



Anaerobic degradation of xenobiotic isophthalate by the fermenting bacterium *Syntrophorhabdus aromaticivorans*

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Abstract

Syntrophorhabdus aromaticivorans is a syntrophically fermenting bacterium that can degrade isophthalate (3-carboxybenzoate). It is a xenobiotic compound which has accumulated in the environment for more than 50 years due to its global industrial usage and can cause negative effects on the environment. Isophthalate degradation by the strictly anaerobic *S. aromaticivorans* was investigated to advance our understanding of the degradation of xenobiotics introduced into nature, and to identify enzymes that might have ecological significance for bioremediation. Differential proteome analysis of isophthalate- vs benzoate-grown cells revealed over 400 differentially expressed proteins of which only four were unique to isophthalate-grown cells. The isophthalate-induced proteins include a phenylacetate:CoA ligase, a UbiD-like decarboxylase, a UbiX-like flavin prenyltransferase, and a hypothetical protein. These proteins are encoded by genes forming a single gene cluster that putatively codes for anaerobic conversion of isophthalate to benzoyl-CoA. Subsequently, benzoyl-CoA is metabolized by the enzymes of the anaerobic benzoate degradation pathway that were identified in the proteomic analysis. In vitro enzyme assays with cell-free extracts of isophthalate-grown cells indicated that isophthalate is activated to isophthalyl-CoA by an ATP-dependent isophthalate:CoA ligase (IPCL), and subsequently decarboxylated to benzoyl-CoA by a UbiD family isophthalyl-CoA decarboxylase (IPCD) that requires a prenylated flavin mononucleotide (prFMN) cofactor supplied by UbiX to effect decarboxylation. Phylogenetic analysis revealed that IPCD is a novel member of the functionally diverse UbiD family (de)carboxylases. Homologs of the IPCD encoding genes are found in several other bacteria, such as aromatic compound-degrading denitrifiers, marine sulfate-reducers, and methanogenic communities in a terephthalate-degrading reactor. These results suggest that metabolic strategies adapted for degradation of isophthalate and other phthalate are conserved between microorganisms that are involved in the anaerobic degradation of environmentally relevant aromatic compounds.

Introduction

Aromatic compounds represent the second most abundant class of organic compounds in nature that are mainly

produced by plants [1, 2] and serve as growth substrates for microorganisms. In addition, industrial activities are an important source of synthetic aromatic compounds that do not occur naturally in the environments, referred to as “xenobiotics” [3]. Due to a lack of essential enzymes for their degradation, these compounds often represent a challenge to microbial decomposition, and many of them persist in the environment [4]. For example, plastics are composed of synthetic or semi-synthetic organic polymers and plasticizers that are non-covalently bound additives and increase the plasticity of materials. Commonly, phthalic acid esters are used as additives in polyvinyl chloride (PVC) applications and are a major cause of environmental pollution.

Phthalic acid esters were introduced at the beginning of the 1920s as a new class of plasticisers which are produced by a reaction of aryl/alkyl alcohols with phthalic acids [5, 6]. The term “phthalic acids” is used here to refer three

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isomers of benzenedicarboxylic acid, i.e., *o*-phthalate (*ortho*), isophthalate (*meta*), and terephthalate (*para*). Besides their main use as plasticisers, phthalic acid esters are employed for various other applications such as the manufacturing of solvents, resins, textiles, paints, or fibers [7]. In the last 30 years, the annual global production of phthalic acid esters has increased rapidly from 1.8 million tons in 1975 to over 8 million tons in 2011 [8]. Their consumption is expected to grow at an average annual rate of 1.3% during 2017–22 (Chemical Economics Handbook, IHS Markit, 2018). After *o*-phthalic acid, isophthalic acid is a commercially important synthetic compound produced at the billion kilogram/year scale by oxidizing meta-xylene [9]. The world's largest producer of isophthalic acid is a Lotte Chemical Corporation (<http://pulsenews.co.kr/view.php?year=2017&no=315343>). Commercially, it is mainly used as a feedstock for the production of unsaturated polyesters [9].

Due to their high global utilization, phthalic acid esters enter the environment during production, leaching, transport, and disposal of plastics, and are polluting various environments such as air, water, sediments, soils, and wastewaters [8, 10–12]. Because phthalic acid esters and their metabolites negatively affect humans and animals [13–15], their removal from the environment is important to maintain the health of the biosphere and to protect wildlife and humans.

In the environment, phthalic acid esters are subject to abiotic degradation, e.g., photolysis. However, this degradation is very slow, with half-lives ranging from a few weeks to a full year [6]. Some specialized aerobic microorganisms have adapted to use phthalic acid esters (partially or completely) as growth substrate and mineralize them to non-toxic products [16–18]. Thus, biodegradation of phthalic acid esters is of interest not only for its benign effect on the environment. It may also allow us to learn how microbes in nature have evolved and adapted new pathways (enzymes) to degrade such xenobiotic compounds [19].

In aerobic and anaerobic biodegradation, phthalic acid esters are first hydrolyzed by esterases to release the side-chain alcohols that are readily utilized by microorganisms, but the phthalic acid residue often accumulates [7, 20, 21]. In the overall degradation of phthalic acid esters, metabolism of the phthalic acid residue is considered as the rate-limiting step as it involves a critical decarboxylation step [22, 23]. The ease of phthalic acid biodegradation in nature follows the order: *o*-phthalate > terephthalate > isophthalate [7, 24]. Aerobic bacteria use molecular oxygen as a co-substrate to attack the stable aromatic ring of phthalate through dioxygenase-catalyzed addition of hydroxyl groups that facilitate decarboxylation to protocatechuate, a key intermediate of aerobic degradation of aromatic compounds

[25–29]. Aerobic phthalate degradation has been studied in detail. However, much less is known about their anaerobic degradation.

In the absence of molecular oxygen, anaerobic bacteria have to use entirely different metabolic strategies. About 30 years ago, anaerobic degradation of *o*-phthalate was assumed to proceed through decarboxylation to benzoate [22, 23]. Only recently, the initial enzymes involved in the anaerobic degradation pathway of *o*-phthalate have been identified in pure cultures of four denitrifying bacteria: *Thauera chlorobenzoica*, *Azoarcus* sp. strain PA01, “*Aromatoleum aromaticum*” strain EbN1, and *Azoarcus evansii* [30–32]. In denitrifying bacteria, *o*-phthalate is first activated to *o*-phthalyl-CoA by a succinyl-CoA:*o*-phthalate CoA transferase (SPT) which facilitates its subsequent decarboxylation to benzoyl-CoA by an UbiD family *o*-phthalyl-CoA decarboxylase (PCD) that uses a novel prenylated flavin mononucleotide (prFMN) cofactor [33, 34]. Benzoyl-CoA is then degraded through the well-studied benzoyl-CoA pathway [35].

Unlike denitrifiers, almost nothing is known about phthalic acid degradation by defined cultures of syntrophically fermenting or sulfate-reducing bacteria, even though a number of studies have been carried out describing the anaerobic degradation of one of the phthalates by mixed cultures [16, 36–38]. Fermentative degradation of phthalate in the absence of external electron acceptors is energetically unfavorable ($C_8H_6O_4 + 8 H_2O \rightarrow 3 CH_3COO^- + 3H^+ + 2 HCO_3^- + 3 H_2$, $\Delta G^0 = +43.2 \text{ kJ mol}^{-1}$; [39]) and requires H_2 or formate-consuming methanogenic or sulfate-reducing partners to render the degradation energetically feasible ($4 C_8H_6O_4 + 35 H_2O \rightarrow 17 HCO_3^- + 9H^+ + 15 CH_4$, $\Delta G^0 = -151.9 \text{ kJ mol}^{-1}$). Isolation of syntrophically fermenting bacteria is difficult, and only three defined co-cultures have been obtained so far which utilize one of the phthalate as a sole source of carbon and energy [40, 41]. A detailed biochemical characterization and ecological impact of syntrophic metabolism in the global anaerobic carbon cycle is largely missing [42, 43].

Syntrophorhabdus aromaticivorans strain UI degrades isophthalate and other aromatic compounds only in syntrophic association with a H_2 -consuming partner organism [40]. Strain UI belongs to the family *Syntrophorhabdaceae* (the class *Deltaproteobacteria*) and was originally isolated from a methanogenic isophthalate-degrading enrichment culture [44]. There are only two other defined co-cultures of strictly anaerobic, syntrophically isophthalate- and terephthalate-fermenting strains known which belong to the genus *Pelotomaculum* (family *Peptococcaceae*), i.e., *Pelotomaculum isophthalicum* strain JI and *Pelotomaculum terephthalicum* strain JT [41] whose genome have not been sequenced so

far. To the best of our knowledge, there is no report on a phthalate-utilizing pure culture of sulfate-reducing bacteria so far.

The availability of the draft genome sequence of *S. aromaticivorans* [45] allows to use it as a syntrophic model organism to study the pathway of anaerobic isophthalate degradation. Our current understanding is that phthalate isomers are anaerobically degraded through the benzoyl-CoA pathway [35]. In the present study, we provide experimental evidence on the identification of genes, key enzymes and intermediates involved in anaerobic degradation of the xenobiotic isophthalate by the syntrophically fermenting bacterium *S. aromaticivorans*, using a combined approach of differential proteomics and in vitro enzyme assays.

Materials and methods

Materials

Chemicals and biochemicals were obtained from Roche Diagnostics (Mannheim, Germany), Fluka (Neu-Ulm, Germany), Merck (Darmstadt, Germany), Roth (Karlsruhe, Germany), Sigma-Aldrich (Deisenhofen, Germany), Thermo Fisher Scientific (Germany), Applichem (Darmstadt, Germany), and Bio-Rad (Munich, Germany). Phthalyl-CoAs were prepared as described earlier [32]. A detailed preparation method is given in the Supplementary Information.

Syntrophic cultivation of *Syntrophorhabdus aromaticivorans*

S. aromaticivorans strain UI (DSM 17771) was purchased as an actively growing syntrophic co-culture with the H₂-consuming sulfate reducer *Desulfovibrio* sp. SI from Deutsche Sammlung von Mikroorganismen und Zellkulturen GmbH (DSMZ, Braunschweig, Germany). The defined syntrophic co-culture was sub-cultured in anoxic mineral medium containing 10 mM sulfate and 0.01% (w/v) yeast extract [46] plus 2 mM of either isophthalate, *o*-phthalate, terephthalate, or benzoate (acids neutralized with sodium hydroxide). Cultures were always inoculated in a fresh medium using 5% (v/v) inoculum via N₂-flushed sterile syringes (Omnifix-F, Germany). For biomass production, cultivations were performed under strict anoxic conditions using 1.2 L anoxic infusion glass bottles filled with about 1 L growth medium. Cultures were incubated at 37 °C for about 40–60 days in the dark. Purity of *S. aromaticivorans* in co-culture with *Desulfovibrio* sp. SI was routinely monitored by light microscopy.

Preparation of cell-free extracts

For cell-free extract preparations, exponentially grown cultures (OD₆₀₀ = 0.08–0.10) were harvested anoxically inside an anoxic chamber by centrifugation (8000×g, 20 min, 4 °C; Dupont Sorvall). The cell pellet obtained from 3–4 L of culture was washed twice with anoxic 50 mM Tris-HCl buffer (pH 7.5, containing 0.2 M NaCl, 50 mM KCl, and 3 mM dithiothreitol). Finally, cell pellets (wet weight ~0.1–0.15 g) were re-suspended anoxically (under N₂-gas phase) in 3 ml of the same buffer in 8 ml glass vials sealed with rubber septa and aluminium caps. Cell suspensions were kept frozen at –20 °C until further use. Prior to cell disruption, suspensions were supplemented with EDTA-free protease inhibitor cocktail tablets (1 mg/ml, Roche; initial experiments), Halt™ protease inhibitor cocktail (10 µl/ml; Thermo Scientific), and Pefabloc® SC-Protease-Inhibitor (1 mM; Roth). Cells were mechanically disrupted by two to three passages through an ice-cold French pressure cell (SLM Aminco) operated at 137 MPa. The cell lysate was centrifuged anoxically (27,000×g, 30 min, 4 °C) and the supernatant was either used immediately for enzyme assays or stored at –20 °C under N₂ until further use. Protein concentrations were determined by the method of Bradford using bovine serum albumin as standard [47].

Differential proteomics of isophthalate- vs benzoate-grown cells

In order to identify the differentially induced cellular proteins in isophthalate- vs benzoate-grown cells, the proteins were separated in one-dimensional polyacrylamide gels (12.5%; SDS-PAGE) as described previously [32]. Coomassie-stained protein bands of interest were excised from the SDS-gels. Excised bands were destained, reduced with dithiothreitol (DTT), and alkylated with iodoacetamide, followed by protein digestion with trypsin. The digested peptides were analyzed by liquid chromatography-tandem mass spectrometry (LC-MS/MS) using a Bruker Esquire 3000+ connected to an Agilent 1100 HPLC (Proteomic Facility Centre, University of Konstanz, Germany). For the analysis of membrane proteins, cell debris (after 27,000×g, 30 min, 4 °C) were solubilised using dodecyl β-D-maltoside (0.5% w/v) as described [32].

Similarly, for total protein analysis, cellular proteins from cell-free extracts of isophthalate- and benzoate-grown cells were treated with dithiothreitol, iodoacetamide, and digested with trypsin. The analysis of cleaved peptides was performed using a Thermo LTQ Orbitrap Discovery connected with an Eksigent 2D-nano HPLC (San Jose, CA, USA). Identification of proteins based on MS/MS spectral data from one or more peptides was matched against the database containing 3632 proteins obtained from the draft

genome of *S. aromaticivorans* strain UI (<https://img.jgi.doe.gov/cgi-bin/mer/main.cgi>). Mass spectra of peptides generated from proteins were searched using the Mascot search engine (Matrix Science, Boston, MA, USA) with parameters set to in-silico protein digestion with trypsin, alkylation with iodoacetamide, and variable methionine oxidation [48]. Identified proteins were validated based on Mascot score (protein searches generating a score below 20 were discarded). The qualitative abundance of identified proteins was deduced from the total peak area of their corresponding peptide ions using the extracted ion chromatogram search. For the analysis of differentially expressed total cellular protein of isophthalate- vs benzoate-grown cells, the samples were equally treated, analyzed, and their peak areas were compared.

Enzyme assays with cell-free extracts

All enzyme activities were determined anoxically under N₂ gas atmosphere (unless otherwise mentioned) in rubber-sealed 4 ml glass vials or in quartz cuvettes incubated at 37 °C. Effect of oxygen on enzyme activity was determined by pre-incubating cell-free extracts under air (10–30 min) and followed by assays in oxic buffer; anoxically incubated extracts served as a control. For discontinuous assays, samples were collected at different time intervals (0, 3, 5, 10, or 15 min). Proteins were precipitated by mixing assay samples with the same volume of organic solvent (90% methanol, 10% acetonitrile, 0.1% acetic acid, v/v/v), and centrifuged (13,000×g, 15 min). Supernatants were analyzed for isophthalyl-CoA and benzoyl-CoA formation by LC-MS/MS (see below). Specific activities are expressed in units (U) defined as 1 μmol substrate consumed/product formed per min and per mg of protein. Protein concentration in cell-free extracts ranged at 4–5 mg/ml. Enzyme assays were performed with the addition of 40–60 μl cell-free extract. Assays were performed at least in three independent replicates.

Isophthalyl-CoA ligase activity

Conversion of isophthalate to isophthalyl-CoA was measured using both spectrophotometric continuous (indirect) and discontinuous (direct) enzyme assays with cell-free extracts of isophthalate- or benzoate-grown cells. The spectrophotometric assay couples the formation of 1 mol of AMP to the oxidation of 2 mol of NADH per mol of substrate as described previously [49]. Oxidation of NADH to NAD⁺ was monitored at $\lambda = 340$ nm ($\epsilon = 6.2 \times 10^3$ M⁻¹ cm⁻¹) using a v-630 UV-VIS spectrophotometer (Thermo Fisher Scientific). The standard photometric assay contained (1 ml): cell-free extract (~protein, 0.3 mg), 100 mM potassium phosphate buffer (pH 7.6, containing 3 mM DTT), 5 mM MgCl₂, 10 mM phosphoenol-pyruvate, 1 mM ATP, 0.25 mM coenzyme A

(CoA), 0.2 mM NADH, 1 U myokinase, 1 U pyruvate kinase, 1 U lactate dehydrogenase. The reaction was routinely started by the addition of neutralized substrate, e.g., isophthalate, *o*-phthalate, terephthalate, or benzoate. Intrinsic activity of NADH oxidation was always subtracted in final calculations. The discontinuous assay mix (0.5 ml) contained: 100 mM potassium phosphate buffer (pH 7.6, containing 3 mM DTT), 5 mM MgCl₂, 1 mM ATP, 0.25 mM coenzyme A, and 0.5 mM isophthalate. Alternatively, CoA transferase reactions were determined discontinuously using succinyl-CoA or acetyl-CoA as CoA donor [32].

Isophthalyl-CoA decarboxylase activity

Decarboxylation of isophthalyl-CoA to benzoyl-CoA was measured in 100 mM potassium phosphate buffer (pH 7.6, +3 mM DTT) using cell-free extracts plus freshly prepared phthalyl-CoAs as substrate. Additionally, decarboxylation of enzymatically formed isophthalyl-CoA was followed in an assay containing (0.5 ml): 100 mM potassium phosphate buffer (pH 7.6, containing 3 mM DTT), 5 mM MgCl₂, 1 mM ATP, 0.5 mM CoA, and 0.5 mM isophthalate. The enzyme reaction was started by the addition of cell-free extract or substrate. The formation of isophthalyl-CoA and benzoyl-CoA was monitored discontinuously by collecting samples at different time points. To enhance the decarboxylase activity, excess KCl (50 mM) was added to the enzyme assay as described previously [34]. Inhibition of decarboxylase activity was performed by the addition of 10 mM EDTA. Specificity of CoA ligase and decarboxylase activities was tested individually by adding *o*-phthalate, terephthalate or their corresponding CoA esters in cell-free extracts.

LC-MS/MS analysis of CoA esters

The formation of CoA esters was monitored using a Thermo Finnigan Surveyor HPLC connected to a Finnigan LTQ ion trap mass spectrometer fitted with an electrospray ion (ESI) source and operated in the MS/MS positive mode to specifically detect CoA esters [50]. CoA and CoA esters were separated with a Phenomenex Synergi polar-RP (250 × 2 mm, 4 μm) column using solvent A (100 mM ammonium acetate buffer 10% acetonitrile, v/v) and solvent B (acetonitrile 0.1% acetic acid). HPLC programme: 2 min isocratic 2% B, in 20 min to 100% B, in 1 min and back to 2% B, 7 min isocratic 2% B equilibration, flow rate 0.2 ml/min and 50 μl injection volume. Metabolites were identified by their retention times and specific MS/MS spectra [32]. Isophthalyl-CoA formation was quantified following the consumption of coenzyme A using coenzyme A as standard (peak area). Similarly, the formation of benzoyl-CoA was quantified by comparison with a benzoyl-CoA standard.

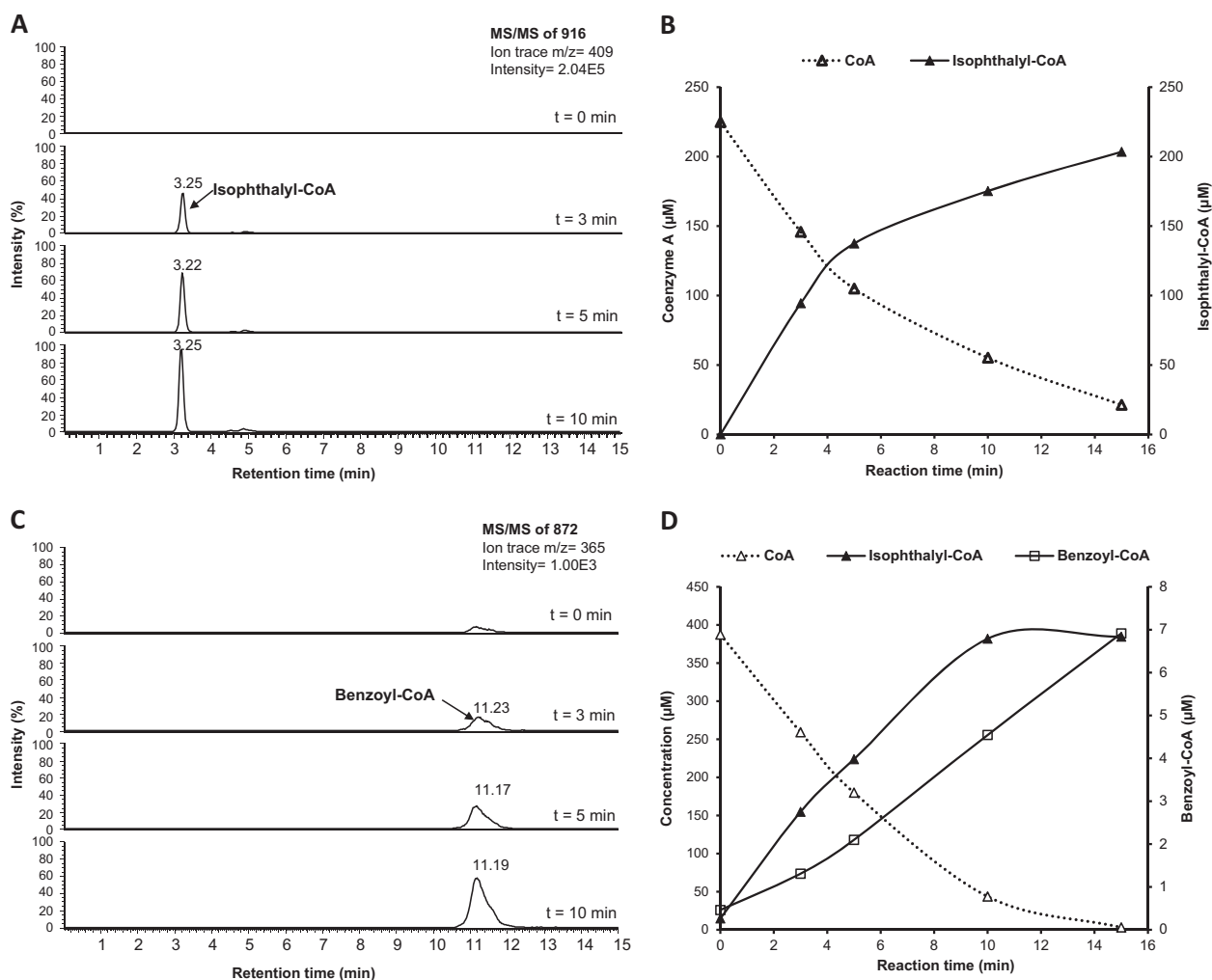


Fig. 1 In vitro enzyme assays performed with cell-free extracts of isophthalate-grown cells of *S. aromaticivorans*. **a** Representative LC-MS/MS chromatograms showing a time course of isophthalyl-CoA formation with coenzyme A, ATP, and isophthalate, monitoring the specific ion trace of m/z 409 of the quasimolecular ion m/z 916. **b** Kinetics of coenzyme A (0.25 mM) consumption and isophthalyl-CoA

formation (no benzoyl-CoA was detected). **c** Representative LC-MS/MS chromatograms exhibiting the formation of benzoyl-CoA by decarboxylation of enzymatically formed isophthalyl-CoA, monitoring the specific ion trace of m/z 365 of the quasimolecular ion m/z 872. **d** Kinetics with excess amount of coenzyme A (0.5 mM) consumption, isophthalyl-CoA and benzoyl-CoA formation

Identification of the prFMN cofactor

To screen for the prenylated flavin mononucleotide (prFMN) cofactor, about 0.8–1.2 mg protein from 100 μl cell-free extracts of isophthalate- and benzoate-grown cells was precipitated by mixing (20 min) with an equal volume of solvent (90% methanol, 10% acetonitrile, 0.1% acetic acid v/v/v) and centrifuged (13,000 $\times g$, 15 min). The supernatant was analyzed using a Waters Acquity UPLC with a Phenomenex Kinetex[®] XB C18 (100 mm \times 2.1 mm, 1.7 μm , 100 \AA) column. HPLC conditions: solvent A (100 mM ammonium acetate buffer containing 10% (v/v) acetonitrile) and solvent B (acetonitrile containing 0.1% (v/v) acetic acid), programmed elution: from 2 to 100% B in 10 min, 2 min 100% B, in 0.5 min 2% B, 5 min 2% B for equilibration, flow rate 0.2 ml/min and 5 μl sample

injection. prFMN was identified with an Exactive Orbitrap mass spectrometer (Thermo Scientific) fitted with a heated electrospray ion source (HESI), positive ionization, mass resolution (100,000, FWHM) and higher-energy collisional dissociation (HCD: 48 eV).

Computational analysis

To obtain closely related homologs of isophthalate-induced proteins of *S. aromaticivorans*, non-redundant protein (nr) or model organisms (landmark) sequences database search was performed using PSI-BLAST (Position-Specific Iterated BLAST) at the NCBI online platform. Alternatively, the search was performed using BLAST UniProt protein identifier (<https://www.uniprot.org/blast/>). Multiple sequence alignment of amino acid sequences was performed

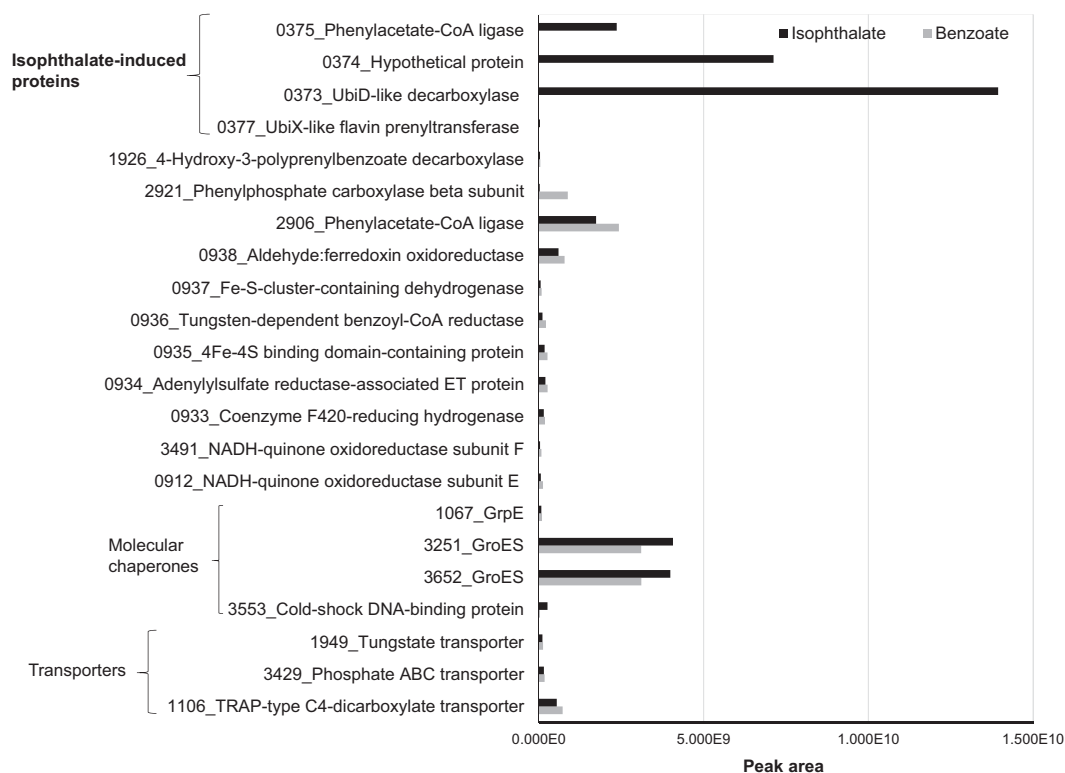


Fig. 2 Comparative analysis of induced-proteins of *S. aromaticivorans* cells grown with isophthalate (black bars) or benzoate (gray bars)

using the Clustal Omega alignment tool with default parameters (<https://www.ebi.ac.uk/Tools/msa/clustalo/>). Aligned sequences were edited in the ESPript 3.0 program (<http://esprict.ibcp.fr/ESPript/cgi-bin/ESPript.cgi>) [51].

Phylogenetic analysis of UbiD-like/UbiX-like decarboxylases and related sequences was performed using the Maximum Likelihood statistical method based on the Poisson correction model [52]. Sequences were aligned automatically with ClustalW set to default parameters, and initial tree(s) searches were performed automatically by applying Neighbor-Joining and BioNJ algorithms. Phylogenetic trees were constructed using the MEGA7 software [53].

Results

Syntrophic growth of *S. aromaticivorans* strain UI

Growth tests with *S. aromaticivorans* in our lab revealed that the bacterium grows syntrophically with the H_2 -consuming sulfate-reducing bacterium *Desulfovibrio* sp. SI with isophthalate or benzoate as substrate, but not with *o*-phthalate or terephthalate, even after extending the incubation time for about 1 year. Our results are in accordance with the previous study that demonstrated syntrophic growth of *S. aromaticivorans* with isophthalate [40].

However, Qiu et al. provided no information about growth tests with *o*-phthalate or terephthalate. Molar growth yields with isophthalate and benzoate were nearly identical.

Activation of isophthalate

In vitro enzyme assays performed with cell-free extracts of isophthalate-grown cells, isophthalate and succinyl-CoA or acetyl-CoA exhibited no formation of isophthalyl-CoA, indicating that there was no CoA-transferase enzyme activity. Instead, if isophthalate was added together with coenzyme A, Mg^{2+} ions, and ATP, isophthalyl-CoA was formed (Fig. 1a; retention time (rt) 3.25 min) as identified based on the characteristic MS/MS fragmentation pattern of the $[M + H]^+$ m/z 916 of isophthalyl-CoA (Supporting Information S1). In cell-free extract of isophthalate-grown cells, the formation of isophthalyl-CoA could directly be correlated with the consumption of coenzyme A, corresponding to a CoA-ligase activity of $83 \pm 11 \text{ nmol min}^{-1} \text{ mg}^{-1}$ (Fig. 1b). Furthermore, analysis of the CoA ligase reaction indicated that ATP was hydrolyzed to AMP. Spectrophotometric assays performed using auxiliary enzymes with isophthalate and cell-free extract exhibited a specific CoA-ligase activity of $66 \pm 12 \text{ nmol min}^{-1} \text{ mg}^{-1}$ that corresponds to the activity observed in the discontinuous assay.

Table 1 A summary of common proteins identified from cell-extracts of isophthalate- and benzoate-grown cells of *S. aromaticivorans*

Gene loci ^a	IMG gene annotation and description ^b	pI ^c	Mr (kDa) ^d	MS/MS identification score		Coverage ^e (%)
				Isophthalate	Benzoate	
0938	Aldehyde:ferredoxin oxidoreductase	5.87	76.5	7139	5845	76
0937	Fe-S-cluster-containing dehydrogenase component	5.11	20.1	198	111	27
0936	Tungsten-dependent benzoyl-CoA reductase bamD	6.46	43.6	1498	1399	43
0935	4Fe-4S binding domain-containing protein	5.30	54.9	1647	1375	39
0934	Putative adenylylsulfate reductase ET protein QmoB	6.05	63.2	1680	1945	60
0933	Coenzyme F420-reducing hydrogenase, delta	8.19	31.3	629	711	33
3491	Subunit	7.18	63.9	498	585	29
0912	NADH-quinone oxidoreductase subunit F	6.16	62.1	1034	1588	41
1236	Cyclohexa-1,5-dienecarbonyl-CoA hydratase	6.25	28.4	667	1091	69
1234	6-Hydroxycyclohex-1-ene-1-carbonyl-CoA dehydrogenase	7.24	39.8	5147	4647	74
1235	6-Ketocyclohex-1-ene-1-carbonyl-CoA hydrolase	6.90	42.7	11,667	16,783	84
1233	3-Hydroxy acyl-CoA dehydrogenase	8.35	26.8	4335	4710	75
3465	Acetyl-CoA C-acetyltransferase	6.05	45.3	4853	4808	72
1516	Acetyl-CoA C-acetyltransferase	6.19	42.0	9503	9230	92
1412	Acetyl-CoA C-acetyltransferase	6.90	41.5	6858	8428	89
2257	Glutaryl-CoA dehydrogenase (decarboxylating)	7.46	63.5	21,884	24,664	84
1512	Glutaryl-CoA dehydrogenase (non-decarboxylating)	6.13	42.3	21,884	24,664	84
1515	3-Hydroxybutyryl-CoA dehydratase/enoyl-CoA hydratase	5.60	27.4	4730	4390	70
1514	3-Hydroxybutyryl-CoA dehydrogenase	6.83	31.7	950	1144	53
1015	Acyl-CoA synthetase	6.29	58.9	164	122	14
1198	Butyryl-CoA dehydrogenase	8.22	42.1	771	316	26

^aIMG gene locus tag SynarDRAFT_“0000” from the draft genome of *S. aromaticivorans* strain UI; ^bGene annotation and predicted functions of gene products; ^cpI—isoelectric point; ^dMass calculated by MS/MS measurement and ^eCoverage represents the extent of peptides measured during MS/MS identification of respective protein

If isophthalate was replaced by *o*-phthalate, terphthalate, or benzoate, no CoA-ligase activity was observed. In control experiments performed with cell-free extract of benzoate-grown cells, no isophthalyl-CoA was formed, indicating that the putative isophthalate:CoA ligase (IPCL) enzyme activity was induced specifically in isophthalate-grown cells (Fig. 2). Several attempts to demonstrate benzoyl-CoA ligase activity in cell-free extracts of benzoate-grown cells were unsuccessful. With isophthalate, the formation of isophthalyl-CoA was strictly dependent on coenzyme A, ATP, and Mg²⁺ ions. Coenzyme A was rapidly used to convert isophthalate to isophthalyl-CoA at a rate that linearly depended on coenzyme A availability (first-order reaction). The formation of isophthalyl-CoA was not inhibited by air exposure (2 h), indicating that the enzyme is oxygen-insensitive.

Conversion of isophthalyl-CoA to benzoyl-CoA

During the standard CoA ligase assays with isophthalate, no spontaneous breakdown of isophthalyl-CoA to coenzyme A and isophthalate was observed, not even after prolonged

incubation for up to 40 min. Instead, isophthalyl-CoA was enzymatically decarboxylated to benzoyl-CoA. In order to demonstrate the decarboxylation of isophthalyl-CoA to benzoyl-CoA, we used chemically prepared isophthalyl-CoA as a substrate in the in vitro enzyme assays. However, initial attempts to demonstrate decarboxylase activity with isophthalyl-CoA plus cell-free extracts indicated that the decarboxylase activity was extremely labile and was lost entirely 1 h after extract preparation. On the other hand, the CoA-ligase activity in cell-free extracts remained largely unaffected. In a recent study, the purified oxygen-sensitive PCD of *T. chlorobenzoica* displayed reproducible activity after treating the enzyme with excess KCl, and became inactivated after EDTA-treatment and air exposure [34]. Several attempts to recover decarboxylase activity by treating cell-free extracts with 50 mM KCl, 5 mM dithiothreitol, and 5% glycerol (v/v) remained unsuccessful. However, the addition of EDTA-free halt protease and SC-protease inhibitors during the preparation of fresh cell-free extracts could stabilize the decarboxylase activity for about 10–15 h.

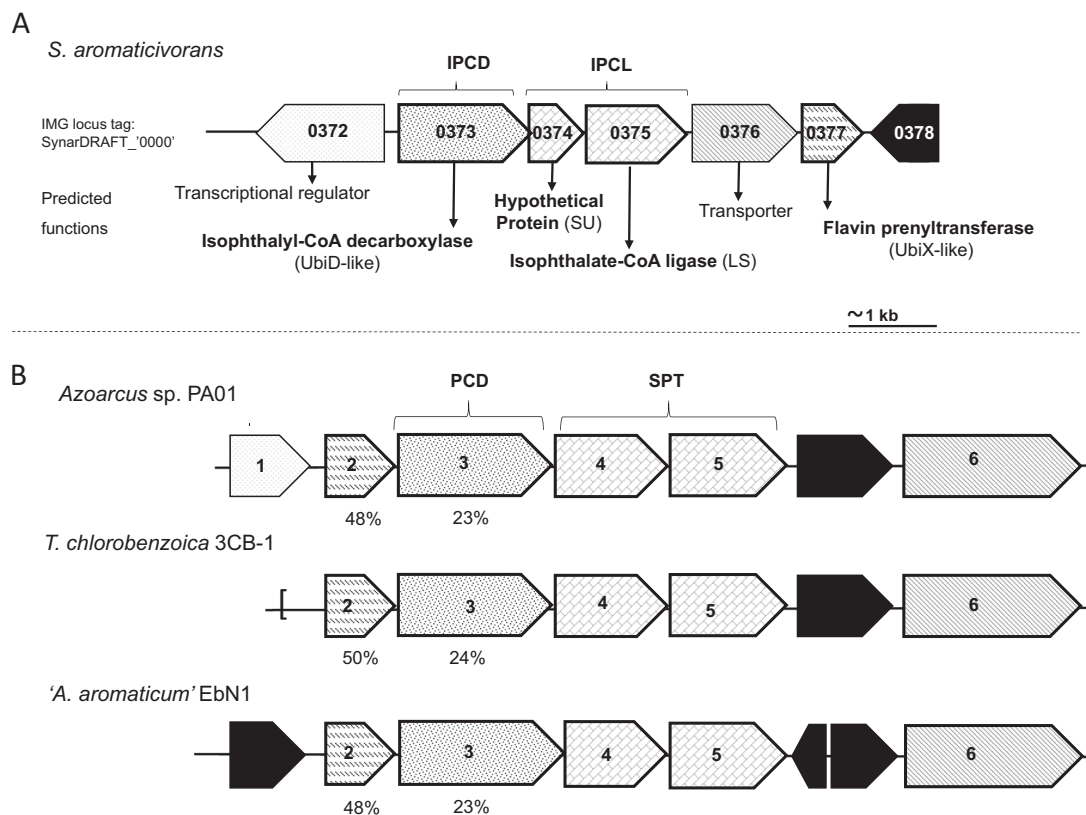


Fig. 3 The identified gene cluster induced specifically during anaerobic degradation of isophthalate to benzoyl-CoA by *S. aromaticivorans*. **a** The predicted functions of induced genes involved in anaerobic isophthalate degradation to benzoyl-CoA included a putative isophthalate:CoA ligase (IPCL), isophthalyl-CoA decarboxylase (IPCD). **b** Representation of *o*-phthalate-induced gene cluster of nitrate-reducing bacteria: IcIR family transcriptional regulator (1), UbiX-

like flavin prenyltransferase (2), UbiD-like *o*-phthalyl-CoA decarboxylase (PCD; 3), succinyl-CoA:*o*-phthalate CoA transferase (SPT; 4 and 5), and TRAP transporters (6). The % identities (BLASTp) with the corresponding products of genes are indicated at the bottom of the respective genes (80–90% coverage). The induced genes are shown with thick frames and black genes have no proposed function (**b** adapted from [30, 32]). SU small subunit, LS large subunit

In assays performed with protease-inhibitor-treated cell-free extract of isophthalate-grown cells, isophthalyl-CoA was converted to benzoyl-CoA (rt: 11.23 min; Fig. 1c) as revealed from identical MS/MS fragmentation patterns of the $[M + H]^+$ m/z 872 ion of benzoyl-CoA (Supporting Information S1). Additionally, in an in vitro assay containing isophthalate (0.5 mM), coenzyme A (0.5 mM), $MgCl_2$ (5 mM), ATP (1 mM), and cell-free extract, the concomitant conversion of isophthalyl-CoA to benzoyl-CoA was found at maximal activity of ca. 0.9–1.1 nmol $min^{-1} mg^{-1}$. This isophthalyl-CoA decarboxylase (IPCD) activity was about 60-fold lower than the determined CoA ligase activity (Fig. 1d). In vitro enzyme tests with chemically prepared *o*-phthalyl-CoA and terephthalyl-CoA showed no benzoyl-CoA formation indicating that they are not accepted as substrate by decarboxylase, in accordance with our growth test results. Control assays performed with cell-free extracts of benzoate-grown cells exhibited no conversion of isophthalyl-CoA to benzoyl-CoA. Unlike isophthalyl-CoA formation, decarboxylation

of isophthalyl-CoA to benzoyl-CoA was strongly impaired if the cell-free extract was exposed to air (~20 min) or if the assays were performed in the oxic buffer. The addition of 10 mM EDTA displayed no significant effect on isophthalyl-CoA formation, but the decarboxylase activity was inhibited.

Identification of proteins involved in isophthalate to benzoyl-CoA conversion

To identify proteins specifically expressed during isophthalate degradation, differential total protein patterns were analyzed with cells grown with either isophthalate or benzoate. In total, more than 400 proteins were identified under both growth conditions by mass spectrometry analysis, which exhibited differences in their abundances (Supporting Table S1). Only four proteins were found to be overexpressed specifically in isophthalate-grown cells, and were completely absent in benzoate-grown cells (Fig. 2). Based on the draft genome of *S. aromaticivorans*,

these isophthalate-induced proteins were identified as putative UbiD-like decarboxylase (SynarDRAFT_0373; in the following section IMG gene locus tag “SynarDRAFT_” is omitted), a hypothetical protein (0374), phenylacetate-CoA ligase (0375), and UbiX-like flavin prenyltransferase (0377). Additionally, we found two similar proteins that were predicted as phenylacetate-CoA ligase (2906) and UbiD-like decarboxylase (1926). However, the latter occurred in both isophthalate- and benzoate-grown cells. The constitutively expressed proteins include several housekeeping genes such as solute transporters (3429; 0686; 1106; 1949) and the GroEL family of molecular chaperons whose proper functioning requires the lid-like co-chaperonin GroE (1067) and GroS (3251, 3652) (Fig. 2). In addition, proteins found in benzoate-grown cells were also detected in isophthalate-grown cells (Table 1) and include mainly the enzymes of the anaerobic benzoyl-CoA pathway in *S. aromaticivorans*, except for benzoate-CoA ligase [54].

Based on the qualitative estimation of the protein abundances, UbiD-like decarboxylase (53.6 kDa) comprises 10% of cellular protein, followed by 5.1% for hypothetical protein (12.1 kDa), 1.7% for phenylacetate-CoA ligase (48.7 kDa), and 0.04% for UbiX-like flavin prenyltransferase (23.8 kDa) (Supporting Information S2). Although the above-mentioned % values did not quantify exact protein abundances under physiological conditions, they were used here to gain a rough idea of representation of isophthalate-induced protein species among identified total proteins. Strikingly, separation of cellular proteins by SDS-PAGE revealed a prominent protein band migrating at about ~57 kDa size that was strongly induced only in isophthalate- compared to benzoate-grown cells. Protein identification of this excised band gave a top hit for a protein with a theoretical mass of 53.6 kDa that was identified as UbiD-like decarboxylase. This result clearly indicates that UbiD-like decarboxylase (0373) is one of the most dominantly expressed cellular proteins during isophthalate degradation. Analysis of additionally excised protein bands migrating in the range of ~50 to ~25 kDa led to the repeated identification of UbiD-like decarboxylase (0373), indicating that this protein was partially degraded by cellular proteases (Supporting Information S2). In agreement with these observations, analysis of the solubilised membrane proteins from isophthalate- and benzoate-grown cells revealed two proteins belonging to the family of serine (0807) and cysteine proteases (1657). This finding indicates that the loss of decarboxylase activity in initial experiments was likely caused by partial protein degradation.

Identification of isophthalate-induced gene cluster

In the genome of *S. aromaticivorans*, the isophthalate-induced four genes 0373, 0374, 0375, and 0377 constitute a

~6 kb gene cluster where all coding genes are arranged in the similar fashion (Fig. 3a). In the identified isophthalate gene cluster, gene 0374 is predicted to code for a hypothetical protein that is located next to gene 0375 which encodes a putative phenylacetate-CoA ligase. Elsewhere in the genome, we found two similar genes 2756 and 2906 which shared 25–34% amino acid sequence identity with the isophthalate-induced gene 0375. The product of gene 0374 had no known homolog, but a BLAST search indicated conserved domains of a γ -subunit of phenylphosphate carboxylase (Ppc). Members of this protein family are the γ -subunit of Ppc that catalyzed phosphorylation of phenol using ATP [55]. Similarly, BLAST analysis of the product of gene 0375 displayed sequence identity to equivalent putative phenylacetate-CoA ligases (Paak superfamily) in other microorganisms, such as *Ardenticatena maritima* (45%), *Azoarcus* sp. KH32C (44%), *Methanothermobacter thermautotrophicus* (31%), *Sulfolobus acidocaldarius* (29%), and *Escherichia coli* strain K-12 (29%). In these organisms, *paak* genes encode a protein that converts phenylacetate to phenylacetyl-CoA, and the encoding gene is often located in a single gene cluster together with other genes involved in phenylacetate catabolism [56, 57, 58]. Based on this homology of induced genes 0374 and 0375, it appears that they likely encode the putative IPCL, enzyme converting isophthalate to isophthalyl-CoA that belongs to the enzyme class of ligases. Noticeably, IPCL shared no sequence identity with the SPT that activates *o*-phthalate to *o*-phthalyl-CoA [33]. The latter enzyme belongs to the transferase enzyme class (Fig. 3a, b).

The isophthalate-induced gene cluster contains two genes 0373 and 0377 that are located at the edge of the cluster coding for an UbiD-like/UbiX-like decarboxylase (Fig. 3a). In the genome of *S. aromaticivorans*, we observed several scattered *ubiD*-like/*ubiX*-like genes (2918, 2921, 3023, 3024, 1926, 2916, and 2915). They shared about 29–60% amino acid sequence identities with the product of isophthalate-specific *ubiD*-like/*ubiX*-like genes (0373 and 0377). The *ubi* genes have been named as constituents of a part of the ubiquinone synthesis pathway in *E. coli* [59]. Over a period of 5 decades, several studies revealed that the bacterial *ubiD/ubiX* genes or the homologous fungal *fdcI/padI* genes product act on aromatic substrate, e.g., cinnamate, in which UbiD(Fdc1) acts as decarboxylase that requires the active form as the prFMN^{iminium} cofactor supplied by the UbiX enzyme [60–63]. Importantly, *o*-phthalate-induced vicinal *ubiD*-like/*ubiX*-like genes in nitrate-reducers that encode a PCD [32, 34] were homologous to the respective *ubiD* (~23%) and *ubiX* (~50%) genes in *S. aromaticivorans* (Fig. 3a, b). This implies that the isophthalate-induced *ubiD*-like/*ubiX*-like genes (0373 and 0377) are likely encoding the proteins that constitute the IPCD, enzyme system requiring the prFMN^{iminium} cofactor

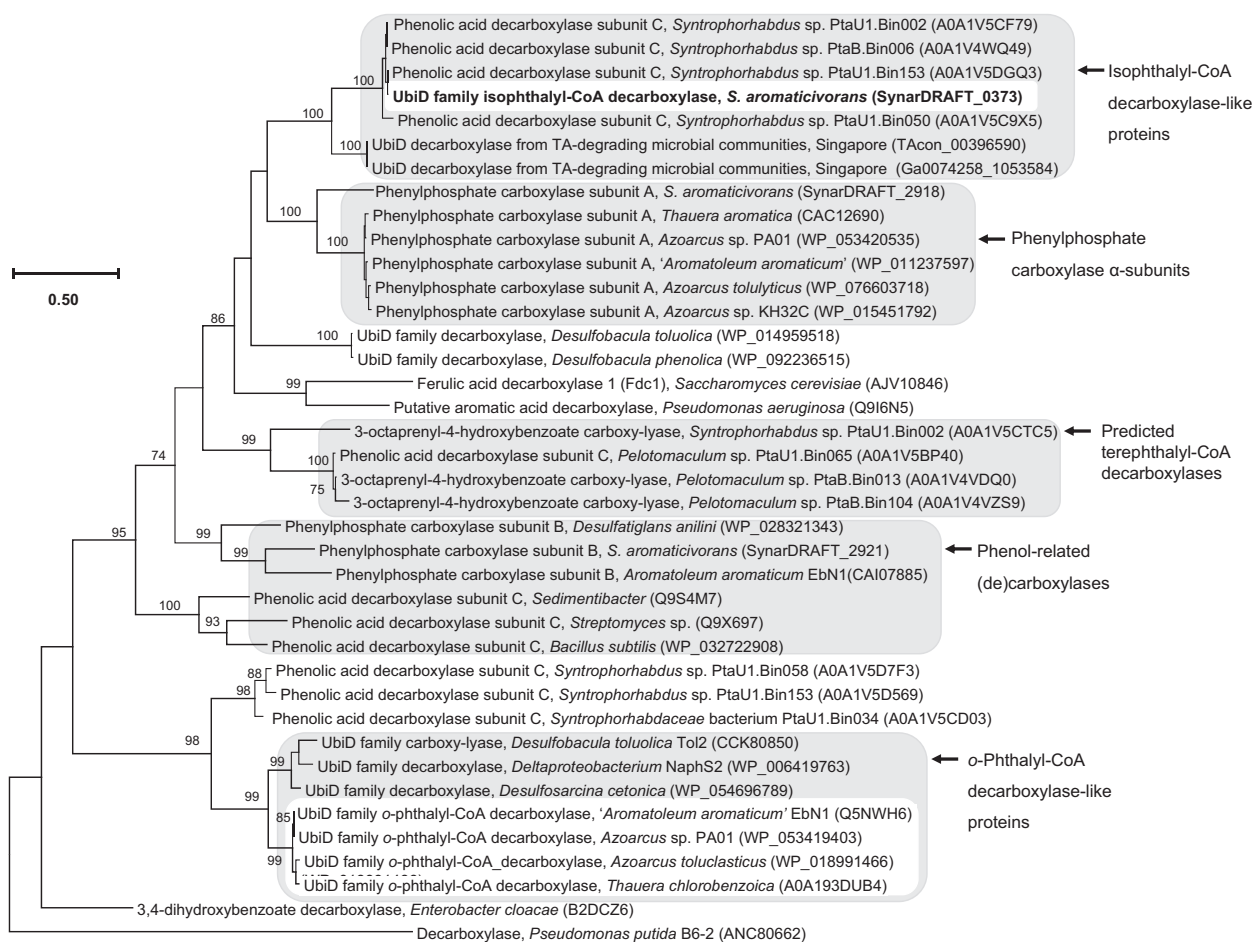


Fig. 4 Phylogenetic tree calculated by the Maximum Likelihood method showing the affiliation of the UbiD family isophthalyl-CoA decarboxylase (IPCD) of *S. aromaticivorans* with the related UbiD-related (de)carboxylase sequences of diverse microorganisms.

Numbers at the nodes are shown only for percentage bootstrap (1000 replicates) values above 50. Accession numbers of sequences are given in parentheses. The scale bar represents 5% sequence divergence

to effect function. Besides this, the gene cluster also contains a gene for a hypothetical benzoate transporter (0376) and a regulatory gene (0372) that were not induced. The presence of a regulatory gene upstream of the gene cluster indicates that gene expression is probably controlled by the specific growth substrate.

Phylogeny of isophthalyl-CoA decarboxylases and related proteins

A PSI-BLAST search restricted to the model organisms' database returned about 12 specific proteins sharing 27–46% identities with the UbiD component (0373) of IPCD of *S. aromaticivorans*. Some of these proteins include the verified UbiD-like decarboxylases, such as ferulic acid decarboxylase 1 (Fdc1) of *Saccharomyces cerevisiae* (30%), aromatic acid decarboxylase of *Pseudomonas aeruginosa* (29%), and 4-hydroxy-3-octaprenyl-benzoate decarboxylase of *E. coli* strain K-12 (29%). The remaining nine

sequences were either UbiD-like or hypothetical proteins (26–33%). However, phylogenetic analysis revealed that the UbiD component (0373) of IPCD is closely related to phenolic acid decarboxylase (UbiD-like) sequences from the isophthalate/terephthalate-degrading metagenome of *Syntrophorhabdaceae* bins that were clustered together (Fig. 4). Other neighboring sub-clusters contained the sequences of α -subunits of putative Ppc of phenol-degrading denitrifiers and aromatic compound-degrading marine sulfate reducers [43, 64–66]. In particular, sequences of the UbiD component of PCD from denitrifiers were among the most distantly related ones that formed a separate phylogenetic sub-cluster (Fig. 4). Furthermore, the presumed UbiD-like terephthalyl-CoA decarboxylase sequences derived from *Pelotomaculum* populations from a terephthalate-degrading metagenome were placed together into a separate clade [43]. Unlike the functional diversity of UbiD decarboxylases, the UbiX-like protein (0377) of *S. aromaticivorans* exhibited significant homology (35–55%)

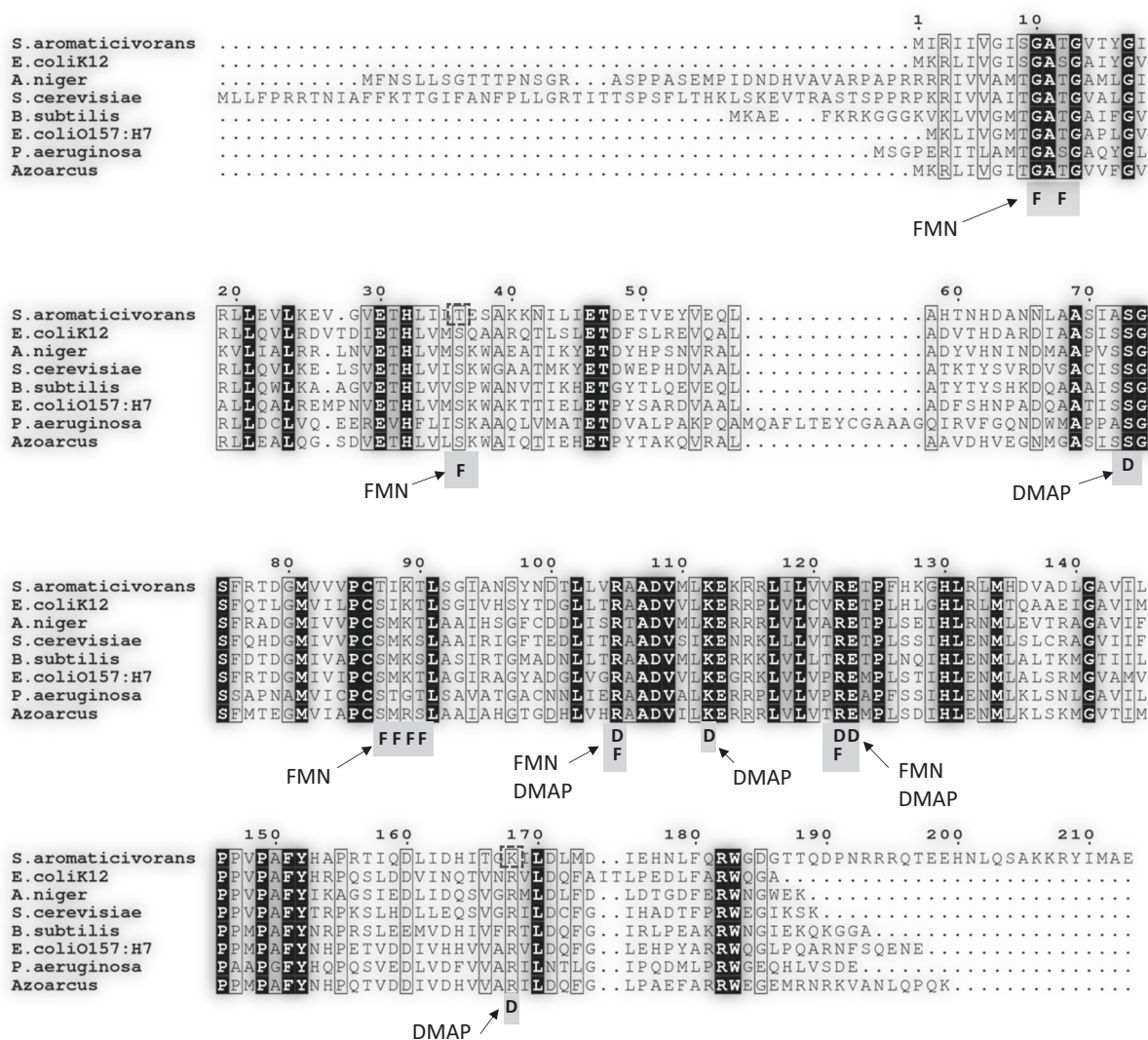


Fig. 5 Multiple sequence alignment of isophthalate-induced UbiX-like putative FMN-binding protein of *S. aromaticivorans* (0377) and related experimentally characterized UbiX-like enzymes of bacterial and fungal strains: *Escherichia coli* strain K-12 (gi2507150), *Aspergillus niger* (ABN13117), *S. cerevisiae* (P33751), *Bacillus subtilis*

(P94404), *E. coli* O157:H7 (P69772), *P. aeruginosa* (Q9HX08), and *Azoarcus* sp. strain PA01 (not characterized). Highly conserved amino acid residues are highlighted and those putatively involved in binding with flavin mononucleotide (FMN) and dimethylallyl phosphate (DMAP) are indicated with arrows (figure adapted from [62, 68])

to UbiX-like proteins of diverse organisms. Phylogenetically it was closely related to the UbiX-like sequences originating from the methanogenic metagenome as well as sequences from marine sulfate-reducers that are known to degrade aromatic compounds. Remarkably, the PCD-associated UbiX-like proteins formed a distinct phylogenetic sub-cluster (Supporting Information S3).

Comparison of FMN-binding proteins

The isophthalate-induced UbiX flavin prenyltransferase (0377) can be grouped in the flavoprotein (FMN/FAD-binding) superfamily (pfam02441) that includes only a small number of known enzymes. A recent study cloned

several UbiX enzymes from phylogenetically distinct organisms and demonstrated that UbiX forms the prFMN cofactor by linking FMN and dimethylallyl phosphate [67]. We compared amino acid sequences of 6 known UbiX enzymes (excluding *Azoarcus* sp. PA01), which all had lengths of ~200 residues and over 30% identity to the UbiX of *S. aromaticivorans*. Importantly, sequence alignment resulted in the identification of 41 conserved residues (Fig. 5). Within the context of function, residues positioned at Gly10, Thr/Ser12, Ser37, Thr/Ser87, and Arg106 that are known to bind FMN in Pad1 (UbiX-like) of *E. coli* O157:H7 [68], were highly conserved among all these enzymes. Noticeably, amino acid residue positioned at 37, Ser (codon: UCU, UCC, UCA, UCG) which is common to the

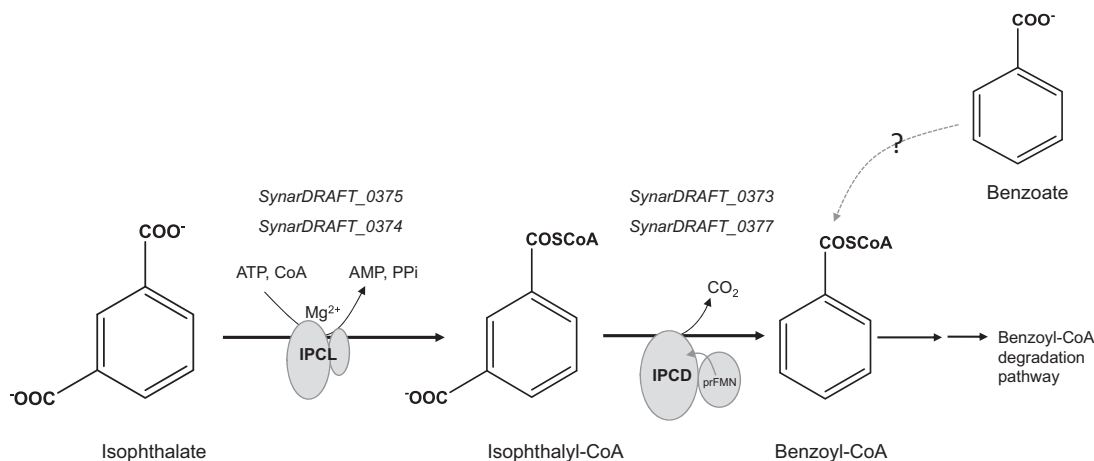


Fig. 6 Pathway of anaerobic isophthalate conversion to benzoyl-CoA reconstructed using in vitro enzyme assays by *S. aromaticivorans* that involve activation of isophthalate to isophthalyl-CoA by an ATP-dependent isophthalate-CoA ligase (IPCL) followed by decarboxylation to benzoyl-CoA which is catalyzed by UbiD family isophthalyl-CoA decarboxylase (IPCD) and associated prFMN forming UbiX-like

flavin prenyltransferase. The identified genes encoding the respective proteins are shown above the arrows. The functions of the gene products have not been directly shown, but inferred by the abundance of the proteins and their annotated functions. The downstream pathway involves the enzymes of the anaerobic benzoyl-CoA pathway (not shown)

compared UbiX was replaced by *Thr* (ACU, ACC, ACA, ACG) in *S. aromaticivorans*, due to a possible point mutation that resulted probably by the change of base U to A (Fig. 5). In addition, the conserved residues *Ser74*, *Arg105*, *Lys112*, *Arg122*, *Glu123*, *Tyr152*, *Arg168*, and *Trp183* have been suggested to play a role either in DMAP binding or in catalysis [62, 68]. In this case, a charged *Arg168* (codon AGA, AGG) was substituted by a similar amino acid residue *Lys168* (AAA, AAG) in UbiX of *S. aromaticivorans* (base change G → A). As it appears, the UbiX enzyme of *S. aromaticivorans* is functionally analogous to flavin prenyltransferase (EC: 2.5.1.129) and prepares an identical, yet functionally versatile prFMN cofactor.

Identification of the catalytically active prFMN in cell-free extracts

Cell-free extracts of isophthalate-grown cells of *S. aromaticivorans* exhibited a faint yellowish color. For identification of the prFMN cofactor, we screened cell-free extracts of isophthalate- and benzoate-grown cells (see Materials and methods). A compound with molecular mass 525.175 Da corresponding to the elemental composition C₂₂H₂₉O₉N₄P (Δ ppm = 0.115) was identified (Supporting Information S4). Collision-induced dissociation (CID) fragmentation of ion 525.175 resulted in the formation of the daughter ions *m/z*: 310, 409, 427, 445, and 507 which fit to the reported catalytically active form of the isolated prFMN cofactor [34, 60, 69]. prFMN was absent in the cell-free extract of benzoate-grown cells.

Discussion

S. aromaticivorans is a strict anaerobe capable of degrading isophthalate but also other aromatic compounds, e.g., phenol, in obligate syntrophic association with a H₂-consuming methanogen or H₂-consuming sulfate-reducer. In this study, the enzymatic steps initiating the anaerobic degradation of the industrially important and environmentally relevant xenobiotic isophthalate to benzoyl-CoA were characterized. It was revealed that *S. aromaticivorans* initially activates isophthalate to isophthalyl-CoA which is subsequently decarboxylated to benzoyl-CoA. This metabolic approach is similar to the one that was discovered in *o*-phthalate degrading nitrate reducers [30, 32]. However, the formation of isophthalyl-CoA in *S. aromaticivorans* is an energetically expensive (ATP-dependent) reaction catalyzed by the AMP-forming IPCL which is different from the transferase reaction utilized by nitrate reducers. The second key step of decarboxylation of isophthalyl-CoA to benzoyl-CoA is catalyzed by a two-component enzyme system consisting of the UbiD family IPCD and the associated UbiX-like flavin prenyltransferase which generates the prFMN cofactor (Fig. 6). Benzoyl-CoA is further degraded through the benzoyl-CoA degradation pathway known from other strictly anaerobic benzoate-fermenting bacteria [70, 71].

ATP-dependent activation of isophthalate

Nitrate reducers use an energetically rather cheap (ATP-independent) reaction for activation of *o*-phthalate to *o*-phthalyl-CoA driven by a SPT with succinyl-CoA as the

CoA donor [30, 32–34]. We expected that fermenting bacteria like *S. aromaticivorans* would make use of a transferase reaction as well, probably with acetyl-CoA as the CoA donor. Instead, *S. aromaticivorans* uses an ATP-dependent CoA ligase reaction, although an AMP-forming ligase means a higher energy investment, depending on the further fate of the pyrophosphate formed from ATP as a side product. This is surprising for a syntrophically fermenting bacterium with an extremely tight energy budget [72]. Nonetheless, many other strict anaerobes including *S. aromaticivorans* use ATP-consuming CoA-ligase reactions for benzoate activation [54, 70, 71]. The fact that the molar growth yields of *S. aromaticivorans* with benzoate and isophthalate were nearly identical indicates that the energy expenditures in substrate activation are similar as well. The ATP-dependent strategy evolved for isophthalate activation by *S. aromaticivorans* is an example of how a microbe compromise over its energy budget to overcome metabolic constraints because a direct decarboxylation of isophthalate to benzoate is chemically unfavorable [30].

In cell-free extracts, the IPCL exhibited a high specific activity of ca. 73 nmol min⁻¹ mg⁻¹, suggesting that the enzyme is quite efficient with isophthalate. This is equivalent to the activity of 76 nmol min⁻¹ mg⁻¹ of the homologous AMP-forming phenylacetate-CoA ligase (29%) in *A. evansii* [73–75]. The activity of SPT was reported to be in a similar range, at 18 to 50 ± 20 nmol min⁻¹ mg⁻¹ [30, 33]. With respect to the phylogenetic and metabolic differences between *S. aromaticivorans* and aromatic compound-degrading *Azoarcus* species, the candidate gene for phenylacetate-CoA ligase of denitrifiers shared high homology to the gene encoding isophthalate-CoA ligase (0375). This finding suggests that the identified isophthalate-induced IPCL is a new member within the AMP-forming CoA-ligase (or synthetase) enzyme family that activates a wide range of acids.

UbiD/UbiX family decarboxylases and their functional versatility

Conversion of isophthalyl-CoA to benzoyl-CoA is catalyzed by the UbiD family IPCD together with the prFMN cofactor-generated by an UbiX-like enzyme (Fig. 6). IPCD appears to be a novel member analogous to the PCD, the only so far known UbiD family decarboxylases which act on CoA-activated substrates, i.e., phthalyl-CoAs [34]. UbiD decarboxylase was first identified in *E. coli* catalysing the decarboxylation reaction in the ubiquinone biosynthesis pathway [59, 76, 77]. In recent years, several studies performed the structural and functional characterization of UbiD decarboxylases of fungal and bacterial origin, which specifically act on non-CoA substrates, e.g., 3-octaprenyl-4-

hydroxybenzoate or cinnamate [50, 62, 63, 68, 69, 78–79]. These observations indicate that members of the UbiD family (de)carboxylases are functionally diverse and versatile. For instance, a number of previous studies have proven the involvement of UbiD enzymes in the carboxylation of a wide range of aromatic substrates such as phenol [55, 65, 80], benzene [81], naphthalene [82, 83], or catechols [84]. Identification of the catalytically active form of the modified flavin-containing prFMN^{riminium} cofactor in cell-free extract of isophthalate-grown cells confirms that all the known UbiD family (de)carboxylases including IPCD and PCD require the identical prFMN cofactor for catalysis [34, 62, 85], independent of their catalytic function or substrate preferences. These findings also explain why the *ubiD/ubiX*-like genes are often located adjacent to each other or in a single gene cluster (Fig. 3).

In cell-free extracts of isophthalate-grown cells, the specific activity of IPCD was unusually low with ca. 1 nmol min⁻¹ mg protein⁻¹, although there was a large pool of isophthalyl-CoA available of which only 2–3% was converted to benzoyl-CoA (Fig. 1d). This may explain why *S. aromaticivorans* grows extremely slowly with isophthalate at a rate of $\mu_{\max} = 0.025 \text{ day}^{-1}$ [40]. The physiological substrate turnover of growing cells under these conditions is about 7 nmol min⁻¹ mg⁻¹, i.e., in the same range as the enzyme activity we measured. Also, one has to take into account that the decarboxylase activity in cell-free extract was extremely labile and was largely hampered by cellular proteases, which may also have weakened the activity. Nonetheless, the low IPCD activity in *S. aromaticivorans* is comparable to that of PCD measured in cell-free extracts of denitrifiers (6–13 nmol min⁻¹ mg⁻¹) which grow significantly faster with $\mu_{\max} = 0.054 \text{ h}^{-1}$ ($t_d = 11\text{--}15 \text{ h}$; [30]). However, purified PCD from *T. chlorobenzoica* displayed significantly higher activity at 104–122 nmol min⁻¹ mg⁻¹ [34].

The low decarboxylase activity in *S. aromaticivorans* is probably compensated by strong over-expression of the UbiD protein (0373) that made up about 10% of the total cellular protein, although this necessitates a high energy investment into producing a rather inefficient enzyme. This phenomenon has been noticed previously also in the *o*-phthalate-degrading *T. chlorobenzoica* in which the UbiD component of PCD represented almost 17% of the total cellular protein [34]. A rough estimation of isophthalate-induced protein abundances in *S. aromaticivorans* revealed that the UbiD (0373) component of IPCD comprised about 10% and the ligase (0375) component of IPCL ca. 2%, which indicates an in vitro ratio of 5:1 that resembles the ratio of 4:1 determined with PCD and SPT in “*A. aromaticum*” [33, 86]. In the latter case, it was hypothesized that a high PCD content is probably needed to bind the highly

unstable intermediate *o*-phthalyl-CoA at low concentration (below 1 μ M) with an apparent K_m of 100 μ M [33, 34]. In analogy to PCD, we suspect that the putative IPCD may also have a rather low affinity for isophthalyl-CoA with low substrate turnover. Therefore, we wonder if the UbiD family *o*-phthalyl- or isophthalyl-CoA decarboxylases may have different, yet unknown (original) functions and might have been adapted for phthalate metabolism only lately. Furthermore, additional experiments—ideally with purified enzymes that are so far not available—are needed to address both mechanistic details and the enzyme specificity.

Ecological relevance of anaerobic phthalate degradation

With their enormous metabolic potential, microorganisms are essential degraders not only of natural products but also of man-made xenobiotics. Phthalates, which increasingly pollute the environment became subject to microbial degradation only lately [86, 87]. Beyond nitrate-, iron-, or sulfate-dependent oxidation, syntrophic fermentation of, e.g., phthalates is an important process in a broad variety of natural and engineered environments, and is driven by a mutualistic interaction of fermenting bacteria with H_2 -consuming anaerobes [36–44]. Thus, syntrophic anaerobes are vital components of the global anaerobic carbon cycles [42]. With the isophthalate-fermenting *S. aromaticivorans* and the H_2 -consuming sulfate reducer *Desulfovibrio* sp. SI as a syntrophic model system, we could now reveal the biochemical reactions in a strictly anaerobic bacterium. Because of the difficulties in cultivating such syntrophic organisms, a detailed biochemical characterization and an analysis of its ecological impact remained largely unknown [40, 41, 43]. The findings of our study confirmed that the enzyme inventory required for anaerobic isophthalate degradation to benzoyl-CoA includes four gene products that constitute the entire necessary enzyme system: isophthalyl-CoA forming IPCD and isophthalyl-CoA decarboxylating IPCD, as key enzymes. Interestingly, the identified isophthalate-induced genes were found to occur in the genomes of pure cultures of denitrifiers, marine sulfate reducers degrading complex aromatic compounds, and also in the metagenome of methanogenic microbial communities degrading phthalate isomers [30, 32, 37, 43]. These findings suggest that the enzymes evolved for anaerobic phthalate degradation appear to be widely prevalent in a wide range of environmental microorganisms that are involved in mineralizing complex aromatic compounds. Considering the synthetic origin and the global occurrence of phthalates, it appears that microbes in nature, have adapted rapidly different metabolic solutions for the degradation of these “xenobiotic” compounds.

Conclusions

Phthalates have accumulated in the environment in the last 5–6 decades, to become available to microorganisms as growth substrates [86, 87]. Here, we provide first in vitro biochemical evidence that the enzymes utilized for isophthalate degradation by strictly anaerobic bacteria are distinct from those used by *o*-phthalate-degrading denitrifiers. However, despite their metabolic differences, both organisms have evolved a common biochemical approach to accomplish the critical step of phthalate decarboxylation. Furthermore, the phylogenetic dissimilarity between the PCD [34] and the IPCD may suggest that the encoding genes could have been acquired from different ancestral routes. This is a good example to learn how microbes in nature adapt their metabolic inventory to benefit from unusual substrates introduced by humans.

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Compliance with ethical standards

Conflict of interest The authors declare that they have no conflict of interest.

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