

Enzymes involved in the anaerobic degradation of *ortho*-phthalate by the nitrate-reducing bacterium *Azoarcus* sp. strain PA01

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Summary

The pathway of anaerobic degradation of *o*-phthalate was studied in the nitrate-reducing bacterium *Azoarcus* sp. strain PA01. Differential two-dimensional protein gel profiling allowed the identification of specifically induced proteins in *o*-phthalate-grown compared to benzoate-grown cells. The genes encoding *o*-phthalate-induced proteins were found in a 9.9 kb gene cluster in the genome of *Azoarcus* sp. strain PA01. The *o*-phthalate-induced gene cluster codes for proteins homologous to a dicarboxylic acid transporter, putative CoA-transferases and a UbiD-like decarboxylase that were assigned to be specifically involved in the initial steps of anaerobic *o*-phthalate degradation. We propose that *o*-phthalate is first activated to *o*-phthalyl-CoA by a putative succinyl-CoA-dependent succinyl-CoA:*o*-phthalate CoA-transferase, and *o*-phthalyl-CoA is subsequently decarboxylated to benzoyl-CoA by a putative *o*-phthalyl-CoA decarboxylase. Results from *in vitro* enzyme assays with cell-free extracts of *o*-phthalate-grown cells demonstrated the formation of *o*-phthalyl-CoA from *o*-phthalate and succinyl-CoA as CoA donor, and its subsequent decarboxylation to benzoyl-CoA. The putative succinyl-CoA:*o*-phthalate CoA-transferase showed high substrate specificity for *o*-phthalate and did not accept isophthalate, terephthalate or 3-fluoro-*o*-phthalate whereas the putative *o*-phthalyl-CoA decarboxylase converted fluoro-*o*-phthalyl-CoA to fluoro-benzoyl-CoA. No decarboxylase activity was observed with isophthalyl-CoA or terephthalyl-CoA. Both enzyme activities were oxygen-

insensitive and inducible only after growth with *o*-phthalate. Further degradation of benzoyl-CoA proceeds analogous to the well-established anaerobic benzoyl-CoA degradation pathway of nitrate-reducing bacteria.

Introduction

Phthalic acids (PAs) are benzoic acids with one additional carboxylic group in either *ortho*, *meta* or *para* position. PA esters are produced massively worldwide and are used in the manufacture of a wide range of plastic products (Vamsee-Krishna *et al.*, 2006; Chen *et al.*, 2007; Liang *et al.*, 2008). Di-esters of *ortho*-phthalic acid (*o*-phthalate) serve mainly as plasticizers, for example, in the production of polyvinyl chloride (Giam *et al.*, 1984).

PA isomers are introduced into the environment from plastics because phthalate esters are noncovalently bound to the plastic polymers in order to maintain the required flexibility of plastic products (Nilsson, 1994). Additionally, phthalates are released into the environment through liquid and solid waste streams generated during the production of phthalates from the corresponding xylenes (Bemis *et al.*, 1982). Therefore, high concentrations of PA isomers can be found in soil around chemical factories (Naumov *et al.*, 1996). PA esters have been detected nearly in every environment, including air (Wensing *et al.*, 2005), soils, sediments, waters (Fatoki and Vernon, 1990) and landfill leachates (Schwarzbauer *et al.*, 2002; Zheng *et al.*, 2007). PAs are the hydrolysis products of phthalate esters and have also been identified as metabolic intermediates in bacterial degradation of polycyclic aromatic hydrocarbons such as phenanthrene (Kiyohara and Nagao, 1978), fluororene (Grifoll *et al.*, 1994) and fluoranthene (Sepic *et al.*, 1998). The metabolic derivatives of phthalates are potentially harmful to humans and wildlife due to their hepatotoxic, teratogenic and endocrine disrupting (carcinogenic) characteristics (Woodward, 1988; Matsumoto *et al.*, 2008), and are listed as priority industrial pollutants (Mayer *et al.*, 1972; Giam *et al.*, 1984). Hence, it is important to remove phthalates from the environment effectively and economically.

Besides slow chemical hydrolysis and photolysis of phthalate esters in the environment, microbial degradation of PAs and their corresponding esters by microorganisms is considered a principal route for removal of phthalates (Liang *et al.*, 2008). Many studies exist on aerobic degradation of phthalates (Chang and Zylstra, 1998; Wang *et al.*, 2003; Vamsee-Krishna *et al.*, 2006; Li and Gu, 2007) and their corresponding esters (Li *et al.*, 2005a,b; Li *et al.*, 2006; Wang and Gu, 2006a,b). The pathway of aerobic phthalate degradation has been well characterized in aerobic bacteria, for example, *Arthrobacter keyseri* 12B and *Burkholderia cepacia* DBO1, and involves either a 3,4-dihydroxyphthalate decarboxylase (Eaton and Ribbons, 1982) or a 4,5-dihydroxyphthalate decarboxylase for decarboxylation of hydroxylated *o*-phthalate to dihydroxybenzoate (Pujar and Ribbons, 1985; Chang and Zylstra, 1998). Since aerobic phthalate degradation uses oxygen as a cosubstrate in oxygenase reactions, anaerobic degradation has to proceed differently. Aftring *et al.*, showed that phthalate isomers can be degraded under anoxic conditions (Aftring *et al.*, 1981). Although less understood than aerobic phthalate degradation, anaerobic degradation of phthalate isomers and phthalate esters was reported for numerous pure and mixed cultures under nitrate- or sulfate-reducing or methanogenic conditions (Battersby and Wilson, 1989; Kleerebezem *et al.*, 1999; Qiu *et al.*, 2006; Cheung *et al.*, 2007; Liang *et al.*, 2008), but no decisive studies on the degradation pathways have been published so far.

In 1983, Taylor and Ribbons proposed a hypothetical pathway for anaerobic conversion of *o*-phthalate to benzoate, which involved two hypothetical enzymatic steps catalyzed by a reductase and a decarboxylase, that is, a reduction and subsequent oxidative decarboxylation (Taylor and Ribbons, 1983). However, no experimental evidence for this hypothetical pathway was provided. Nozawa and Maruyama (1988) proposed a pathway for anaerobic PA degradation by a nitrate-reducing *Pseudomonas* sp. that involves initial activation of *o*-phthalate with coenzyme A (CoA), followed by decarboxylation to benzoyl-CoA (Nozawa and Maruyama, 1988), however, no precise experimental evidence supported this assumption either. Nonetheless, the current view of anaerobic *o*-phthalate degradation involves benzoyl-CoA as a key intermediate, and its further degradation would proceed through the well-described anaerobic benzoyl-CoA degradation pathway (Schink *et al.*, 1992; Fuchs *et al.*, 1994; Harwood *et al.*, 1999). In the present work, we studied the anaerobic *o*-phthalate metabolism by the recent genome sequenced bacterium *Azoarcus* sp. strain PA01 (IMG genome ID: 2596583641, NCBI genome accession: PRJNA279928; Junghare *et al.*, 2015) that degrades *o*-phthalate with nitrate as an electron acceptor. We used a combined proteomic and genomic approach in order to

identify the catabolic genes and enzymes involved in the initial steps of *o*-phthalate degradation.

Results

Anaerobic growth with o-phthalate and physiological characteristics

To compare the growth and substrate utilization by *Azoarcus* sp. strain PA01 (KCTC 15483), cells were grown with 2 mM *o*-phthalate or benzoate as sole source of electrons, supplemented with nitrate (10–12 mM) as the final electron acceptor. Irrespective of the substrate, no pronounced difference in growth was observed. Cells showed doubling times of about 10 to 12 h with both substrates (although cells grew slightly faster with benzoate (Supporting Information Fig. S1). Nitrite accumulated as an intermediate (0.5–4 mM) in the early log phase. Cells consumed 1.7 mM of *o*-phthalate and reduced 7 mM of nitrate, whereas benzoate-grown cells consumed 1.8 mM of benzoate and 8.3 mM of nitrate. Thus, the experimentally observed stoichiometry of *o*-phthalate or benzoate oxidation to nitrate-reduction was approximately 1:4. The biomass produced with benzoate- or *o*-phthalate was nearly the same, that is, 34.5 and 36.5 g dry cell mass per mol, respectively. No intermediate organic degradation products were detected in the growth medium.

Differential proteome analysis of o-phthalate-grown cells versus benzoate-grown cells

The proteome of *o*-phthalate-grown cells was compared to that of benzoate-grown cells using two-dimensional polyacrylamide gel electrophoresis (2D-PAGE). Qualitative comparison of the resolved proteomes displayed significant differences in the protein profiles; approximately 15–20 protein spots were visible specifically in *o*-phthalate-grown cells (Fig. 1A). Of these, 12 protein spots were selected (labelled in Fig. 1A) and identified by mass spectrometry (MS). These protein spots were shown to represent eight different proteins (Table 1). On the other hand, in the proteome of benzoate-grown cells, nine protein spots were selected for MS identification (labelled in Fig. 1B), and the identified spots comprised seven different enzymes/proteins (Table 1). The protein spots SBA3, 4, 6 and 9 from the benzoate-grown cells were identified as proteins homologous to the enzymes of the anaerobic benzoyl-CoA degradation pathway of other denitrifying bacteria, for example *Thauera aromatica* (Breese *et al.*, 1998).

Finally, eight of the identified protein spots (SPA1, 2, 3, 7, 8, 9, 10 and 11) from *o*-phthalate-grown cells were shown to represent three different proteins and were suspected to be involved in the initial steps of anaerobic *o*-phthalate degradation (Fig. 1; Table 1) and were not

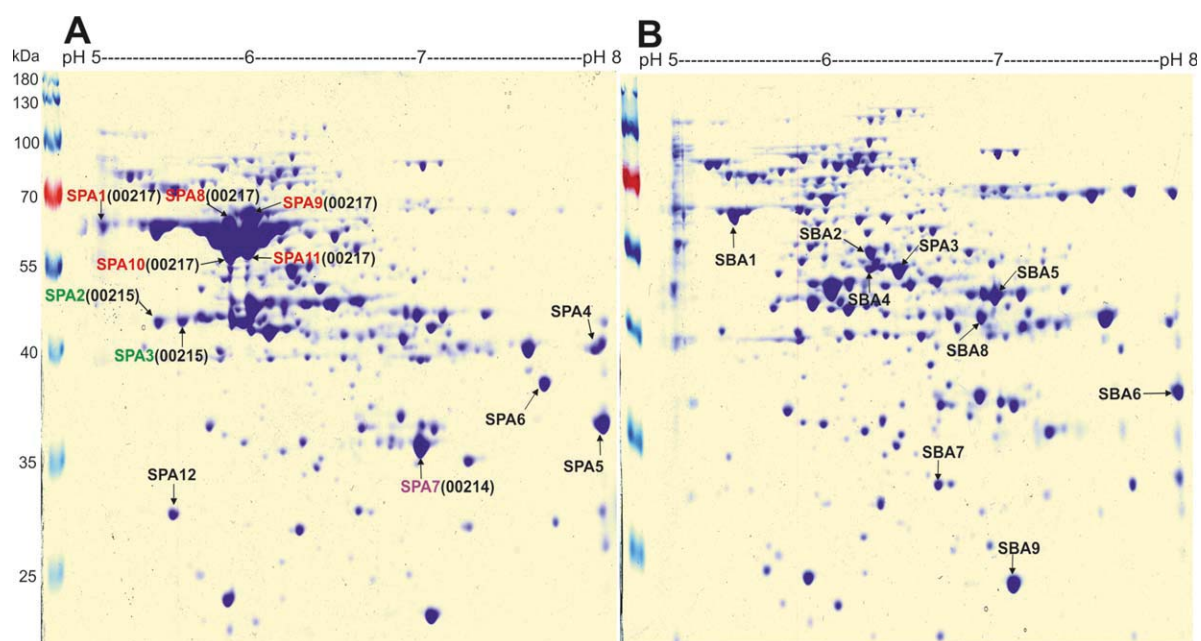


Fig. 1. Differential proteome analysis of cytosolic proteins of *Azoarcus* sp. strain PA01 grown with *o*-phthalate (A) or benzoate (B): Proteins resolved by two-dimensional differential gel electrophoresis (IEF-SDS PAGE). The induced protein spots are labelled SPA (soluble proteins from *o*-phthalic acid-grown cells) and SBA (soluble proteins from benzoic acid-grown cells) followed by the corresponding IMG locus tags (green, CoA transferase; red, decarboxylase). For IEF, a strip with a pH gradient from 5 to 8 was used and a prestained molecular protein marker was used for protein size comparison (kDa) on the gel.

observed in benzoate-grown cells. These proteins were identified as a TRAP (tripartite) transporter (SPA7), a benzylsuccinate CoA-transferase (SPA2-3) and a 3-polyprenyl-4-hydroxybenzoate decarboxylase (SPA1, 8-11) and suggested to be involved in *o*-phthalate metabolism. Furthermore, total proteome analysis of cell-free extracts of *o*-phthalate-grown cells (Fig. 2) and SDS-polyacrylamide gel electrophoresis of membrane proteins (Supporting Information Fig. S2) strongly supported and complemented the results of the differential 2D-gel protein profiling (Fig. 1; Table 1). The identified proteins constituted the most abundant cytosolic proteins (locus tag PA01_00214; PA01_00215; and PA01_00217) that were observed in the 2D-gel proteome of *o*-phthalate-grown cells (Fig. 1; Table 1).

The total proteome results allowed the identification of one extra protein with locus tag PA01_00216 that was identified as the BbsE subunit of benzylsuccinate CoA-transferase (30% sequence similarity) of *T. aromatica* and was similar to the previously identified protein spots SPA2-3 (Fig. 1; Table 1) with the locus tag PA01_00215 that was also identified as the BbsF subunit of benzylsuccinate CoA-transferase (30% sequence similarity). The total proteome analysis of benzoate-grown cells revealed the identification of proteins that were homologous to enzymes involved in the anaerobic benzoyl-CoA degradation pathway comprised of: benzoyl-CoA reductase (2-electron) β -

subunit (locus tag PA01_03236, SBA3-4), 6-ketocyclohex-1-ene-1-carbonyl-CoA hydrolase (locus tag PA01_03225, SBA6), benzoate-CoA ligase (locus tag PA01_03196), 6-hydroxycyclohex-1-ene-1-carbonyl-CoA dehydrogenase (locus tag PA01_03226) and 6-hydroxycyclohex-1-ene-1-carbonyl-CoA hydratase (locus tag PA01_03227). As expected, most of these proteins of the anaerobic benzoyl-CoA degradation pathway were also detected in the proteome of *o*-phthalate-grown cells (Figs 1 and 2; Table 1).

Characterization of the gene cluster involved in anaerobic o-phthalate degradation

Comparative proteome analysis of *o*-phthalate- versus benzoate-grown cells of *Azoarcus* sp. strain PA01 was performed using protein sequences obtained from the genome of the *Azoarcus* sp. strain PA01. The MS-identified proteins include the protein-coding genes with locus tags PA01_00214, PA01_00215, PA01_00216 and PA01_00217 that were only induced in *o*-phthalate-grown cells. To compare and to infer the possible catabolic functions of these genes, similarity searches were performed using the online search programs blastp (www.ncbi.nlm.nih.gov) or UniProtKB (<http://www.uniprot.org/blast/>). The protein blast searches of *o*-phthalate induced genes revealed the following similarities: the gene with the locus tag PA01_00214 was similar to TRAP transporters

Table 1. Identification of selected induced protein spots resolved by two-dimensional difference gel electrophoresis from cell-free extract of *Azoarcus* sp. strain PA01 cells grown with *o*-phthalate or benzoate.

Spot ID ^a	Gene loci ^b	IMG predicted function	Gene ID ^c	Score	Mass ^d	Sequence coverage % ^e
SPA1*	00217	4-hydroxy-3-polyprenylbenzoate decarboxylase (UbiD-decarboxylase)	<i>phtDa</i>	5060	59	69
SPA2*	00215	benzylsuccinate CoA-transferase (CoA-transferase family III)	<i>phtSa</i>	7613	44	74
SPA3*	00215	benzylsuccinate CoA-transferase (CoA-transferase family III)	<i>phtSa</i>	7745	44	66
SPA4	01506	ABC-type amide transporter substrate-binding protein (HAAT family)	<i>livK</i>	7513	44	55
SPA5	00796	TRAP-type mannitol/chloroaromatic compound transport system, periplasmic component	<i>ebA1033</i>	5866	39	79
SPA6*	03412	ABC-type Fe ³⁺ transport system substrate-binding protein	<i>ebA4918</i>	13 245	37	71
SPA7*	00214	TRAP-type transport system, periplasmic component (TAXI family)	<i>sO0456</i>	10 564	35	76
SPA8*	00217	4-hydroxy-3-polyprenylbenzoate decarboxylase (UbiD-decarboxylase)	<i>phtDa</i>	1102	59	69
SPA9*	00217	4-hydroxy-3-polyprenylbenzoate decarboxylase (UbiD-decarboxylase)	<i>phtDa</i>	1183	59	65
SPA10*	00217	4-hydroxy-3-polyprenylbenzoate decarboxylase (UbiD-decarboxylase)	<i>phtDa</i>	1257	59	63
SPA11*	00217	4-hydroxy-3-polyprenylbenzoate decarboxylase (UbiD-decarboxylase)	<i>phtDa</i>	1197	59	61
SPA12	03235	benzoyl-CoA reductase (2-electron) delta subunit	<i>oah</i>	11671	28	91
SBA1	00882	chaperonin GroEL (HSP60 family)	<i>groL1</i>	1308	58	62
SBA2	03692	isocitrate lyase	<i>aceA</i>	916	47	80
SBA3*	03236	benzoyl-CoA reductase (2-electron) beta subunit	<i>bcrB</i>	744	50	71
SBA4*	03236	benzoyl-CoA reductase (2-electron) beta subunit	<i>bcrB</i>	639	50	55
SBA5	02982	citrate synthase	<i>gltA</i>	968	48	79
SBA6*	03225	6-ketocyclohex-1-ene-1-carbonyl-CoA hydrolase	<i>oah</i>	388	42	57
SBA7	02989	malate dehydrogenase (NAD)	<i>mdh</i>	1092	35	71
SBA8*	03412	ABC-type iron(III) transport system substrate-binding protein	<i>ebA4918</i>	802	37	61
SBA9	00613	5-ketocyclohex-1-ene-1-carbonyl-CoA hydratase	<i>ebA722</i>	1016	30	76

a. SPA1-11, protein spots from *o*-phthalate-grown cells and SBA1-9 protein spots from benzoate-grown cells. **b.** IMG gene locus tag PA01_ from the genome of *Azoarcus* sp. strain PA01. **c.** gene name obtained from IMG annotation. **d.** peptide mass calculated by MS-MS identification. **e.** sequence coverage represents the extent of peptides obtained during MS-MS identification of respective protein and. *Indicates proteins that were also identified in the total proteome analyzed by Orbitrap LC-MS analysis. Protein spots that were exclusively induced with *o*-phthalate and likely to be involved in the initial anaerobic degradation of *o*-phthalate are highlighted in bold.

(substrate binding protein), and PA01_00215 and PA01_00216 were similar to CoA-transferases (family III) and PA01_00217 was similar to UbiD-like decarboxylases (UbiD family) respectively.

The genes induced exclusively in *o*-phthalate-grown cells were localized in a single 9.9 kb gene cluster in the genome of *Azoarcus* sp. strain PA01 (Fig. 3A). This indicates that the genes involved in anaerobic *o*-phthalate decarboxylation to benzoyl-CoA are clustered together, co-expressed and up-regulated in one open reading frame (ORF) during growth with *o*-phthalate. Based on the proteomic data (Figs 1 and 2; Table 1), the newly identified *o*-phthalate induced gene cluster is predicted to encode four candidate proteins (locus tags from PA01_00214 to PA01_00217) that were proposed to be involved and catalyze the initial steps of anaerobic *o*-phthalate degradation as shown in Fig. 3B. The abbreviation 'pht' referring to phthalate was introduced to the IMG (Integrated Microbial Genomes) locus tags from PA01_00215 to PA01_00218, for example, 00215_phtSa.

Phylogenetic analysis of the amino acid sequences of the putative CoA transferases of *Azoarcus* sp. PA01 revealed that the proteins PhtSa (PA01_00215) and PhtSb (PA01_00216) clustered together in one clade and belong to the CoA-transferase family III (Supporting Information

Fig. S3A). These two putative CoA-transferases of *Azoarcus* sp. strain PA01 designated as PhtSa and PhtSb showed 26% similarity with each other and shared only about 30% of sequence similarity with the two subunits (BbsEF, AAF89840 and AAF89841) of the previously characterized succinyl-CoA:(*R*)-benzylsuccinate CoA-transferase (family III) from *Thauera aromatica*. However, blastp search analysis revealed that these proteins (PhtSa and PhtSb) shared high (>80%) sequence similarity with uncharacterized CoA-transferases of '*A. aromaticum* EbN1' (Q5NWH8 and Q5NWH7) and *A. toluclasticus* (WP_0189914468 and WP_018991467), respectively (Supporting Information Fig. S3A).

Phylogenetic analysis of the remaining two genes coding for the putative decarboxylases (UbiD- and UbiX-like protein) namely, PhtDa (PA01_00217) and PhtDb (PA01_00218; the latter gene was not detected in the proteome analysis) revealed that they belong to two different clades with the protein sequences of the UbiD- and UbiX-like decarboxylases, respectively (Supporting Information Fig. S3B). The gene *phtDa_00217* (locus tag PA01_00217) is predicted to code for the putative *o*-phthalyl-CoA decarboxylase (PhtDa; see discussion) clustered with the protein sequences of the UbiD-like decarboxylases. On the other hand, the putative flavin-

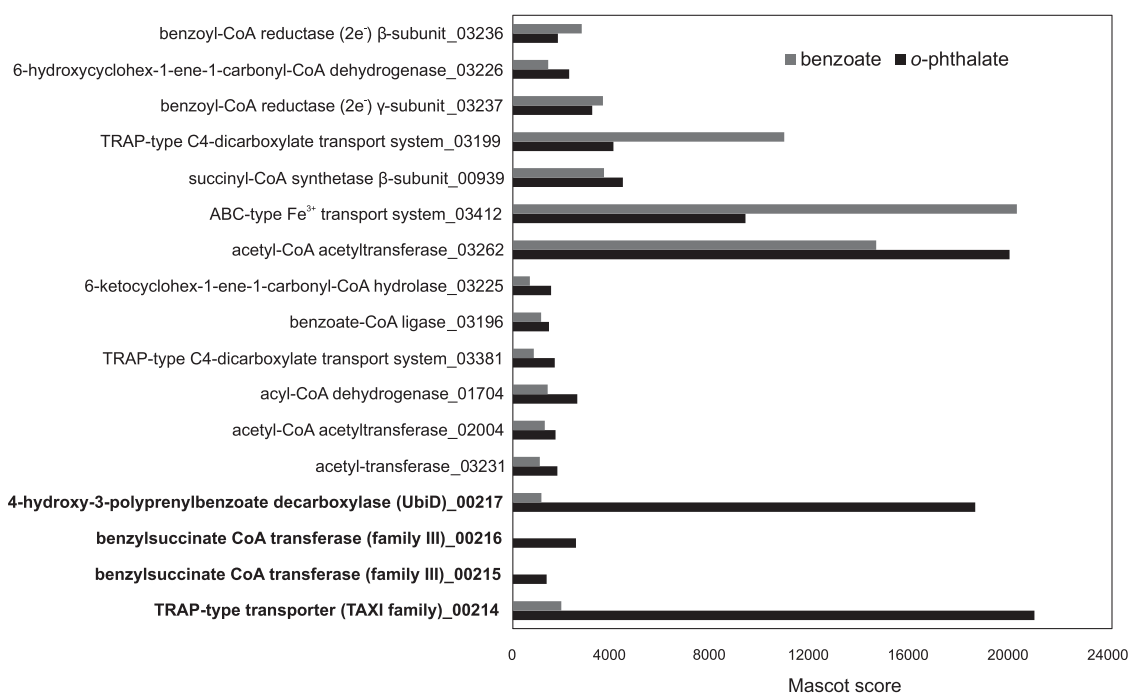


Fig. 2. Total proteome analysis of cell-free extracts from the cells of *Azoarcus* sp. strain PA01 grown with *o*-phthalate (black bars) and benzoate (grey bars). Proteins were identified by Orbitrap LC-MS analysis showing the *o*-phthalate specific induced genes with locus tags 00214, 00215, 00216 and 00217 (in bold) during growth on *o*-phthalate.

binding decarboxylase (UbiX-like protein) designated as PhtDb (locus tag PA01_00218) clustered with the protein sequences of the UbiX-like decarboxylases of other bacteria (Supporting Information Fig. S3B). Blastp searches showed that the amino acid sequence of PhtDa (PA01_00217) and PhtDb (PA01_00218) shared >95% sequence similarity with the uncharacterized UbiD/UbiX-like decarboxylase/carboxy-lyase-like proteins of '*A. aromaticum* EbN1' (Q5NWH6 and Q5NWX7) and *A. toluclasticus* (WP_018991466 and WP_040395783), respectively.

The gene with the locus tag PA01_00214 (So0456) in the identified *o*-phthalate gene cluster is predicted to encode a putative TRAP transporter (periplasmic binding protein) which is likely to be involved in *o*-phthalate transport (substrate transport). Finally, the identified *o*-phthalate degradation gene cluster comprised a gene with locus tag PA01_00219 (IcIR) coding for a protein that showed high similarity with the transcriptional regulator of the DNA-binding IcIR family protein that could hence act as a regulatory protein for expression/repression of these genes in the *o*-phthalate gene cluster (Fig. 3A). A similar distribution of genes as in the *o*-phthalate-induced gene cluster in *Azoarcus* sp. strain PA01 (Fig. 3A) was also found to be present in the genomes of other aromatic compound-degrading nitrate reducers such as '*A. aromaticum* EbN1' (Rabus *et al.*, 2005), *A. toluclasticus* and *Thauera*

chlorobenzoica (Liolios *et al.*, 2008). In all cases, the putative genes encoding CoA-transferases (00215_ *pht*Sa and 00216_ *pht*Sb) and decarboxylases (00217_ *pht*Da and 00218_ *pht*Da) were located adjacent to each other in a single gene cluster (Supporting Information Fig. S4).

The genes coding for the enzymes catalyzing the anaerobic degradation of benzoate/benzoyl-CoA to 3-hydroxy-pimeloyl-CoA were identified in the proteome of benzoate-grown cells as well as in the proteome of *o*-phthalate-grown cells of *Azoarcus* sp. strain PA01 (Figs. 1 and 2; Table 1). These genes coding for the enzymes of the anaerobic benzoate degradation were located in the single gene cluster (17.5 kb) in the genome of *Azoarcus* sp. strain PA01 (Fig. 4A). The enzymatic steps of anaerobic benzoate degradation by *Azoarcus* sp. strain PA01 were deduced based on the results of our proteome and genome data, and are similar to those of the known anaerobic benzoate degradation pathway (Breese *et al.*, 1998; Carmona *et al.*, 2009) (Fig. 4B).

Enzyme activity measurements with cell-free extracts of Azoarcus sp. strain PA01

Interpretation of our proteomics data suggested an activation of *o*-phthalate to *o*-phthalyl-CoA by a putative succinyl-CoA-dependent CoA-transferase followed by its decarboxylation to benzoyl-CoA by a putative *o*-phthalyl-CoA

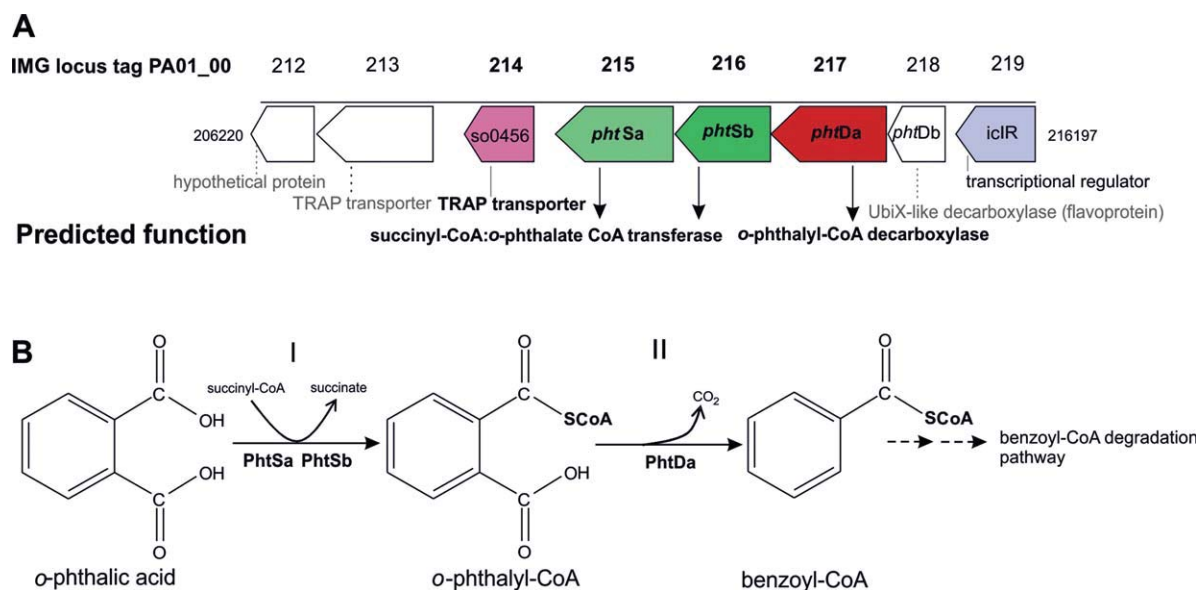


Fig. 3. Organization of the genes in the *o*-phthalate degradation gene cluster and the proposed pathway of anaerobic *o*-phthalate degradation by *Azoarcus* sp. strain PA01. **A.** The gene organization and putative function of individual genes induced with *o*-phthalate-grown cells are highlighted in bold and different colour. **B.** Proposed pathway based on proteomics and *in vitro* enzyme assay results showing reaction steps in the initial degradation of *o*-phthalate: (1) activation of *o*-phthalate to *o*-phthalyl-CoA by the putative succinyl-CoA: *o*-phthalate CoA-transferase (green, *PhtSa*; light green, *PhtSb*); and (2) subsequent decarboxylation of *o*-phthalyl-CoA to benzoyl-CoA by the putative *o*-phthalyl-CoA decarboxylase (red, *PhtDa*). The dotted arrows indicate further degradation of benzoyl-CoA via the anaerobic benzoyl-CoA degradation pathway.

decarboxylase. To establish the assumed pathway, *in vitro* enzyme activity assays were performed with cell-free extracts of *o*-phthalate or benzoate-grown cells (the latter as a control) using *o*-phthalate, succinyl-coenzyme A (Succinyl-CoA, Supporting Information Fig. S5; SigmaAldrich), synthesized *o*-phthalyl-CoA (rt: 4.9 min, Supporting Information Fig. S6) or fluoro-*o*-phthalyl-CoA (Supporting Information Fig. S7) as the substrates.

Succinyl-CoA dependent formation of *o*-phthalyl-CoA from *o*-phthalate was observed in cell-free extracts of *o*-phthalate-grown cells and *o*-phthalate was converted to *o*-phthalyl-CoA (Fig. 5A and B). No activity was observed when succinyl-CoA was replaced by free CoA (sodium salt of CoA) or acetyl-CoA as possible CoA donor. Furthermore, no activity was detected with either isophthalate and terephthalate or with 3-fluoro-*o*-phthalate. Cell-free extracts of benzoate-grown cells of *Azoarcus* sp. strain PA01 did not exhibit any activity with all three isomers of phthalate or 3-fluoro-*o*-phthalate. In the coupled enzyme assay performed with the addition of *o*-phthalate and succinyl-CoA in cell-free extract of *o*-phthalate-grown cells, formation of benzoyl-CoA was also observed, that is, enzymatically formed *o*-phthalyl-CoA was subsequently decarboxylated to benzoyl-CoA (Fig. 5C and D). Further, the decarboxylase activity was tested individually in cell-free extracts by the addition of synthesized *o*-phthalyl-CoA (Supporting Information Fig. S6), isophthalyl-CoA (Supporting Information Fig. S8), terephthalyl-CoA (Supporting Information Fig. S9)

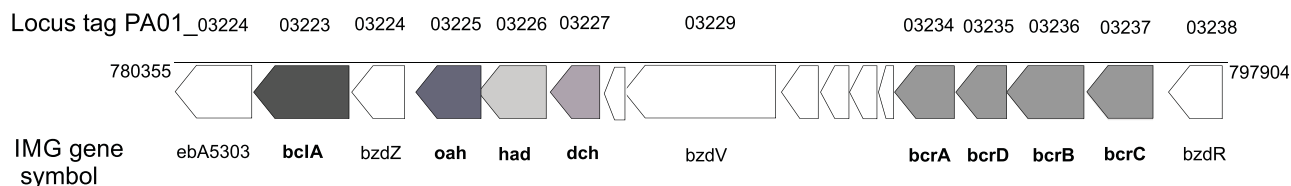
and fluoro-*o*-phthalyl-CoA (Supporting Information Fig. S7). Cell-free extracts of *o*-phthalate-grown cells converted synthesized *o*-phthalyl-CoA to benzoyl-CoA (rt: 11.4 min, Supporting Information Fig. S10) and similarly fluoro-*o*-phthalyl-CoA was also efficiently decarboxylated to fluoro-benzoyl-CoA (rt: 11.5 min, Supporting Information Fig. S11). No decarboxylase activity was observed with either isophthalyl-CoA or terephthalyl-CoA.

Benzoate-grown cell-free extract or heat-inactivated cell-free extract of *o*-phthalate-grown cells did not form *o*-phthalyl-CoA or benzoyl-CoA. Exposure of cell-free extract to air did not affect the activities of *o*-phthalyl-CoA formation or decarboxylation of the *o*-phthalyl-CoA to benzoyl-CoA (Supporting Information Fig. S12). Storage of cell-free extract for 1–2 day at 4°C did not result in a loss of enzyme activity. Synthesized *o*-phthalyl-CoA standing overnight at room temperature was not decarboxylated and was found to be stable for at least 1–2 days.

Discussion

In the present study, we elucidated the pathway of anaerobic *o*-phthalate degradation in *Azoarcus* sp. strain PA01 using differential proteomics and *in vitro* enzyme assays. Based on the draft genome sequence of *Azoarcus* sp. strain PA01 (IMG genome ID: 2596583641, NCBI genome accession: PRJNA279928), we identified the genes coding for the enzymes involved in the anaerobic conversion of *o*-phthalate to benzoyl-CoA. The combined proteome and

A



B

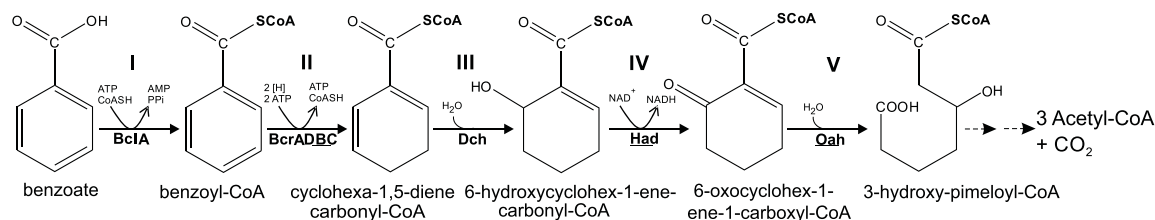


Fig. 4. Scheme of the proposed anaerobic benzoate degradation gene cluster (A), and scheme of the proposed pathway of anaerobic benzoate/benzoyl-CoA degradation (B) by *Azoarcus* sp. strain PA01: I) ATP-dependent benzoate-CoA ligase (**bclA**); II) ATP-dependent benzoyl-CoA reductase-class I (**BcrADBC**); III) cyclohexa-1,5-diene carbonyl-CoA hydratase (**Dch**); IV) 6-hydroxycyclohex-1-ene-1-carbonyl-CoA dehydrogenase (**Had**); V) 6-hydroxycyclohex-1-ene-1-carbonyl-CoA dehydrogenase (**Oah**) and further degradation (dotted arrows) furnishing three molecules of acetyl-CoA and carbon dioxide. The enzymes which were detected in the proteome analysis are in bold.

genome analysis of *Azoarcus* sp. strain PA01 identified an *o*-phthalate-induced gene cluster coding for the enzymes likely involved in the initial steps of anaerobic degradation of *o*-phthalate to benzoyl-CoA (Fig. 3; Table 1). They comprised enzymes homologous to a dicarboxylic acid transporter (TRAP transporter, PA01_00214), the CoA-transferases (putative succinyl-CoA:*o*-phthalate CoA-transferase) encoded by two adjacent genes *phtSa* and *phtSb* (locus tag PA01_00215 and PA01_00216) and a putative *o*-phthalyl-CoA decarboxylase (UbiD-like decarboxylase) encoded by *phtDa* (locus tag PA01_00217), respectively.

Although the putative succinyl-CoA:*o*-phthalate CoA-transferase of *Azoarcus* sp. strain PA01 was only distantly related (30% similarity) to the succinyl-CoA:(*R*)-benzylsuccinate CoA-transferase of *T. aromatica* which belongs to the CoA-transferase family III (Leutwein and Heider, 2001; Leutwein and Heider, 1999), both enzyme showed similar function, that is, transfer of CoA to a free acids. The succinyl-CoA:(*R*)-benzylsuccinate CoA-transferase of *T. aromatica* consists of two subunits BbsEF (28% identical) of similar sizes (44 and 45 kDa) constituting an $\alpha_2\beta_2$ tetramer (Heider, 2001; Leutwein and Heider, 2001). Similarly, *Azoarcus* sp. strain PA01 grown with *o*-phthalate induced the expression of the putative CoA-transferases (*PhtSa*, PA01_00215 and *PhtSb*, PA01_00216) of similar sizes (44 kDa and 41 kDa). Thus, the two *o*-phthalate-induced CoA-transferases, *PhtSa* and *PhtSb* (26% similarity) might work together as a single enzyme entity, similar to the BbsEF subunits of the succinyl-CoA:(*R*)-benzylsuccinate CoA-

transferase of *T. aromatica* (Heider, 2001; Leutwein and Heider, 2001).

Phylogenetic analysis supported our view that the putative succinyl-CoA:*o*-phthalate CoA-transferase of *Azoarcus* sp. strain PA01 belongs to the enzymes of CoA-transferase family III. No significant sequence similarity was observed with the proteins of CoA-transferase families I and II. The currently characterized CoA-transferases of family III are known to transfer CoA to free acids in anaerobic degradation pathways and activate organic acids for subsequent metabolization (Heider, 2001). Therefore, we suspected that the putative succinyl-CoA:*o*-phthalate CoA-transferase of *Azoarcus* sp. strain PA01 activates *o*-phthalate to *o*-phthalyl-CoA with succinyl-CoA as a CoA donor, followed by decarboxylation of *o*-phthalyl-CoA to benzoyl-CoA (Fig. 3B).

Our conclusion was experimentally proven by observing the conversion of *o*-phthalate to *o*-phthalyl-CoA and its subsequent decarboxylation to benzoyl-CoA in cell-free extracts of *o*-phthalate-grown cells in the presence of succinyl-CoA (Fig. 5). This is in contrast to an earlier report on the anaerobic phthalate degradation by *Pseudomonas* sp. strain P136, in which formation of phthalyl-CoA and its subsequent decarboxylation to benzoyl-CoA was reported in the presence of free CoA plus ATP (adenine triphosphate) by an acyl-CoA synthetase (Nozawa and Maruyama, 1988). However, our enzyme assay results did not show *o*-phthalyl-CoA formation when succinyl-CoA was replaced with free CoA or acetyl-CoA as a possible CoA donor. Moreover, earlier enzyme assays with ATP and

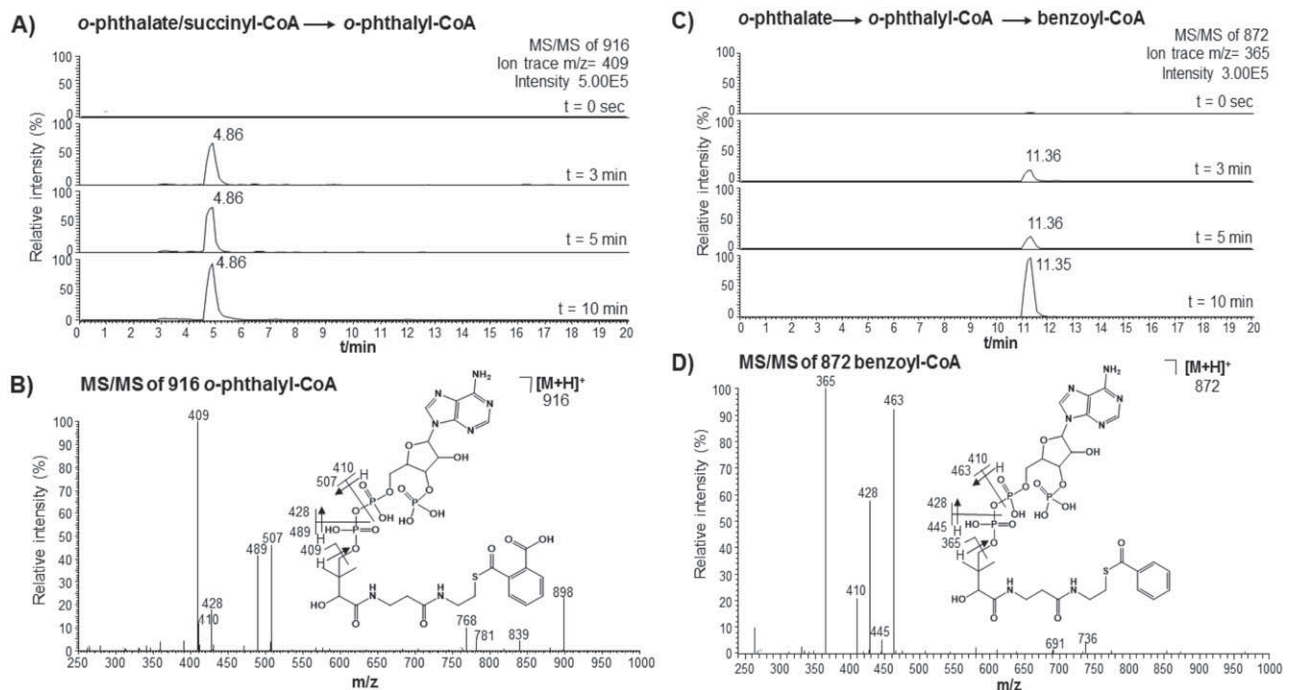


Fig. 5. LC-MS/MS ion traces following the product formation in a coupled enzyme assay performed with cell-free extracts of *o*-phthalate-grown cells of *Azoarcus* sp. strain PA01. A. Time course of the formation of *o*-phthalyl-CoA from *o*-phthalate in the presence of succinyl-CoA (succinyl-CoA:*o*-phthalate CoA-transferase) monitoring the specific ion traces m/z 409 of the MS/MS fragmentation of *o*-phthalyl-CoA (quasimolecular ion m/z 916). B. ESI-MS/MS of the quasimolecular ion m/z 916 of *o*-phthalyl-CoA. C. Time course of enzymatic benzoyl-CoA formation due to subsequent decarboxylation of formed *o*-phthalyl-CoA by the putative *o*-phthalyl-CoA decarboxylase monitoring the specific ion trace m/z 365 of the MS/MS of benzoyl-CoA (quasimolecular ion m/z 872). D. ESI-MS/MS of the quasimolecular ion m/z 916 of benzoyl-CoA (Park *et al.*, 2007).

o-phthalate supplied with free CoA did not show the formation of *o*-phthalyl-CoA (Junghare and Schink, 2014; unpublished results). Also in the proteome analysis of *o*-phthalate-grown cells of *Azoarcus* sp. strain PA01, no induced acyl-CoA synthetase was found (Figs 1 and 2; Table 1). Thus, we conclude that in *Azoarcus* sp. strain PA01 *o*-phthalate is activated by a CoA-transferase rather than an ATP-dependent CoA-ligase or synthetase.

Isophthalate or terephthalate were not converted to the corresponding phthalyl-CoAs by the putative succinyl-CoA:*o*-phthalate CoA-transferase. Moreover, *Azoarcus* sp. strain PA01 was unable to grow with isophthalate or terephthalate (Junghare *et al.*, 2015). The observed high substrate specificity of the putative CoA-transferase is in line with the observation that the enzymes of CoA-transferase family III catalyse the transfer of the CoA moiety in a highly substrate- and stereospecific manner (Heider, 2001). The activity of the CoA-transferase was insensitive to air exposure, similar to the previously studied CoA-transferase BbsEF (Leutwein and Heider, 2001). However, *Azoarcus* sp. strain PA01 was unable to grow aerobically with *o*-phthalate or benzoate.

The *o*-phthalyl-CoA formed in the first step of *o*-phthalate degradation is subsequently decarboxylated

to benzoyl-CoA by a putative *o*-phthalyl-CoA decarboxylase (Fig. 5C and D). In the *o*-phthalate-induced gene cluster (Fig. 3A), there are two alternative genes predicted to code for putative decarboxylases (*phfDa*, PA01_00217 and *phfDb*, PA01_00218). Of these, only the *PhfDa* was found to be highly induced exclusively in *o*-phthalate-grown cells of *Azoarcus* sp. strain PA01 and thus was suspected to catalyze the decarboxylation of *o*-phthalyl-CoA to benzoyl-CoA as shown in Fig. 3B. In contrast, the gene with locus tag PA01_00218 (*phfDb*) is predicted to encode an UbiX-like decarboxylase (flavo-protein) of about 22 kDa size that was never detected in our proteome analyses (Figs 1 and 2 and Supporting Information Fig. S2). Therefore, the role of gene *phfDb* (PA01_00218; UbiX-like protein) in *o*-phthalate metabolism is still unclear. However, recently White *et al.* (2015) reported that the UbiX-like protein in bacterial ubiquinone biosynthesis acts as a flavin mononucleotide (FMN)-binding protein with no decarboxylase activity. The FMN-binding protein (UbiX-like protein) is a metal-independent flavin prenyltransferase involved in the formation of a novel flavin-derived cofactor that is required for the decarboxylase activity of the UbiD-like protein (Lin *et al.*, 2015; White *et al.*, 2015).

Phylogenetic analysis of the putative *o*-phthalyl-CoA decarboxylase revealed that PhtDa (PA01_00217) was affiliated with UbiD-like decarboxylases (Bhuiya *et al.*, 2015) that are known to decarboxylate, for example 3-polyprenyl-4-hydroxybenzoate in *Escherichia coli* (Baba *et al.*, 2006). UbiD-like decarboxylases are commonly found in bacteria (Jacewicz *et al.*, 2013) and are involved in ubiquinone biosynthesis (Zhang and Javor, 2000). The protein designated as PhtDb (00218) is affiliated to flavin-prenyltransferases of the UbiX-like protein family (Supporting Information Fig. S3B). It is possible that PhtDb might play a similar role in the decarboxylation of *o*-phthalyl-CoA.

Enzyme assays with synthesized *o*-phthalyl-CoA resulted in the formation of benzoyl-CoA (Supporting Information Fig. S10) or conversion of the synthesized fluorinated *o*-phthalyl-CoA analogue fluoro-*o*-phthalyl-CoA to fluoro-benzoyl-CoA (Supporting Information Fig. S11). No decarboxylase activity was observed with isophthalyl-CoA and terephthalyl-CoA or with extracts of benzoate-grown cells, indicating that the putative *o*-phthalyl-CoA decarboxylase (00217_PhtDa) is rather selective for its substrate.

The newly identified *o*-phthalate-induced gene cluster comprised also a gene with the locus tag PA01_00214 (So0458) encoding a protein homologous to a subunit of TRAP transporters (TRAP: transporter-tripartite ATP-independent periplasmic) that are specialised in the transport of dicarboxylic acids, for example, malate or succinate (Forward *et al.*, 1997). Thus, the TRAP transporter subunit that was highly induced in *o*-phthalate-grown cells is most likely involved in the transport of *o*-phthalate. This conclusion is further backed by the observation that this protein was not induced in benzoate-grown cells (Figs 1 and 2; Table 1). Finally, the *o*-phthalate gene cluster contains a gene with the locus tag 00214_IcIR that is highly similar to the proteins of the IcIR family. Members of this family include transcriptional regulators, which act as both activators and repressors (Molina-Henares *et al.*, 2006). Therefore, this protein might be involved in the regulation of genes of the *o*-phthalate gene cluster. Future experiments are needed to address mechanistic details of the enzymes involved in *o*-phthalate degradation by *Azoarcus* sp. strain PA01.

Experimental procedures

Bacterial strain and growth conditions

Azoarcus sp. strain PA01 (KCTC 15483) was isolated from the wastewater treatment plant in Constance, Germany. It is a mesophilic, nonmotile, Gram-negative, short rod-shaped bacterium that grows optimally at 30°C and pH 7.3 ± 0.2 (Junghare *et al.*, 2015). Cells were cultured anaerobically without shaking in 50 or 100 ml infusion bottles in nonreduced, bicarbonate-buffered mineral medium under a N₂/CO₂ (80:20) atmosphere. Benzoate or *o*-phthalate was added anoxically from sterile anoxic stock solutions to final concentration of 2 mM as growth substrate plus nitrate (10–12 mM) as terminal

electron acceptor. Cultures were inoculated from pre-cultures adapted over >20 generations on the respective substrates (*o*-phthalate or benzoate) in 100 ml serum bottles containing 75 ml of culture medium.

Bacterial growth analysis

Cell growth was monitored by measuring the optical density at 600 nm wavelength (OD₆₀₀) in a spectrophotometer (Uvikon 860, Zürich, Switzerland). At different time intervals, samples were removed for growth measurement and substrate utilization analysis. Samples were centrifuged (8000 × *g* for 10 min) and the supernatant was applied to HPLC analysis for quantification of benzoate, *o*-phthalate, nitrate and nitrite (data not shown). Fifty microliter of sample supernatant was injected into an HPLC (LC prominence Shimadzu, Japan) equipped with a Grom™ Sii 120 ODS-5 ST, column (4 mm × 150 mm, 5 μm) using an UV detector (SPD-M20A prominence) at 230 nm for signal detection. Eluent A (acetonitrile) and eluent B (20 mM H₃PO₄) were used at a flow rate of 0.8 ml/min at 30°C. A gradient of eluent B was applied for 30 min: 5 min isocratic of 92%; 3 min gradient to 89%; 10 min gradient to 80%; 5 min isocratic of 80%; 2 min gradient to 95%; 3 min gradient to 92%; 2 min isocratic 92%.

Preparation of cell-free extracts

Cultivation of bacterial cell mass for proteomics or enzyme activity measurements was performed in 1 l infusion bottles inoculated with adapted precultures grown with benzoate or *o*-phthalate. Parallel cultures were grown on 2 mM of benzoate or *o*-phthalate and harvested in identical physiological states. All steps for preparation of cell-free extracts were performed under anoxic conditions. Cells of *Azoarcus* sp. strain PA01 were harvested in the late exponential growth phase (OD₆₀₀ = 0.2–0.3) by centrifugation (7000 × *g* for 20 min at 4°C, Dupont Sorvall). Cell pellets from 1 l growth medium were washed with about 300 ml of anoxic Tris-HCl buffer (0.1 M pH 7.6) and re-suspended in 4–5 ml of the same buffer by adding 0.5 mg of DNase and RNase (Sigma-Aldrich). Prior to cell disruption, approximately 2 mg of protease inhibitor (Complete Mini tablets, Roche Diagnostics, Germany) was added. Cells were broken anoxically by two to three passages through a cooled MiniCell French pressure cell (SLM Aminco, Cat. No. FA003) operated at 137 MPa. Cell debris was removed by centrifugation at 27 000 × *g* for 30 min at 4°C to obtain the crude extract. The soluble protein fraction was obtained by high speed centrifugation (60 000 × *g* for 60 min) of the crude extract and insoluble membrane proteins were pelleted. The supernatant containing the soluble cytosolic proteins was filtered through a Sephadex NAP-25 column (GE Healthcare, Germany) for proteomic analysis. For enzyme assays, 3–4 ml of cell-free extract was processed further using vivaspin centrifugal concentrators (GE Healthcare Life Sciences) with a 10 000 molecular weight cut-off (MWCO) filter to remove smaller molecules. The cytosolic soluble protein fraction was washed at least 3 times with 3–4 ml of Tris-HCl buffer (0.1 M, pH 7.6). The protein concentration was determined with the Bradford assay using bovine serum albumin as standard (Bradford, 1976).

Differential proteome analysis

Two-dimensional (2D) gel electrophoresis (isoelectric focusing (IEF) and SDS-PAGE) of cytosolic soluble proteins was carried out with cells grown with *o*-phthalate or benzoate. IEF was performed using 17 cm long immobilized pH gradient (IPG) strips (BioRad ReadyStrip™) of pH gradient range 5–8 (results of pH range of 3–10 and 5.7–6.7 strips are not shown). Approximately 1 mg of total protein from the soluble fraction was precipitated by the addition of at least 5 volumes of ice-cold acetone and incubated overnight at –20°C. The protein precipitate was collected by centrifugation (13 000 *g* × 10 min, 4°C), the protein pellet was air-dried at room temperature and solubilized in 600 µl of rehydration buffer (8 M urea, 2 M thiourea and 0.2% CHAPS (3-[(3-cholamidopropyl)dime-thylammonio]-1-propanesulfonate). Prior to use, 60 mM of dithiothreitol (DTT) and 0.2% ampholyte (Sigma-Aldrich) were added to rehydration buffer. The IPG strips (pH 5–8) were rehydrated using 300 µl of rehydration buffer (protein load 500 µg) overnight in a rehydration tray covered with mineral oil. IEF was performed using a maximal current of 50 mA per strip at 20°C. It was started for 1 h at a maximal voltage of 500 V (desalting), followed by a voltage ramp (rapid) to a maximal voltage of 10 000 V within 3 h and additional focusing at 10 000 V until a total of 40 000 Volt-hours (Vh) were reached (Schmidt *et al.*, 2013). The second dimension, that is, SDS-PAGE was performed using the BioRad Protean II system (17 × 20 cm). After focusing, the strips were equilibrated in SDS-equilibration buffers I (6 M urea; 0.375 M Tris-HCl, pH 8.8; SDS 2%; glycerol 20%, and DTT 2% w/v) and II (6 M urea, 0.375 M Tris-HCl pH 8.8, SDS 2% glycerol 20%, and iodoacetamide 2.5% w/v) for 10 min, respectively. Strips were rinsed with 1 × Tris-glycine-SDS buffer (0.025 M Tris-HCl, pH 8.6; 0.192 M glycine and 0.1% SDS). IPG Strips were mounted onto 12% acrylamide gel and kept firm using an overlay of agarose (0.5% w/v) solution. Electrophoresis was performed at 40 mA for 15–16 h under cooling conditions (8–10°C). One-dimensional (1D) SDS-PAGE was performed for analysis of membrane proteins (see above). The membranes were washed with Tris-HCl (50 mM pH 7.6) and solubilised in 0.5 ml of Tris-HCl (20 mM pH 8.0) containing 0.5% dodecyl β-D-mal-toside, and incubated on ice for 2 h. The suspension was centrifuged at 60 000 × *g* for 30 min to remove remaining insoluble debris. The supernatant was mixed with 2 volumes of SDS-gel loading buffer containing 5% β-mercaptoethanol and incubated at 100°C for 5 min. Approximately 50 µg of protein (20 µl) per sample was loaded onto the SDS-gel (8 × 6 cm × 1.0 mm BioRad Protean Mini cell, 12% resolving and 4% stacking gel). Electrophoresis was performed for about 2 h at 120 V. 1D and 2D gels were stained with a colloidal solution of Coomassie Brilliant Blue R-250 (Neuhoff *et al.*, 1988) and analyzed for specific protein spots. All protein gel electrophoresis experiments were repeated at least three times.

Protein identification by MS

Coomassie-stained gels were scanned (HP Scanjet G4050) and the gel images were printed on transparency film (InkJet). Specifically induced protein spots of *o*-phthalate-grown and benzoate-grown cells were identified by manual overlapping of the printed gel images. Protein spots of interest were excised

and submitted for MS analysis to the Proteomics Center of the University of Konstanz. Protein spots were destained, reduced with DTT, alkylated with iodoacetamide and digested with trypsin. Trypsinized peptides were analyzed by liquid chromatography–tandem mass spectrometry (LC–MS/MS) using a Bruker Esquire 3000+ with an Agilent 1100 HPLC or a Bruker amaZon Speed ETD with a Bruker Advance nano-HPLC. Total proteome analysis of the cells grown with *o*-phthalate or benzoate was performed using a LTQ Orbitrap Discovery with an Eksigent 2D-nano HPLC (Thermo Fisher Scientific). Short peptide sequences obtained by MS (peptide spectral data) were matched using the Mascot search engine [v2.2.2 from Matrix Science] (Perkins *et al.*, 1999) with the locally established protein database of the IMG annotated genome of *Azoarcus* sp. strain PA01 (Junghare *et al.*, 2015) for the identification of protein spots. Search results were validated on the basis of top hits and scores obtained in the Mascot search engine. MS identification of protein spots and Mascot search were performed at least twice from independent protein spots analysis.

Identification of gene clusters and phylogenetic analysis

To identify the *o*-phthalate gene cluster, we analyzed the genome of *Azoarcus* sp. strain PA01 and selected protein sequences were BLASTp-searched using the NCBI (<https://www.ncbi.nlm.nih.gov/>) or UniProt (<http://www.uniprot.org/blast/>) online search tool. The relative positions of the genes within the genome and putative transcription direction of the genes were determined using online tools provided by the IMG, JGI (<http://genome.jgi.doe.gov/>, Markowitz *et al.*, 2009). Phylogenetic analysis of predicted amino acid sequences of the *o*-phthalate induced genes with the IMG gene loci PA01_00215 to PA01_00218 were obtained from the *Azoarcus* sp. strain PA01 genome were aligned using ClustalW from the MEGA 7 software (Kumar *et al.*, 2016) with closely related and characterized CoA-transferases or decarboxylases from other bacteria, respectively. Protein sequences for the alignments were obtained from GenBank and UniProtKB/Swiss-Prot or from the IMG database. Phylogenetic analysis was performed using the neighbor-joining algorithm (Saitou and Nei, 1987), and Poisson correction method (Zuckermandl and Pauling, 1965). The phylogenetic tree was constructed using MEGA 7 (Kumar *et al.*, 2016). Bootstrap values were calculated as a percentage of 1000 replicates (Felsenstein, 1985).

Determination of enzyme activities in cell-free extracts

Cell-free extracts (~2–3 g wet weight grown either with *o*-phthalate or benzoate) were prepared anoxically as described. Enzyme activities were measured anoxically (unless mentioned otherwise) in 5 ml serum vials closed with butyl rubber septa. Vials were flushed with nitrogen and all additions and samples were taken with gas-tight Unimatrix microliter syringes (Macherey-Nagel, Düren, Germany). All enzyme assays were run at least in triplicates at 28–30°C.

- (i) Succinyl-CoA:*o*-phthalate CoA-transferase assay: CoA-transferase activity catalyzing the conversion of *o*-phthalate to *o*-phthalyl-CoA with succinyl-CoA as CoA donor

was measured discontinuously by LC-MS/MS screening for the formation of *o*-phthalyl-CoA and benzoyl-CoA. The standard assay mixture (500 μ l) contained: triethanolamine buffer (0.1 M pH 7.6), 0.2–0.3 mg protein, succinyl-CoA (2 mM, Sigma-Aldrich) and *o*-phthalate (6 mM, Fluka Chemie). After initial incubation for about 5 min, the enzyme reactions were started by the addition of the CoA acceptor (*o*-phthalate) or CoA donor (succinyl-CoA, free CoA or acetyl-CoA). Occasionally 0.1–0.2 mM of *N, N*-dicyclohexylcarbodiimide was added to inhibit ATPase activity to minimize succinyl-CoA hydrolysis. For determination of the substrate specificity, free acids; such as isophthalate, terephthalate (Fluka Chemie) or 3-fluoro-*o*-phthalate (Sigma) were used, or succinyl-CoA was replaced by free CoA or acetyl-CoA (Sigma-Aldrich) as a possible alternative CoA donor.

(ii) *o*-Phthalyl-CoA decarboxylase assay: The standard enzyme assay mixture (500 μ l) contained; triethanolamine buffer (0.1 M, pH 7.6) and 0.2–0.3 mg protein extract. After initial incubation of cell-free extract for about 5 min in buffer, the enzyme reaction was started by the addition of 50 μ l synthesized *o*-phthalyl-CoA (approximately 20–30 mM). Alternatively, isophthalyl-CoA, terephthalyl-CoA or fluoro-*o*-phthalyl-CoA were used in enzyme assays in order to investigate the substrate specificity of the putative decarboxylase. Formation of benzoyl-CoA/fluoro-benzoyl-CoA was measured by LC-MS/MS analysis. Control assays were performed either after heat inactivation of the cell-free extract (90°C, 10 min) or by excluding one key substrate in the enzyme assay, for example, succinyl-CoA or *o*-phthalate or *o*-phthalyl-CoA. The effect of oxygen exposure on the enzyme activities was studied by incubating the enzyme assays exposed to air. For analysis of the enzyme products, 70 μ l of the samples was withdrawn at different time points 0 min (before the start of the reaction), 3, 5, 10 or 20 min and the reaction was stopped by addition of an equal volume of methanol and centrifuged (13 000 \times *g* for 5 min). The supernatant was transferred into 200 μ l glass vial inserts and analyzed for the respective enzyme product formation by LC-MS/MS.

Synthesis of *o*-phthalyl-CoAs

Phthalic anhydride (3 mg, 20 μ mol; Sigma-Aldrich) was dissolved in 100 μ l of acetone and mixed with 200 μ l of NaOH (0.3 N) containing coenzyme A trilithium salt (10 mg, 13 μ mol) for 3 min. Acetone was removed by a gentle stream of nitrogen and the formation of *o*-phthalyl-CoA was analyzed by LC-MS/MS (Supporting Information Fig. S4). Isophthalyl-CoA and fluoro-*o*-phthalyl-CoA were generated using 16 μ mol of each phthalate dissolved in 100 μ l of dry tetrahydrofuran in a dried 4 ml glass vial fitted with a stirring bar under nitrogen. To this solution, ethyl chloroformate (2 μ l, 21 μ mol) and trimethylamine (2 μ l, 15 μ mol) were added. The reaction mixture was stirred for 1 min and coenzyme A trilithium salt (10 mg, 13 μ mol) dissolved in 200 μ l of NaOH (0.3N) was added and mixed for 3 min. Tetrahydrofuran was removed by blowing it off in a gentle nitrogen stream. Terephthalyl-CoA was synthesized analogously, but terephthalate (16 μ mol) was dissolved

in dimethylformate (DMF, 500 μ l) and activated with ethyl chloroformate (2 μ l, 21 μ mol) and trimethylamine (2 μ l, 15 μ mol) to the anhydride that was reacted with free CoA in 500 μ l of NaOH (0.3N). DMF was removed by freeze drying the sample. The formation of phthalyl-CoAs was checked by LC-MS/MS (Supporting Information Fig. S5). Phthalyl-CoAs were obtained in about 70–90% yield as determined by the peak area ratio of free-CoA/CoA-ester, and were used directly for the enzyme assays.

LC-MS/MS analysis of coenzyme A esters

LC-MS/MS analysis of coenzyme A esters (CoAs) was performed with an Agilent 1100 HPLC system fitted with a Phenomenex Synergi polar-RP (250 \times 2 mm, 4 μ m) column. The mobile phase consisted of solvent A, 30 mM ammonium acetate buffer (pH 7.4), and solvent B, acetonitrile with 0.1% acetate. The CoAs were separated by programmed elution: 2 min isocratic 2% B, in 20 min gradient elution to 100% B at a flow rate of 0.25 ml/min. The assay samples of 30–90 μ l was injected. For detection of the respective CoAs, the HPLC was connected to a Finnigan LCQ ion trap mass spectrometer fitted with an electrospray ion (ESI) source and operated in the MS/MS mode to specifically detect CoA esters (Park *et al.*, 2007). In addition, reference compounds were measured for all CoA esters studied except for fluoro-benzoyl-CoA.

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References

- Aftring, R.P., Chalker, B.E., and Taylor, B.F. (1981) Degradation of phthalic acids by denitrifying, mixed cultures of bacteria. *Appl Environ Microbiol* **41**: 1177–1183.
- Baba, T., Ara, T., Hasegawa, M., Takai, Y., Okumura, Y., Baba M., *et al.* (2006) Construction of *Escherichia coli* K-12 in-frame, single-gene knockout mutants: the keio collection. *Mol Syst Biol* **2**: 2006.0008.
- Battersby, N.S., and Wilson, V. (1989) Survey of the anaerobic biodegradation potential of organic chemicals in digesting sludge. *Appl Environ Microbiol* **55**: 433–439.
- Bemis, A.G., Dindorf, J.A., Horwood, B., and Samans, C. (1982) Phthalic acids and other benzenepolycarboxylic acids. In *Kirk Othmer Encyclopedia of Chemical Technology*. Mark H. F., Othmer D. F., Overberg C. G., Seaborg G. T., Grayson M., and Eckroth D. (eds). New York, NY: Wiley, Vol. 17, pp. 732–777.
- Bhuiya, M.W., Lee, S.G., and Jez, J.M. (2015) Structure and mechanism of ferulic acid decarboxylase (FDC1) from *Saccharomyces cerevisiae*. *Appl Environ Microbiol* **81**: 1216–1223.
- Breese, K., Boll, M., Alt-Morbe, J., Schagger, H., and Fuchs, G. (1998) Genes coding for the benzoyl-CoA pathway of

- anaerobic aromatic metabolism in the bacterium *Thauera aromatica*. *Eur J Biochem* **256**: 148–154.
- Carmona, M., Zamarro, M.T., Blázquez, B., Durante-Rodríguez, G., Juárez, J.F., Valderrama, J.A., *et al.* (2009) Anaerobic catabolism of aromatic compounds: a genetic and genomic view. *Microbiol Mol Biol Rev* **73**: 71–133.
- Chang, H.K., and Zylstra, G.J. (1998) Novel organization of the genes for phthalate degradation from *Burkholderia cepacia* DBO1. *J Bacteriol* **180**: 6529–6537.
- Chen, J., Li, X., Li, J., Cao, J., Qiu, Z., Zao, Q., *et al.* (2007) Degradation of environmental endocrine disruptor di-2-ethylhexyl phthalate by a newly discovered bacterium, *Microbacterium* sp. Strain CQ0110Y. *Appl Microbiol Biotechnol* **74**: 676–782.
- Cheung, J.K.H., Lam, R.K.W., Shi, M.Y., and Gu, J.D. (2007) Environmental fate of endocrine-disrupting dimethyl phthalate esters (DMPE) under sulfate-reducing condition. *Sci Total Environ* **381**: 126–133.
- Eaton, R.W., and Ribbons, D.W. (1982) Metabolism of dibutylphthalate and phthalate by *Micrococcus* sp. strain 12B. *J Bacteriol* **151**: 48–57.
- Fatoki, O.S., and Vernon, F. (1990) Phthalate esters in rivers of the greater manchester area, U.K. *Sci Total Environ* **95**: 227–232.
- Felsenstein, J. (1985) Confidence limits on phylogenies: an approach using the bootstrap. *Evol* **39**: 783–791.
- Forward, J.A., Behrendt, M.C., Wyborn, N.R., Cross, R., and Kelly, D.J. (1997) TRAP transporters: a new family of periplasmic solute transport systems encoded by the dctPQM genes of *Rhodobacter capsulatus* and by homologs in diverse gram-negative bacteria. *J Bacteriol* **179**: 5482–5493.
- Fuchs, G., Mohamed, M.E.S., Altenschmidt, U., Koch, J., Lack, A., Brackmann, R., *et al.* (1994) Biochemistry of anaerobic biodegradation of aromatic compounds. In *Biochemistry of Microbial Degradation*. Ratledge, C. (ed.). Dordrecht, The Netherlands: Kluwer Academic Publishers, pp. 513–553.
- Giam, C.S., Atlas, E., Powers, M.A., Jr., and Leonard, J.E. (1984) Phthalic acid esters. In *Handbook of Environmental Chemistry*. Hutzinger, O. (ed.) Berlin: Springer Verlag, Vol. 3, pp. 67–142.
- Grifoll, M., Selifonov, S.A., and Chapman, P.J. (1994) Evidence for a novel pathway in the degradation of fluorene by *Pseudomonas* sp. strain F274. *Appl Environ Microbiol* **60**: 2438–2449.
- Harwood, C.S., Burchhardt, G., Herrmann, H., and Fuchs, G. (1999) Anaerobic metabolism of aromatic compounds via the benzoyl-CoA pathway. *FEMS Microbiol Rev* **22**: 439–458.
- Heider, J. (2001) A new family of CoA-transferases. *FEBS Lett* **509**: 345–349.
- Jacewicz, A., Izumi, A., Brunner, K., Schnell, R., and Schneider, G. (2013) Structural insights into the UbiD protein family from the crystal structure of PA0254 from *Pseudomonas aeruginosa*. *PLoS One* **8**: e63161.
- Junghare, M., Patil, Y., and Schink, B. (2015) Draft genome sequence of a nitrate-reducing, o-phthalate degrading bacterium, *Azoarcus* sp. strain PA01. *Stand Genomic Sci* **10**: 90.
- Kiyohara, H., and Nagao, K. (1978) The catabolism of phenanthrene and naphthalene by bacteria. *J Gen Microbiol* **105**: 69–75.
- Kleerebezem, R., Hulshoff, P.L.W., and Lettinga, G. (1999) Anaerobic degradation of phthalate isomers by methanogenic consortia. *Appl Environ Microbiol* **65**: 1152–1160.
- Kumar, S., Stecher, G., and Tamura, K. (2016) MEGA7: molecular evolutionary genetics analysis version 7.0 for bigger datasets. *Mol Biol Evol* **33**: 1870–1874.
- Leutwein, C., and Heider, J. (1999) Anaerobic toluene catabolic pathway in denitrifying *Thauera aromatica*: activation and b-oxidation of the first intermediate, (R)-(1)-benzylsuccinate. *Microbiology* **145**: 3265–3271.
- Leutwein, C., and Heider, J. (2001) Succinyl-CoA:(R)-benzylsuccinate CoA-transferase: an enzyme of the anaerobic toluene catabolic pathway in denitrifying bacteria. *J Bacteriol* **183**: 4288–4295.
- Li, H., and Gu, J.D. (2007) Complete degradation of dimethyl isophthalate requires the biochemical cooperation between *Klebsiella oxytoca* sc and *Methylobacterium mesophilicum* sr isolated from wetland sediment. *Sci Total Environ* **380**: 181–187.
- Li, J., Chen, J.A., Zhao, Q., Li, X., and Shu, W.Q. (2006) Bioremediation of environmental endocrine disruptor di-n-butyl phthalate ester by *Rhodococcus ruber*. *Chemosphere* **65**: 1627–1633.
- Li, J.X., Gu, J.D., and Pan, L. (2005a) Transformation of dimethyl phthalate, dimethyl isophthalate and dimethyl terephthalate by *Rhodococcus ruber* sa and modeling the processes using the modified gompertz model. *Int Biodeterior Biodegradation* **55**: 223–232.
- Li, J.X., Gu, J.D., and Yao, J.H. (2005b) Degradation of dimethyl terephthalate by *Pasteurella multocida* sa and *Sphingomonas paucimobilis* sy isolated from mangrove sediment. *Int Biodeterior Biodegradation* **56**: 158–165.
- Lin, F., Ferguson, K.L., Boyer, D.R., Lin, X.N., and Marsh, E.N.G. (2015) Isofunctional enzymes PAD1 and UbiX catalyze formation of a novel cofactor required by ferulic acid decarboxylase and 4-hydroxy-3-polyprenylbenzoic acid decarboxylase. *ACS Chem Biol* **10**: 1137–1144.
- Liang, D., Zhang, T., Fang, H.H.P., and He, J. (2008) Phthalates biodegradation in the environment. *Appl Microbiol Biotechnol* **80**: 183–198.
- Liolios, K., Mavromatis, K., Tavernarakis, N., and Kyrpides, N.C. (2008) The genomes on line database (GOLD) in 2007: status of genomic and metagenomic projects and their associated metadata. *Nucleic Acids Res* **36**: D475–D479.
- Markowitz, V.M., Mavromatis, K., Ivanova, N.N., Chen, I.M.A., Chu, K., and Kyrpides, N.C. (2009) IMG-ER: a system for microbial genome annotation expert review and curation. *Bioinformatics* **25**: 2271–2278.
- Matsumoto, M., Hirata-Koizumi, M., and Ema, M. (2008) Potential adverse effects of phthalic acid esters on human health: a review of recent studies on reproduction. *Regul Toxicol Pharmacol* **50**: 37–49.
- Mayer, F.L.J., Stalling, D.L., and Johnson, J.L. (1972) Phthalate esters as environmental contaminants. *Nature* **238**: 411–413.
- Molina-Henares, A.J., Krell, T., Eugenia-Guazzaroni, M., Segura, A., and Ramos, J.L. (2006) Members of the IclR family of bacterial transcriptional regulators function as activators and/or repressors. *FEMS Microbiol Rev* **30**: 157–186.
- Naumov, A.V., Gafarov, A.B., and Boronin, A.M. (1996) A novel *ortho*-phthalate utilizer- an acetic acid bacterium *Acetobacter* sp. *Microbiology* **65**: 182–186.

- Neuhoff, V., Arold, N., Taube, D., and Ehrhardt, W. (1988) Improved staining of proteins in polyacrylamide gels including isoelectric focusing gels with clear background at nanogram sensitivity using coomassie brilliant blue G-250 and R-250. *Electrophoresis* **9**: 255–262.
- Nilsson, C. (1994) *Phthalic Acid Esters Used as Plastic Additives Comparisons of Toxicological Effects*. Solna: Swedish National Chemicals Inspectorate.
- Nozawa, T., and Maruyama, Y. (1988) Anaerobic metabolism of phthalate and other aromatic compounds by a denitrifying bacterium. *J Bacteriol* **170**: 5778–5784.
- Park, J.W., Jung, W.S., Park, S.R., Park, B.C., and Yoon, Y.J. (2007) Analysis of intracellular short organic acid-coenzymeA esters from actinomycetes using liquid chromatography-electrospray ionization-mass spectrometry. *J Mass Spectrom* **42**: 1136–1147.
- Perkins, D.N., Pappin, D.J.C., Creasy, D.M., and Cottrell, J.S. (1999) Probability-based protein identification by searching sequence databases using mass spectrometry data. *Electrophoresis* **20**: 3551–3567.
- Pujar, B.G., and Ribbons, D.W. (1985) Phthalate metabolism in *Pseudomonas fluorescens* PHK: purification and properties of 4, 5-dihydroxyphthalate decarboxylase. *Appl Environ Microbiol* **49**: 374–376.
- 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., Kube, M., Heider, J., Beck, A., Heitmann, K., Widdel, F., et al. (2005) The genome sequence of an anaerobic aromatic-degrading denitrifying bacterium, strain EbN1. *Arch Microbiol* **183**: 27–36.
- Saitou, N., and Nei, M. (1987) The neighbor-joining method: a new method for reconstructing phylogenetic trees. *Mol Biol Evol* **4**: 406–425.
- Schink, B., Brune, A., and Schnell, S. (1992) Anaerobic degradation of aromatic compounds. In *Microbial Degradation of Natural Products*. Winkelmann, G. (ed.). Germany: VCH Weinheim, pp. 219–242.
- Schmidt, A., Müller, N., Schink, B., and Schleheck, D. (2013) A proteomic view at the biochemistry of syntrophic butyrate oxidation in *Syntrophomonas wolfei*. *PLoS One* **8**: 56905.
- Schwarzbauer, J., Heim, S., Brinker, S., and Littke, R. (2002) Occurrence and alteration of organic contaminants in seepage and leakage water from a waste deposit land fill. *Water Res* **36**: 2275–2287.
- Sepic, E., Bricelj, M., and Leskovsek, H. (1998) Degradation of fluoranthene by *Pasteurella* sp. IFA and *Mycobacterium* sp. PYR-1, isolation and identification of metabolites. *J Appl Microbiol* **85**: 746–754.
- Taylor, B.F., and Ribbons, D.W. (1983) Bacterial decarboxylation of *o*-phthalic acids. *Appl Environ Microbiol* **46**: 1276–1281.
- Vamsee-Krishna, C., Mohan, Y., and Phale, P.S. (2006) Biodegradation of phthalate isomers by *Pseudomonas aeruginosa* PP4, *Pseudomonas* sp. PPD and *Acinetobacter lwoffii* ISP4. *Appl Microbiol Biotechnol* **72**: 1263–1269.
- Wang, Y.P., and Gu, J.D. (2006a) Degradability of dimethyl terephthalate by *Variovorax paradoxus* T4 and *Sphingomonas yanoikuyae* DOS01 isolated from deep-ocean sediments. *Ecotoxicology* **15**: 549–557.
- Wang, Y.P., and Gu, J.D. (2006b) Degradation of dimethyl isophthalate by *Variovorax paradoxus* strain T4 isolated from deep-ocean sediment of the south china sea. *Human Ecol Risk Assess* **12**: 236–247.
- Wang, Y.Y., Fan, Y.Z., and Gu, J.D. (2003) Aerobic degradation of phthalic acid by *Comamonas acidovorans* Fy-1 and dimethyl phthalate ester by two reconstituted consortia from sewage sludge at high concentrations. *World J Microbiol Biotechnol* **19**: 811–815.
- Wensing, M., Uhde, E., and Salthammer, T. (2005) Plastics additives in the indoor environment-flame retardants and plasticizers. *Sci Total Environ* **339**: 19–40.
- 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.
- Woodward, K.N. (1988) *The Adipates in Phthalate Esters: Toxicity and Metabolism*. Boca Raton, FL: CRC press, Vol. II, pp. 168–173.
- Zhang, H., and Javor, G.T. (2000) Identification of the ubiD gene on the *Escherichia coli* chromosome. *J Bacteriol* **182**: 6243–6246.
- Zheng, Z., He, P.J., Shao, L.M., and Lee, D.J. (2007) Phthalic acid esters in dissolved fractions of land fill leachates. *Water Res* **41**: 4696–4702.
- Zuckerandl, E., and Pauling, L. (1965) *Evolutionary divergence and convergence in proteins*. In *Evolving Genes and Proteins*. Bryson, V., Vogel, H.J. (eds). New York: Academic Press, pp. 97–166.

Supporting information

Additional Supporting Information may be found in the online version of this article at the publisher's web-site:

Fig. S1. Anaerobic growth of *Azoarcus* sp. strain PA01 with 2 mM *o*-phthalate or 2 mM benzoate plus nitrate (12 mM) as electron acceptor.

Fig. S2. SDS-PAGE analysis of solubilized membrane proteins treated with dodecyl β -maltoide. Lane 1, -prestained protein ladder; lane 2, benzoate-grown and lane 3, *o*-phthalate-grown cells. The table shows the LC-MS/MS identification of the respective protein spots and their predicted functions are shown.

Fig. S3. A. Phylogenetic neighbor-joining tree of the putative succinyl-CoA:*o*-phthalate CoA-transferase of the *Azoarcus* sp. strain PA01 and representative protein sequences from members of the CoA-transferase families I, II and III from the bacteria. The evolutionary distances were computed using the Poisson correction method (Zuckerandl and Pauling, 1965) and tree generated using MEGA7 software (Kumar et al., 2016). The numbers at the corresponding nodes show bootstrap support (1000 replicates) (Felsenstein, 1985) and accession numbers are given in parenthesis. The bar represents 20% estimated sequence divergence.

Fig. S3. B. Phylogenetic neighbor-joining tree of the putative *o*-phthalyl-CoA decarboxylase from *Azoarcus* sp. strain PA01 and putative amino acid sequences from related bacterial UbiD- or UbiX-like decarboxylases. The evolutionary

distances were computed using the Poisson correction method (Zuckerandl and Pauling, 1965) and tree generated using MEGA7 software (Kumar *et al.*, 2016). The numbers at the corresponding nodes show bootstrap support (1000 replicates) (Felsenstein, 1985) and accession numbers are given in parenthesis. The bar represents 20% estimated sequence divergence.

Fig. S4. Comparison of the genes from the phthalate gene cluster from the genome of *Azoarcus* sp. strain PA01 and distribution of genes with similar functions in the genomes of the other nitrate-reducing bacteria '*A. aromaticum* EbN1', *Azoarcus toluclasticus* ATCC 700605 and *Thauera chlorobenzoica*. The neighborhoods of the genes from the genome with the same top COG hit (Clusters of Orthologous Groups of protein) are compared. Genes having significant similar IMG predicted function are shown in the same colour (top COG hit). Grey, 4-hydroxy-3-polyprenylbenzoate decarboxylase (UbiX-like decarboxylase/flavin binding protein); red, 4-hydroxy-3-polyprenylbenzoate decarboxylase (UbiD-like decarboxylase); green, benzylsuccinate CoA-transferase (CoA-transferase family III); purple, TRAP-type uncharacterized transport system periplasmic component; blue, TRAP-type uncharacterized transport system, fused permease components and black, hypothetical protein respectively.

Fig. S5. ESI MS/MS of the $[M+H]^+$ m/z 868 of succinyl-CoA (Sigma-Aldrich).

Fig. S6. ESI-MS/MS of the $[M+H]^+$ m/z 916 of synthesized *o*-phthalyl-CoA.

Fig. S7. ESI-MS/MS of the $[M+H]^+$ m/z 934 of synthesized fluoro-*o*-phthalyl-CoA.

Fig. S8. ESI-MS/MS of the $[M+H]^+$ m/z 916 of isophthalyl-CoA.

Fig. S9. ESI-MS/MS of the $[M+H]^+$ m/z 916 of terephthalyl-CoA.

Fig. S10. The time course of benzoyl-CoA formation (rt: 11.4 min) by the enzymatic decarboxylation of the synthesized *o*-phthalyl-CoA in the enzyme assay performed with cell-free extract of *o*-phthalate-grown cells, monitoring the specific ion trace m/z 365 of the MS/MS fragmentation of benzoyl-CoA (quasimolecular ion m/z 872).

Fig. S11. The time course of fluoro-benzoyl-CoA formation (rt: 11.5 min) by the enzymatic decarboxylation of the synthesized fluoro-*o*-phthalyl-CoA in the enzyme assay performed with cell-free extract of *o*-phthalate-grown cells, monitoring the specific ion trace m/z 383 of the MS/MS fragmentation of fluoro-benzoyl-CoA (quasimolecular ion m/z 890).

Fig. S12. The time course of benzoyl-CoA formation (rt: 11.4 min) in a coupled enzyme assay with *o*-phthalate and succinyl-CoA by the cell-free extract of *o*-phthalate-grown cells monitoring the specific ion trace m/z 365 of the MS/MS fragmentation of benzoyl-CoA (quasimolecular ion m/z 872) under aerobic incubation conditions.