^{-1} RNase A. Cells were lysed by incubating suspension for 1 h with lysozyme 1 mg g^{-1} cells followed by ultra-centrifugation as described above.

LC analyses coupled to MS and UV/Vis detection

Metabolites were analysed by LC/MS using a Waters Acquity I-class UPLC with a Waters C18 HSS T3 column (2.1 mm × 100 mm, 1.8-μm particle size) coupled to a Waters Synapt G2-S*i* HDMS ESI/Q-TOF system. For analysis of CoA thioesters, an 8 min linear gradient of 2% to 30% acetonitrile in 10 mM ammonium acetate pH 6.8 at a flow rate of 0.35 ml min^{-1} was applied. For analysis of prFMN/FMN, a 20 min linear gradient of 5% to 25% acetonitrile/0.1% formic acid (v/v) in water/0.1% formic acid (v/v) was used at a flow rate of 0.35 ml min^{-1}. Both, CoA esters and prFMN/FMN were measured in MS positive mode with a capillary voltage of 3 kV, 150 °C source temperature, 450 °C desolvation temperature, 1000 l min^{-1} N_2

desolvation gas flow and 100 l min^{-1} N_2 cone gas flow. Collision induced dissociation of precursor ions was performed using a collision energy ramp from 10 to 50 V. LC analyses coupled to UV/visible detection using a Waters Acquity photo diode array detector were conducted with a Waters Acquity H-class UPLC with a Knauer Eurospher 100–2 C18 column (2 mm × 100 mm, 2-μm particle size). CoA esters were separated using a 10 mM potassium phosphate buffer pH 7.0 applying the same gradient and same flow rate as described above for MS-based analyses. Evaluation of LC/MS metabolite data was performed using MassLynx (Waters); for evaluation of LC-UV/visible data MassLynx or Empower (Waters) was used.

For protein identification, protein bands from SDS-PAGE were excised, cysteine residues were reduced using dithiothreitol and alkylated by treatment with iodoacetamide. After in-gel digestion with trypsin (Sigma-Aldrich), the resulting peptides were separated on a Waters Acquity I-class UPLC with a Waters Peptide CSH C18 column (2.1 mm × 150 mm, 1.7-μm particle size) with a gradient from 1% to 40% ACN/0.1% formic acid (v/v) in water/0.1% formic acid (v/v) at a flow rate of 0.04 ml min^{-1}. The source conditions used were the same as described above except that the source temperature was lowered to 80 °C, the desolvation temperature to 400 °C and the desolvation gas flow to 800 l h^{-1}. The instrument was operated in positive HD-MSE mode. The resulting spectra were analysed with ProteinLynx Global Server (Waters) by matching with the UniProt database (minimal fragment ion matches per peptide = 3, minimal fragment ion matches per protein = 3, minimal peptide matches per protein = 7, false discovery rate 3%).

Enzyme assays

Coupled, discontinuous PCL activity assays were performed in a glove box (95% N_2, 5% H_2) at 30 °C in 100 mM potassium phosphate buffer (pH 8). It followed the PCD (isolated from *Thauera chlorobenzoica* grown phthalate) (Mergelsberg *et al*., 2017), phthalate (0.5 mM), MgATP (5 mM) and CoA (0.5 mM)-dependent formation of benzoyl-CoA by UPLC coupled to diode array detection at 260 nm as described (Mergelsberg *et al*., 2017). For detecting maximal PCL activities, PCD was added at saturating amounts to guarantee that only PCL was rate-limiting during benzoyl-CoA formation from phthalate and CoA. Coupled PCD activity assay was performed under the identical conditions. Here, PCD activity-free, enriched PCL from *D. cetonica* was used as phthaloyl-CoA synthesizing enzyme. Prior detection of PCD activities, the required saturating PCL activity to be added was determined in control experiments. For all assays proteins were precipitated in 0.9 M HCl/10% acetonitrile (v/v). Product formation was quantified based on calibration curves of standards. Oxygen inactivation was tested by pre-incubating cell-free extracts in the presence or absence of oxygen at 4 °C. The assay was then performed in the absence of oxygen and started with coenzyme A. Potassium dependence was tested using 20 mM Tris/HCl (pH 8) buffer instead of 100 mM potassium phosphate buffer and varying concentrations (0–200 mM) of potassium chloride.

Enrichment of PCD and PCL

All steps were carried out under anoxic conditions (95% N_2, 5% H_2). Around 3 g of phthalate grown cells (wet mass) were suspended in 9–12 ml buffer A and lysed using a French pressure cell. After ultracentrifugation, the supernatant was filtered through a 0.2-μm sterile filter (Filtropur S 0.2, Sarstedt) and applied to a 12 ml DEAE-Sepharose column (GE Healthcare), equilibrated with buffer A at a flow rate of 0.5 ml min^{-1}. The column was washed with two bed volumes of buffer A and with buffer A supplemented with 15 mM KCl (PCD) or 20 mM KCl (PCL). Fractions eluting in buffer A at higher KCl concentrations as indicated in the results section were tested for PCL/PCD activities. Activity containing fractions were concentrated and used for SDS-PAGE analysis and enzymatic assays. Other chromatography materials tested for PCL/PCD enrichment were Resource Q-Sepharose anionic exchanger, Superdex 200 10/300 Gl gel filtration (all GE Healthcare), and the affinity dyes Reactive Green 5 Agarose, Reactive Red 120, and Cibacron Blue 3GA (1 ml columns each, all Sigma-Aldrich). All columns were equilibrated with buffer A, and elution was by varying KCl, or in case of the affinity dyes, phthalate concentrations (0.1–1 M).

Extraction of CoA esters and prFMN for MS analyses

Intracellular CoA esters and intracellular prenylated FMN were extracted from 100 ml *D. cetonica* cells in exponential growth phase with an OD_{578} 0.1–0.2. Cells were harvested through filtration with regenerated cellulose filters 0.2 μm (Sartorius). Filters were prewashed in a volume of 50 ml ddH_2O at 60 °C. After filtration filters were treated twice with ultrasonic in 10-ml extraction solution (0.1 M formic acid, 80% acetonitrile (v/v). Filters were washed with 2 ml of extraction solution afterwards to remove remaining cells. Total volume of 12-ml extraction solution containing cells was freeze dried and stored at −80 °C until use. Directly before measurements, the samples were suspended in 100 μl 10 mM ammonium acetate (volume normalized depending on OD_{578} before harvesting). For relative quantification via LC/MS 1 mM 2-Cl-benzoyl-CoA was added to the samples.

Prenylated FMN was extracted from 1 g frozen *D. cetonica* cells. Cells were anaerobically lysed as

described and ultracentrifuged (1 h, 200,000 g, 4°C). Supernatant was incubated with 50% aqueous acetonitrile (v/v) followed by incubation for 10 min at 70 °C. The mix was centrifuged and resulting supernatant was freeze dried. The product was dissolved in 100 µl 10% acetonitrile/ddH_2O (v/v) and analysed via LC/MS. Absolute prFMN concentrations were estimated in reference to a calibration curve using FMN as a standard.

Mass spectrometry-based proteome analyses

For proteome analysis cells were grown in biological replicates (n = 6 for phthalate and benzoate, and n = 5 for butyrate). Preparation of protein samples from cells for mass spectrometric analyses including separation by SDS-PAGE and tryptic digestion were as previously described (Warnke *et al*., 2017). The resulting peptides were separated by UHPLC (Ultimate 3000, Dionex/Thermo Fisher Scientific) using a C18 column with reverse phase (25 cm) in a linear gradient from 2% to 55% acetonitrile in 0.1% formic acid (v/v) (120 min). Proteome analysis was performed using a Q Exactive HF mass spectrometer (Thermo Fisher Scientific). Proteome Discoverer (version 1.4.1.14 Thermo Scientific) was used for protein identification, and the acquired MS/MS spectra were searched with SEQUEST HT against NCBI available *Desulfosarcina cetonica* database. Only peptides with a false discovery rate < 0.01 calculated by Percolator and peptide rank = 1 were considered as identified. The abundance of one protein was quantified using the average of the top-3 peptides assigned to this protein.

Computational analyses

Gene cluster of *Desulfosarcina cetonica* was analysed using SnapGene© (version 3.2.1, GSL Biotech LLC) and NCBI available shotgun genome sequences of *Desulfosarcina cetonica.* Homology of the corresponding gene products was analysed using the blastp algorithm (Altschul *et al*., 1990).

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References

Altschul, S.F., Gish, W., Miller, W., Myers, E.W., Lipman, D.J. (1990) Basic local alignment search tool. *J Mol Biol* **215**: 403–410.

Anantharaman, K., Brown, C.T., Hug, L.A., Sharon, I., Castelle, C.J., Probst, A.J., *et al*. (2016) Thousands of microbial genomes shed light on interconnected biogeochemical processes in an aquifer system. *Nat Commun* **7**: 13219.

Benjamin, S., Pradeep, S., Josh, M.S., Kumar, S., and Masai, E. (2015) A monograph on the remediation of hazardous phthalates. *J Hazard Mater* **298**: 58–72.

Bergmann, M., Tekman, M.B., and Gutow, L. (2017) Marine litter: sea change for plastic pollution. *Nature* **544**: 297.

Boll, M., Löffler, C., Morris, B.E.L., and Kung, J.W. (2014) Anaerobic degradation of homocyclic aromatic compounds via arylcarboxyl-coenzyme a esters: organisms, strategies and key enzymes. *Environ Microbiol* **16**: 612–627.

Buckel, W., Kung, J.W., and Boll, M. (2014) The benzoyl-coenzyme a reductase and 2-hydroxyacyl-coenzyme a dehydratase radical enzyme family. *Chembiochem* **15**: 2188–2194.

Caldwell, J.C. (2012) DEHP: genotoxicity and potential carcinogenic mechanisms-a review. *Mutat Res* **751**: 82–157.

Chang, B.V., Liao, C.S., and Yuan, S.Y. (2005) Anaerobic degradation of diethyl phthalate, di-n-butyl phthalate, and di-(2-ethylhexyl) phthalate from river sediment in Taiwan. *Chemosphere* **58**: 1601–1607.

Cousins, I.T., Mackay, D., and Parkerton, T.F. (2003) Physical-chemical properties and evaluative fate modelling of phthalate esters. In *Series Anthropogenic Compounds*, Hutzinger, O., and Staples, C.A. (eds). Berlin, Heidelberg: Springer Berlin Heidelberg, pp. 57–84.

DiDonato, R.J., Jr., Young, N.D., Butler, J.E., Chin, K.J., Hixson, K.K., Mouser, P., *et al*. (2010) Genome sequence of the deltaproteobacterial strain NaphS2 and analysis of differential gene expression during anaerobic growth on naphthalene. *PLoS One* **5**: e14072.

Eaton, R.W., and Ribbons, D.W. (1982) Metabolism of dibutylphthalate and phthalate by micrococcus sp. strain 12B. *J Bacteriol* **151**: 48–57.

Ebenau-Jehle, C., Mergelsberg, M., Fischer, S., Bruls, T., Jehmlich, N., von Bergen, M., and Boll, M. (2017) An unusual strategy for the anoxic biodegradation of phthalate. *ISME J* **11**: 224–236.

Fuchs, G., Boll, M., and Heider, J. (2011) Microbial degradation of aromatic compounds - from one strategy to four. *Nat Rev Microbiol* **9**: 803–816.

Galushko, A., and Rozanova, E.P. (1991) *Desulfobacterium cetonicum* sp. nov.: a sulfate-reducing bacterium which oxidizes fatty acids and ketones. *Microbiology* **60**: 742–746.

Gao, D.-W., and Wen, Z.-D. (2016) Phthalate esters in the environment: a critical review of their occurrence, biodegradation, and removal during wastewater treatment processes. *Sci Total Environ* **541**: 986–1001.

Giam, C.S., Atlas, E., Powers, J.M.A., and Leonard, J.E. (1984) *Phthalate Esters. Anthropogenic Chemicals*. Berlin, Germany: Springer-Verlag, pp. 67–142.

Haward, M. (2018) Plastic pollution of the world's seas and oceans as a contemporary challenge in ocean governance. *Nat Commun* **9**: 667.

Huang, J., Nkrumah, P.N., Li, Y., and Appiah-Sefah, G. (2013) Chemical behavior of phthalates under abiotic conditions in landfills. *Rev Environ Contam Toxicol* **224**: 39–52.

Janssen, P.H., and Schink, B. (1995a) Metabolic pathways and energetics of the acetone-oxidizing, sulfate-reducing bacterium *Desulfobacterium cetonicum*. *Arch Microbiol* **163**: 188–194.

Janssen, P.H., and Schink, B. (1995b) Pathway of butyrate catabolism by *Desulfobacterium cetonicum*. *J Bacteriol* **177**: 3870–3872.

Junghare, M., Spiteller, D., and Schink, B. (2016) Enzymes involved in the anaerobic degradation of ortho-phthalate by the nitrate-reducing bacterium *Azoarcus* sp. strain PA01. *Environ Microbiol* **18**: 3175–3188.

Junghare, M., Spiteller, D., and Schink, B. (2019) Anaerobic degradation of xenobiotic isophthalate by the fermenting bacterium *Syntrophorhabdus aromaticivorans*. *ISME J* **13**: 1252–1268.

Kleerebezem, R. (1999) Energetics of product formation during anaerobic degradation of phthalate isomers and benzoate. *FEMS Microbiol Ecol* **29**: 273–282.

Kleerebezem, R., Hulshoff Pol, L.W., and Lettinga, G. (1999) Anaerobic degradation of phthalate isomers by methanogenic consortia. *Appl Environ Microbiol* **65**: 1152–1160.

Koelschbach, J.S., Moutakki, H., Merl-Pham, J., Arnold, M. E., and Meckenstock, R.U. (2019) Identification of naphthalene carboxylase subunits of the sulfate-reducing culture N47. *Biodegradation* **30**: 147–160. https://doi.org/10.1007/s10532-019-09872-z

Kung, J.W., Baumann, S., von Bergen, M., Müller, M., Hagedoorn, P.L., Hagen, W.R., and Boll, M. (2010) Reversible biological birch reduction at an extremely low redox potential. *J Am Chem Soc* **132**: 9850–9856.

Leys, D. (2018) Flavin metamorphosis: cofactor transformation through prenylation. *Curr Opin Chem Biol* **47**: 117–125.

Leys, D., and Scrutton, N.S. (2016) Sweating the assets of flavin cofactors: new insight of chemical versatility from knowledge of structure and mechanism. *Curr Opin Struct Biol* **41**: 19–26.

Liang, D.-W., Zhang, T., Fang, H.H.P., and He, J. (2008) Phthalates biodegradation in the environment. *Appl Microbiol Biotechnol* **80**: 183–198.

Liu, S.-M., and Chi, W.-C. (2003) CO_2–H_2-dependent anaerobic biotransformation of phthalic acid isomers in sediment slurries. *Chemosphere* **52**: 951–958.

Liu, S.M., Lin, Y.L., and Chi, W.C. (2005a) The effects of benzoate, cyclohex-1-ene carboxylate, and cyclohexane carboxylate on biotransformation of o-phthalate in sediment slurries under sulfate-reducing conditions. *Chemosphere* **59**: 41–48.

Liu, S.M., Lin, Y.L., and Tsai, T.L. (2005b) Growth dynamics of major microbial populations during biodegradation of o-phthalate in anaerobic sediment slurries under a CO_2/H_2 atmosphere. *Chemosphere* **59**: 91–98.

Mergelsberg, M., Willistein, M., Meyer, H., Stark, H.J., Bechtel, D.F., Pierik, A.J., and Boll, M. (2017) Phthaloyl-coenzyme a decarboxylase from *Thauera chlorobenzoica*: the prenylated flavin-, K(+) - and Fe(2+) -dependent key enzyme of anaerobic phthalate degradation. *Environ Microbiol* **19**: 3734–3744.

Mergelsberg, M., Egle, V., and Boll, M. (2018) Evolution of a xenobiotic degradation pathway: formation and capture of the labile phthaloyl-CoA intermediate during anaerobic phthalate degradation. *Mol Microbiol* **108**: 614–626.

Musat, F., Galushko, A., Jacob, J., Widdel, F., Kube, M., Reinhardt, R., *et al*. (2009) Anaerobic degradation of naphthalene and 2-methylnaphthalene by strains of marine sulfate-reducing bacteria. *Environ Microbiol* **11**: 209–219.

Net, S., Sempéré, R., Delmont, A., Paluselli, A., and Ouddane, B. (2015) Occurrence, fate, behavior and ecotoxicological state of phthalates in different environmental matrices. *Environ Sci Technol* **49**: 4019–4035.

Nomura, Y., Nakagawa, M., Ogawa, N., Harashima, S., and Oshima, Y. (1992) Genes in PHT plasmid encoding the initial degradation pathway of phthalate in *Pseudomonas putida*. *J Ferment Bioeng* **74**: 333–344.

Nozawa, T., and Maruyama, Y. (1988) Anaerobic metabolism of phthalate and other aromatic compounds by a denitrifying bacterium. *J Bacteriol* **170**: 5778–5784.

Payne, K.A.P., White, M.D., Fisher, K., Khara, B., Bailey, S. S., Parker, D., *et al*. (2015) New cofactor supports α,β-unsaturated acid decarboxylation via 1,3-dipolar cycloaddition. *Nature* **522**: 497–501.

Peters, F., Rother, M., and Boll, M. (2004) Selenocysteine-containing proteins in anaerobic benzoate metabolism of *Desulfococcus multivorans*. *J Bacteriol* **186**: 2156–2163.

Qiu, Y.-L., Sekiguchi, Y., Hanada, S., Imachi, H., Tseng, I.-C., Cheng, S.-S., *et al*. (2006) *Pelotomaculum terephthalicum* sp. nov. and *Pelotomaculum isophthalicum* sp. nov.: two anaerobic bacteria that degrade phthalate isomers in syntrophic association with hydrogenotrophic methanogens. *Arch Microbiol* **185**: 172–182.

Rabus, R., Nordhaus, R., Ludwig, W., and Widdel, F. (1993) Complete oxidation of toluene under strictly anoxic conditions by a new sulfate-reducing bacterium. *Appl Environ Microbiol* **59**: 1444–1451.

Rabus, R., Kube, M., Heider, J., Beck, A., Heitmann, K., Widdel, F., and Reinhardt, R. (2005) The genome sequence of an anaerobic aromatic-degrading denitrifying bacterium, strain EbN1. *Arch Microbiol* **183**: 27–36.

Rabus, R., Boll, M., Heider, J., Meckenstock, R.U., Buckel, W., Einsle, O., *et al*. (2016) Anaerobic microbial degradation of hydrocarbons: from enzymatic reactions to the environment. *J Mol Microbiol Biotechnol* **26**: 5–28.

Rabus, R., Wohlbrand, L., Thies, D., Meyer, M., Reinhold-Hurek, B., and Kampfer, P. (2019) *Aromatoleum* gen. Nov., a novel genus accommodating the phylogenetic lineage including *Azoarcus evansii* and related species, and proposal of *Aromatoleum aromaticum* sp. nov., *Aromatoleum petrolei* sp. nov., *Aromatoleum bremense* sp. nov., *Aromatoleum toluolicum* sp. nov. and *Aromatoleum diolicum* sp. nov. *Int J Syst Evol Microbiol* **69**: 982–997.

Schauder, R., Eikmanns, B., Thauer, R.K., Widdel, F., and Fuchs, G. (1986) Acetate oxidation to CO_2 in anaerobic bacteria via a novel pathway not involving reactions of the citric acid cycle. *Arch Microbiol* **145**: 162–172.

Schmid, G., Rene, S.B., and Boll, M. (2015) Enzymes of the benzoyl-coenzyme a degradation pathway in the hyperthermophilic archaeon *Ferroglobus placidus*. *Environ Microbiol* **17**: 3289–3300.

Taylor, B.F., and Ribbons, D.W. (1983) Bacterial decarboxylation of *o*-phthalic acids. *Appl Environ Microbiol* **46**: 1276–1281.

Tiedt, O., Fuchs, J., Eisenreich, W., and Boll, M. (2018) A catalytically versatile benzoyl-CoA reductase, key enzyme in the degradation of methyl- and halobenzoates in denitrifying bacteria. *J Biol Chem* **293**: 10264–10274.

Warnke, M., Jacoby, C., Jung, T., Agne, M., Mergelsberg, M., Starke, R., *et al*. (2017) A patchwork pathway for

oxygenase-independent degradation of side chain containing steroids. *Environ Microbiol* **19**: 4684–4699.

White, M.D., Payne, K.A.P., Fisher, K., Marshall, S.A., Parker, D., Rattray, N.J.W., *et al*. (2015) UbiX is a flavin prenyltransferase required for bacterial ubiquinone biosynthesis. *Nature* **522**: 502–506.

Wöhlbrand, L., Jacob, J.H., Mussmann, M., Jarling, R., Beck, A., Amann, R., *et al*. (2013) Complete genome, catabolic subproteomes and key-metabolites of *Desulfobacula toluolica* Tol2, a marine, aromatic compound-degrading, sulfate-reducing bacterium. *Environ Microbiol* **15**: 1334–1355.