Physiological and molecular investigation of the anaerobic 4-methylbenzoate and benzoate degradation in *Magnetospirillum* sp. strain pMbN1

Physiologische und molekulare Untersuchung des anaeroben 4-Methylbenzoat- und Benzoat-Abbaus in *Magnetospirillum* sp. Stamm pMbN1

Von der Fakultät für Mathematik und Naturwissenschaften der Carl von Ossietzky Universität Oldenburg zur Erlangung des Grades und Titels eines

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SUMMARY

The benzene ring is one of the most abundant chemical structures encountered in terrestrial organic material. The resonance stabilized ring structure of aromatic compounds confers exceptional (bio-)chemical stabilities for this class of organic molecules. The high stability and toxicity of most aromatic compounds contribute to their persistence in the environment. Aerobic microorganism use reactive oxygen species (O_2-derived) to activate and cleave the aromatic ring. However, rapid depletion of oxygen yields anoxic conditions in many habitats (e.g. freshwater and marine sediments, groundwater aquifers or deep soil layers). Thus, anaerobic degradation of aromatic compounds is an important environmental process. In recent years, several biochemical strategies for the oxygen-independent activation and degradation of aromatic compounds have been elucidated. Similar to aerobic bacteria, anaerobes channel the broad diversity of aromatic compounds into only a few central aromatic intermediates. A prominent example is the anaerobic benzoyl-CoA pathway. Central to this pathway is the reductive dearomatization catalyzed by benzoyl-CoA reductases (BCR). Facultative and strict anaerobes employ for energetic reasons two different types of BCRs, the ATP-dependent (class I) and -independent (class II) enzyme, respectively. Contrasting class II BCRs of strict anaerobes, the mechanism of class I enzymes is already fairly well understood. Notably, known class I enzymes are unable to convert *para*-substituted benzoyl-CoA analogues (e.g. 4-methylbenzoyl-CoA).

In the present study physiological and quantitative molecular approaches were combined to **(1)** physiologically characterize strain pMbN1 and its capability to anaerobically degrade 4-methylbenzoate and benzoate, **(2)** discriminate two distinct pathways for 4-methylbenzoate and benzoate degradation and **(3)** study effects of aliphatic and aromatic acids on the anaerobic degradation of benzoate and 4-methylbenzoate in binary and ternary substrate mixtures.

(1) The freshwater sediment isolate strain pMbN1 affiliates with the genus *Magnetospirillum* within the *Alphaproteobacteria*. Mesophilic strain pMbN1 is heterotrophic and capable of facultative anaerobic growth by denitrification. Despite affiliation with the genus *Magnetospirillum*, strain pMbN1 is non-magnetotactic and was shown to utilize 26 (13 aromatic) organic substrates for growth under nitrate-reducing conditions. In contrast, only five of the aromatic compounds were also utilized

under oxic conditions, suggesting a preference of strain pMbN1 for anaerobic growth. Benzoate and 4-methylbenzoate were both completely oxidized to CO_2 under anoxic conditions. The higher growth rate and molar growth yield observed with benzoate suggest higher efficiency of anaerobic benzoate utilization compared to that of 4-methylbenzoate.

(2) Adaptation studies indicate the substrate-specific induction of anaerobic benzoate and 4-methylbenzoate degradation, respectively. The differential protein profile (2D-DIGE) of 4-methylbenzoate-adapted cells revealed the specific formation of a protein set, which was absent in benzoate- or succinate-adapted cells. This set contained amongst others, a putative 4-methylbenzoyl-CoA reductase (MbrBCAD) and a distinct set of modified β-oxidation enzymes (Dch-2, Oah-2 and Had-2), that differ from the ones formed in anaerobically benzoate-grown cells. Metabolite analysis demonstrated the 4-methylbenzoate-specific formation of a putative 4-methylcyclohex-1,5-diene-1-carboxylate and 3-methylglutarate, which taken together suggest conservation of the *para*-methyl group beyond dearomatization and ring fission. In agreement, only anaerobically 4-methylbenzoate-adapted cells showed *in vitro* a high ATP-dependent conversion of 4-methylbenzoyl-CoA to 4-methyl-dienoyl-CoA. Protein sequence comparison of MbrBCAD with conventional class I BCRs revealed remarkable sequence differences and suggested the discovery of a novel subclass of class I BCRs. The identification of two 4-methylbenzoate-specific gene clusters in a shotgun sequence database and their functional analysis agree with a specific 4-methylbenzoyl-CoA pathway. The proposed initial reaction sequences are similar to the anaerobic benzoyl-CoA pathway. However, strain pMbN1 apparently employes reactions similar to the leucine/isovalerate pathway to degrade 3-methylglutarate. In accordance, the transcript of a putative 3-hydroxy-3-methylglutaryl-CoA lyase (*orf460*) was significantely increased during anaerobic growth with 4-methylbenzoate.

(3) Anaerobic growth and substrate preferences of strain pMbN1 were studied in mixtures of two or three different substrates, using cells adapted to one of six different aliphatic acids or 4-methylbenzoate. Anaerobically 4-methylbenzoate-adapted cells displayed diauxic growth in a ternary mixture of 4-methylbenzoate, benzoate and succinate, due to a preference for benzoate. Diauxic growth with preferred utilization of benzoate was also observed in binary mixtures comprising an aliphatic acid (succinate, L-malate, fumarate, oxaloacetate or pyruvate) and benzoate. On the other hand, succinate was co-utilization or preferred from mixtures with other aromatic acids

(phenylacetate or 4-hydroxybenzoate, respectively). Diauxic growth and preferred utilization of benzoate (or phenylacetate) was also observed in anaerobic 4-methylbenzoate-adapted cells grown with a binary mixture of 4-methylbenzoate and benzoate (or phenylacetate). In most cases, substrate preferences were uncoupled from growth rates. Transcript analysis and differential protein profiles from the ternary mixture revealed dynamic abundance changes of transcripts and proteins related to the uptake and catabolism of the respective substrates. Utilization of benzoate in the first active growth phase was paralleled by maximal abundances of proteins and transcripts related to the anaerobic catabolism of benzoate (e.g. BcrBCAD) and minimal abundances of transcript and proteins related to the uptake (e.g. DctP, Orf870) and catabolism of succinate (e.g. PdhB) and 4-methylbenzoate (e.g. MclA). Upon benzoate depletion, succinate and 4-methylbenzoate related transcripts and proteins increased and reached maximal values at the end of the diauxic lag phase and/or in the second active growth phase. The results suggested benzoate-mediated transcriptional repression of genes related to uptake and catabolism of 4-methylbenzoate paralleled by unchanged concentrations of 4-methylbenzoate in the medium during the benzoate utilization phase. On the other hand, benzoate seemed to have only a minor effect on succinate uptake indicated by only marginal decreased protein and transcript levels compared to benzoate- or 4-methylbenzoate-adapted cells. This coincided with a decrease of external succinate already during the benzoate utilization phase. However, strongly decreased protein and transcript level during the benzoate utilization phase suggests a direct or indirect effect of benzoate on pyruvate dehydrogenase gene expression, which might be responsible for the observed diauxie in mixtures of aliphatic acids and benzoate.

The results described in this dissertation also include contributions from cooperation partners published in joint papers. Instead of individual citations, my personal contribution is described on page 8 - 9.

ZUSAMMENFASSUNG

Der aromatische Ring ist eine der häufigsten chemischen Strukturen unter natürlich vorkommenden Verbindungen. Die Resonanz-stabilisierte Ringstruktur aromatischer Verbindungen macht diese Klasse organischer Verbindungen besonders inert gegenüber (bio-)chemischen Reaktionen und trägt zusammen mit der Toxizität vieler aromatischer Verbindungen zu deren Persistenz in der Umwelt bei. Aerobe Mikroorganismen verwenden hoch-reaktive Sauerstoff-Spezies (aus O_2) zur Aktivierung und Öffnung des aromatischen Ringes. Jedoch herrschen in vielen Habitaten (z.B. Süßwasser und marine Sedimente oder Grundwasserleitern) durch raschen Verbrauch von Sauerstoff vornehmlich anoxische Bedingungen. Dies macht den anaeroben Abbau aromatischer Verbindungen zu einem wichtigen Umweltprozess. In den vergangenen Jahren konnten biochemische Strategien zur Aktivierung und Abbaus aromatischer Verbindungen unabhängig von Sauerstoff aufgedeckt werden. In Analogie zu aeroben Bakterien, überführen auch die anaeroben die große Vielzahl verschiedener aromatischer Verbindungen in wenige zentrale aromatische Zwischenprodukte. Von besonderer Bedeutung ist der anaerobe Benzoyl-CoA Abbauweg. Von zentraler Bedeutung für diesen Stoffwechselweg ist die reduktive Dearomatisierung durch Benzoyl-CoA Reduktasen (BCR). Fakultativ und obligat anaerobe Bakterien verwenden ATP-abhängige (Klasse I) beziehungsweise -unabhängige (Klasse II) Enzyme. Im Gegensatz zur Klasse II BCR (strikte Anaerobier) ist der Mechanismus des Klasse I Enzymes bereits weitgehend bekannt. Allerdings sind bekannte Enzyme der Klasse I nicht in der Lage, *para*-substituierte Benzoyl-CoA Analoga (z.B. 4-Methylbenzoyl-CoA) umzusetzen.

In der vorliegenden Studie wurden physiologische und molekulare Ansätze verwendet um **(1)** den Stamm pMbN1 und seine Fähigkeit zum anaeroben Abbau von Benzoat und 4-Methylbenzoat zu charakterisieren, **(2)** die anaeroben Abbauwege von Benzoat und 4-Methylbenzoat aufzudecken und **(3)** den Einfluss aliphatischer und aromatischer Säuren auf den anaeroben Abbau von Benzoat und 4-Methylbenzoat in binären und ternären Substratgemischen zu untersuchen.

(1) Isoliert wurde Stamm pMbN1 aus Süßwasser-Sedimenten; phylogenetisch gehört er zur Gattung *Magnetospirillum* der *Alphaproteobacteria*. Stamm pMbN1 ist ein heterotrophes, mesophiles und fakultativ anaerobes (Denitrifikation) Bakterium.

Trotz Zugehörigkeit zu der Gattung *Magnetospirillum*, zeigte Stamm pMbN1 keine Magnetotaxis. Insgesamt 26 (13 aromatische) organische Substrate unterstützen das anaerobe Wachstum von Stamm pMbN1. Hingegen wurde mit nur fünf der aromatischen Verbindungen aerobes Wachstum beobachtet. Dies deutet auf eine Spezialisierung von Stamm pMbN1 auf anaerobes Wachstum mit aromatischen Verbindungen hin. Benzoat sowie 4-Methylbenzoat wurden anaerob vollständig zu CO_2 umgesetzt. Die höhere Wachstumsrate und molare Wachstumsausbeute mit Benzoat, verglichen mit 4-Methylbenzoat könnten eine höhere Effizienz des anaeroben Benzoat-Abbaus implizieren.

(2) Anpassungsstudien deuteten auf eine Substrate-spezifische Induktion des anaeroben Benzoat- bzw. 4-Methylbenzoat-Abbaus hin. Das differenzielle Protein Profil (2D-DIGE) 4-Methylbenzoat-angepasster Zellen zeigte die spezifische Bildung einer Gruppe von Proteinen, welche in Benzoat- oder Succinat-angepassten Zellen fehlten. In dieser Gruppe konnte unter anderem eine mögliche 4-Methylbenzoyl-CoA Reduktase (MbrBCAD) sowie ein Enzymsatz der modifizierten β-Oxidation (Dch-2, Oah-2 und Had-2) identifiziert werden, welche sich von denen des anaeroben Benzoat-Abbaus unterschieden. In anaerob 4-Methylbenzoat-gewachsenen Zellen wurden 4-Methylcyclohex-1,5-Diene-1-Carboxylat sowie 3-Methylglutarat als mögliche Intermediate nachgewiesen. Dies spricht für eine Konservierung der *para*-Methylgruppe über die Dearomatisierung und Ringspaltung hinaus. Zusätzlich wurde eine hohe *in vitro* Aktivität der 4-Methylbenzoyl-CoA Reduktase und die ATP-abhängige Umsetzung zu 4-Methyl-Dienoyl-CoA ausschließlich in 4-Methylbenzoat-angepassten Zellen nachgewiesen. Die deutlichen Sequenzunterschiede zwischen MbrBCAD und konventionellen Klasse I BCRs deuten auf eine neue Untergruppe von Klasse I Enzymen hin. Die Identifizierung zweier 4-Methylbenzoate-spezifischer Gencluster in einer Shotgun-Sequenz Datenbank sowie deren funktionelle Vorhersage steht im Einklang mit einem spezifischen 4-Methylbenzoyl-CoA Abbauweg. Die vorgeschlagenen initialen Reaktionen verlaufen in Analogie zum anaeroben Benzoyl-CoA-Abbau. Beim Abbau von 3-Methylglutarat scheint Stamm pMbN1 jedoch Reaktionen zu verwenden, die denen des Leucin/Isovalerat-Abbaus ähneln. Tatsächlich zeigte das Transkript einer möglichen 3-Hydroxy-3-Methylglutaryl-CoA Lyase (*orf460*) eine signifikant erhöhte Abundanz in 4-Methylbenzoat-gewachsenen Zellen.

(3) Anaerobes Wachstum und Substratpreferenzen des Stammes pMbN1 wurden in Gemischen mit zwei oder drei verschiedenen Substraten untersucht. Die verwendeten

Zellen wurden an eine von sechs verschiedenen aliphatischen Säuren oder 4-Methylbenzoat angepasst. Anaerob 4-Methylbenzoat-angepasste Zellen zeigten diauxisches Wachstum mit einer Präferenz für Benzoat in einem ternären Gemisch aus 4-Methylbenzoat, Benzoat und Succinat. Ein präferenzieller Verbrauch von Benzoat wurde ebenfalls in binären Gemisch einer aliphatischen Säure (Succinat, L-Malat, Fumarat, Oxaloacetat oder Pyruvat) und Benzoat beobachtet. Auf der anderen Seite wurde Succinat in binären Gemischen mit anderen aromatischen Säuren entweder gemeinsam verbraucht (Phenylacetat) oder präferiert (4-Hydroxybenzoat). Eine Bevorzugung von Benzoat bzw. Phenylacetat und diauxisches Wachstum wurde auch in anaerob 4-Methylbenzoat-angepassten Zellen beobachtet, denen ein binäres Gemisch aus 4-Methylbenzoat und Benzoat bzw. Phenylacetat angeboten wurde. In den meisten Fällen waren die beobachteten Substratpräferenzen unabhängig von den Wachstumsraten der Einzelsubstrate. Differentielle Transkript- und Proteinprofile des ternären Gemisches belegten dynamische Abundanzänderungen von Proteinen für Aufnahme und Abbaus der einzelnen Substrate. Der Verbrauch von Benzoat während der ersten aktiven Wachstumsphase ging einher mit maximalen Abundanzen von Transkripten und Proteinen des Abbaus von Benzoat (z.B. BcrBCAD), wohingegen minimale Abundanzen für Transkripte und Proteine der Aufnahme (z.B. DctP, Orf870) und des Abbaus von Succinat (e.g. PdhB) und 4-Methylbenzoat (e.g. MclA) beobachtet wurden. Nachdem Benzoat vollständig verbraucht war, stiegen die Abundanzen der Succinat- und 4-Methylbenzoat-spezifischen Transkripte und Proteine stark an und erreichten maximale Werte am Ende der diauxischen Lagphase und/oder in der zweiten aktiven Wachstumsphase. Die Ergebnisse deuten auf eine durch Benzoat vermittelte Repression der Transkription von Genen für die Aufnahme und den Abbaus von 4-Methylbenzoat hin. Dies korreliert mit einem konstanten 4-Methylbenzoatgehalt im Medium während der anfänglichen Benzoatverbrauchsphase. Im Gegensatz dazu deuten die nur gering reduzierten Protein- und Transkriptabundanzen verglichen mit Benzoat- oder 4-Methylbenzoat-angepassten Zellen auf eine weniger starke Repression der Succinataufnahme hin. Dies korreliert zudem mit einem geringen Verbrauch von Succinat schon während der Benzoatverbrauchsphase. Die geringen Protein- und Trankriptabundanzen während der Benzoatverbrauchsphase implizieren möglicherweise einen direkten oder indirekten Einfluss von Benzoat auf die Genexpression der Pyruvat Dehydrogenase. Dies könnte die Ursache für die beobachtete Diauxie in Gegenwart von Gemischen aus aliphatischen Säuren mit Benzoat sein.

Die in dieser Studie beschriebenen Ergebnisse beinhalten auch Beiträge anderer Kooperationspartner aus gemeinsamen Publikationen. Mein persönlicher Beitrag wird auf den Seiten 8 – 9 erläutert, anstelle von Einzelnachweisen im Text.

LIST OF PUBLICATIONS

This dissertation comprises three research articles; two of which are published and one is in preparation. My contribution to the manuscripts is outlined below.

Research articles

1. **Anaerobic degradation of 4-methylbenzoate by a newly isolated denitrifying bacterium, strain pMbN1**

   Sven Lahme, Jens Harder, and Ralf Rabus

   Applied and Environmental Microbiology (2012) **78** (5): 1606-1610

   Performance of physiological experiments (together with Daniela Lange), analytical methods (HPLC, GC), electron microscopy and image analysis (together with Erhard Rhiel). Data analysis, experimental design and manuscript preparation with Ralf Rabus and Jens Harder. Sequence analysis was performed by Michael Kube and Richard Reinhardt. Phylogenetic analysis was performed by Jens Harder. Strain pMbN1 was enriched by Ralf Rabus and isolated by Christina Probian and Ramona Appel. Dirk Schüler tested for magnetotaxis.

2. **Anaerobic degradation of 4-methylbenzoate via a specific 4-methylbenzoyl-CoA pathway**

   Sven Lahme, Christian Eberlein, René Jarling, Michael Kube, Matthias Boll, Heinz Wilkes, Richard Reinhardt, and Ralf Rabus

   Environmental Microbiology (2012) **14** (5): 1118–1132

   Performance of physiological experiments and HPLC analysis, metabolite extractions (together with Daniela Lange), 2D-DIGE, 2DE (together with Daniela Lange), phylogenetic analysis, sample preparation for protein analysis (together with Daniela Lange) and enzymatic assays, data analysis, experimental design and manuscript preparation with Ralf Rabus. René Jarling and Heinz Wilkes performed metabolite analysis. Christian Eberlein and

Matthias Boll performed enzyme assays with benzoyl-CoA reductases. Michael Kube and Richard Reinhardt performed sequencing.

3. *Magnetospirillum* sp. pMbN1 prefers benzoate from binary and ternary substrate mixtures during anaerobic growth

Sven Lahme, Kathleen Trautwein, Annemieke Strijkstra, Marvin Dörries, Lars Wöhlbrand, and Ralf Rabus

Manuscript in preparation

Performance of physiological experiments (together with Kathleen Trautwein, Annemieke Strijkstra and Daniela Lange) and HPLC analysis (together with Marvin Dörries and Christina Hinrichs), sample preparation for RNA and proteomic analysis (together with Kathleen Trautwein and Daniela Lange), reverse transcription real-time PCR, data analysis (except 2D-DIGE), experimental design and manuscript preparation with Kathleen Trautwein and Ralf Rabus. Kathleen Trautwein performed 2D-DIGE and 2DE experiments and analysis. Lars Wöhlbrand performed protein identification by MALDI-TOF MS/MS and LC-ESI MS/MS. Daniela Lange prepared SDS-PAGE with membrane protein-enriched fraction.

Abbreviations

2D-DIGE	Two-dimensional difference gel electrophoresis
AC	Adenylate cyclase
BCR	Benzoyl-CoA reductase
BTEX	Benzene, toluene, ethylbenzene and xylenes
CCR	Carbon catabolite repression
CoA	Coenzyme A
FAD	Flavin adenine dinucleotide
GC-MS	Gas chromatography mass spectrometry
HPLC	High pressure liquide chromatography
PEP	Phosphoenolpyruvate
PET	Polyethylene terephthalate
PHA	Polyhydroxyalkanoates
PTS	PEP-Phosphotransferase system
RTqPCR	Reverse transcription real-time polymerase chain reaction
TCA	Tricarboxylic acid
THNCoA	5,6,7,8-Tetrahydro-2-naphthoyl-CoA
TRAP	Tripartite ATP-independent periplasmic (transporter)
$Y_{X/S}$	Molar growth yield calculated per substrate consumed
$Y_{X/C}$	Molar growth yield calculated per carbon consumed
μ_{max}	Maximum specific growth rate

Part I: Presentation of the results in the overall context

A INTRODUCTION

1. Properties and environmental distribution of aromatic compounds

1.1 Physico-chemical characteristics of monocyclic aromatic compounds

The benzene ring (1,3,5-cyclohexatriene) represents the simplest aromatic compound, with a planar structure due to a fully conjugated double bond system (Fig. 1A). Benzene consists exclusively of carbon and hydrogen atoms and the low difference in electronegativity between carbon (2.55) and hydrogen (2.20) results in apolar bonds. Taken together, symmetric structure, resonance stabilization and apolar bonds yield the extraordinary stability of this molecule. Any (bio-)chemical reaction at the aromatic ring must overcome the high resonance stabilization (~151 kJ mol^{-1} for benzene) (Wilkes and Schwarzbauer 2010). Substitutions at the ring (e.g. carboxyl-, hydroxyl-, amino- or alkyl-moieties) increase the structural diversity of aromatic compounds, and may also distort the delocalized π-electron system (Möbitz and Boll 2002; Wilkes and Schwarzbauer 2010). The latter promote the biochemical reactivity (Fig. 1B). Moreover, polar groups (e.g. carboxyl or hydroxyl) increase the water solubility of the molecules, and thereby the bioavailability.

1.2 Sources of aromatic compounds in the environment

Aromatic compounds are highly diverse and abundant in nature, mainly due to their role as building blocks of biomacromolecules (e.g. lignin) (Leeuw *et al*. 2006) and proteins as well as their abundance in crude oil (Tissot and Welte 1984). Considering their abundance and energy-richness aromatic compounds are attractive substrates for aerobic and anaerobic microorganisms. Despite this, the capacity to degrade aromatic compounds is mainly restricted to aerobic and anaerobic bacteria (see chapter 2 and 3). In the environment aromatic compounds can be of biological, geological or anthropogenic origin.

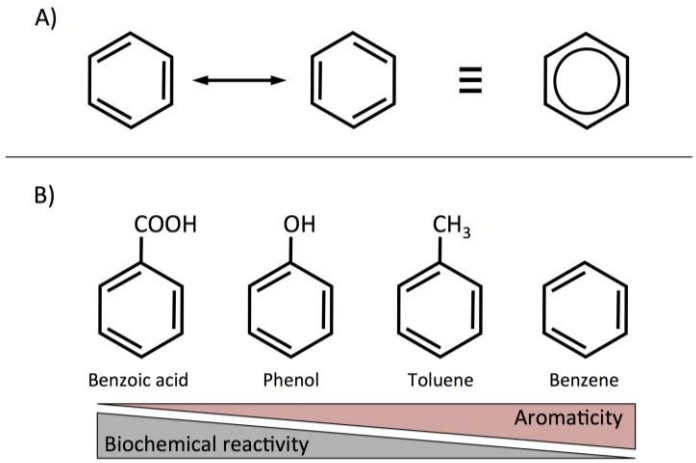

Fig. 1 The special character of aromatic compounds. A) Resonance structures of benzene (resonance between two structures is indicated by a double arrow). The aromaticity in benzene rings may also be depicted by an inner circle (modified from Wilkes and Schwarzbauer 2010). B) Relationship between ring substituents, aromaticity and assumed biochemical reactivity.

1.2.1 Biological formation

Aromatic compounds are important constituents of biomass and are produced via various biosynthetic routes. The most important one is the shikimate pathway. Here the precursor chorismate is generated from one molecule D-erythrose-4-phosphate and two molecules phosphoenolpyruvate (for a review see Maeda and Dudareva 2012). Specific biosynthetic pathways convert chorismate into L-phenylalanine, L-tyrosine and L-tryptophane, respectively. The shikimate pathway is present in plants, bacteria and fungi, but not in higher animals (Maeda and Dudareva 2012). Starting from the three aromatic amino acids, plants can produce a large variety of aromatic secondary metabolites such as flavonoids, alkaloids and polyphenolic proanthrocyanidins (tannins), as well as diverse 3-phenylpropanoids (from phenylalanine) as precursors of the heteropolymer lignin (for an overview see Singer *et al*. 2003).

Next to cellulose, lignin is one of the most abundant terrestrial biopolymer, accounting for about one third of the total organic carbon in the biosphere (Boerjan *et al*. 2003). The phenylpropanoids (4-hydroxyphenyl, guaiacyl and syringyl phenyl-propanoids) are usually connected at the β-position of the alkyl side-chain in the lignin polymer (Boerjan *et al*. 2003). The β-O-4 (β-aryl ether) linkage is the most frequent

chemical bond present in hard- and softwood (up to 80%) (Achyuthan *et al*. 2010). Lignin biodegradation is mainly performed by fungi, which employ several extracellular oxidative enzymes (e.g. laccases and lignolytic peroxidases). Their catalytical key feature is the formation of reactive molecules (e.g. H_2O_2, Mn^{3+}) to initially attack the stable lignin polymer (Bugg *et al*. 2011).

1.2.2 Geochemical formation and anthropogenic input

Aromatic hydrocarbons are prominent constituents of crude oil, with the aromatic fraction representing about 28% of the total hydrocarbons (Tissot and Welte 1984). Crude oil and natural gas are formed from organic matter buried deeply in marine sediments, forming a so-called source-bed. Geochemical hydrocarbon formation requires timescales of several million up to several hundred million years (Tissot and Welte 1984). During the first few thousand years after burial, most of the organic matter is anaerobically biotransformed at temperatures <50°C, including depolymerization and removal of heteroatoms (oxygen, sulfur and nitrogen). Further biodegradation and other abiotic reactions (e.g. polymerization or free-radical reactions) yield kerogen, a structurally complex bio- and geopolymer (Libes 2009). At ~3–5 km depth the kerogen is thermogenically (60–200°C) transformed into natural gases (e.g. methane, ethane, propane) and crude oil (aliphatic and aromatic hydrocarbons). Petroleum reservoirs form at locations where hydrocarbon migration is hindered by impermeable layers forming a cap rock (Libes 2009). Petroleum hydrocarbons can enter the biosphere via natural seeps (e.g. cold seeps and asphalt pits), but also anthropogenic activities (e.g. production and transport accidents) introduce hydrocarbons into the environment. It is estimated that about 1.3–8.8 million tons of petroleum hydrocarbons enter marine environments per year (Berthe-Corti and Nachtkamp 2010). E.g. in April 2010 an estimate of 4.9 million barrel crude oil were released into the Gulf of Mexico by the Deepwater Horizon accident (http://www.noaa.gov).

Aromatic hydrocarbons are also important starting materials for the chemical industry. A very prominent example is terephthalic acid derived from *p*-xylene. Terephthalic acid is the basis for the polyethylene terephthalate (PET) production. The wastewaters from such petrochemical plants contain high amounts of diverse aromatic compounds (e.g. terephthalic acid, benzoic acid, 4-methylbenzoic acid) (Macarie *et al*.

1992; Zhang *et al*. 2006). E.g. 4-methylbenzoic acid can reach concentrations of up to 0.5 g/l in petrochemical wastewaters and was proven to be particularly recalcitrant to biodegradation (Kleerebezem *et al*. 1999).

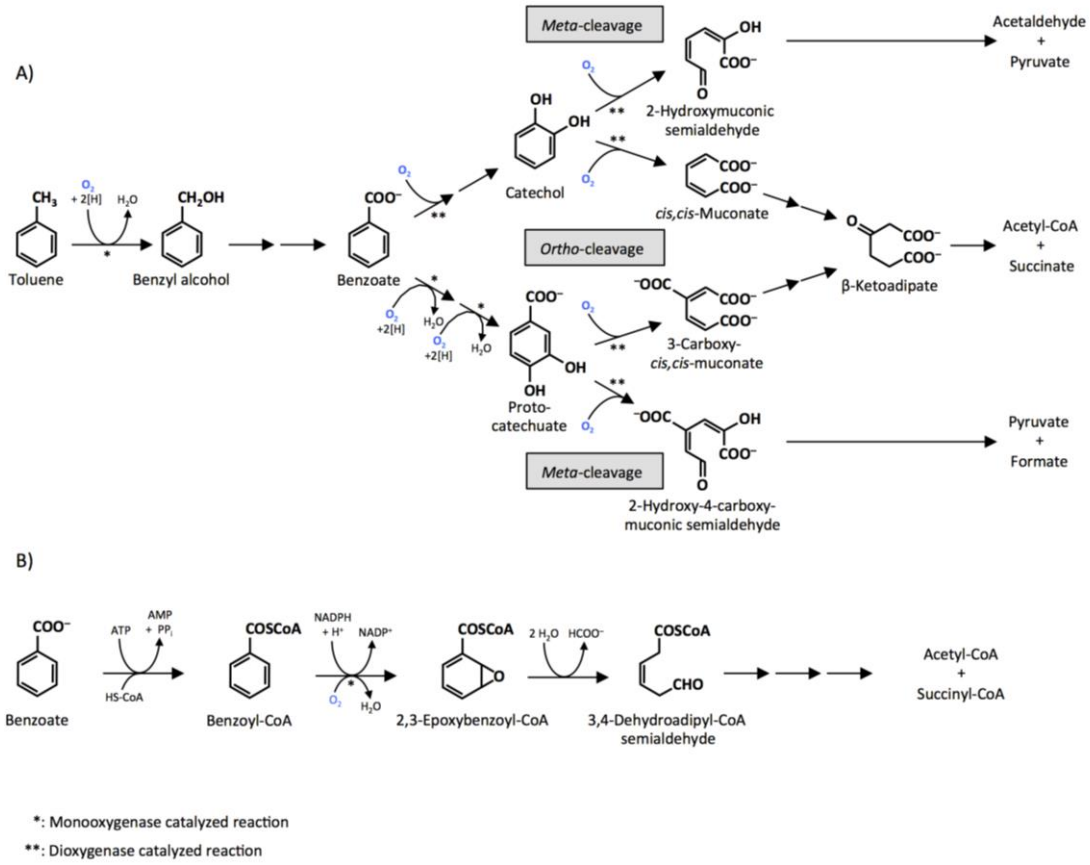

Fig. 2 The aerobic degradation pathways of aromatic compounds. A) Conversion of toluene to benzoate and *meta*- and *ortho*-cleavage of catechol and protocatechuate. B) Aerobic degradation of benzoate by activation to benzoyl-CoA, epoxidation and subsequent hydrolytic cleavage of the ring (modified from Pérez-Pantoja *et al*. 2010; Fuchs *et al*. 2011).

2. Aerobic degradation of monocyclic aromatic compounds

The ability of fungi, yeast and bacteria to degrade monocyclic aromatic compounds by employing highly reactive oxygen species (O_2-derived) has been known since many years (for reviews see Pérez-Pantoja *et al*. 2010; Widdel and Musat 2010b; Fuchs *et al*. 2011). The benzene ring is first activated and then cleaved by specific mono- and dioxygenases (Fig. 2A) (Boyd *et al*. 2001; Ullrich and Hofrichter 2007;

Pérez-Pantoja *et al*. 2010). Oxygenases reductively activate the inert triplet oxygen ($^3O_2$) to form reactive oxygen species (e.g. superoxide or hydrogen peroxide). The latter can react with the inert C–H bonds of the aromatic ring. Catalysis of most oxygenases requires metal cofactors (mostly iron(II) or iron(III)), but some contain a flavin cofactor instead (Bugg 2003; Widdel and Musat 2010b). While dioxygenases incorporate both atoms of the dioxygen into the reactant, monooxygenases incorporate only one atom and reduce the second one to H_2O. Only recently, so-called hybrid-pathways were discovered, where coenzyme A (CoA) thioesters of aromatic compounds are activated by oxygenases, while the ring is non-oxygenolytically cleaved (Fig. 2B) (for a review see Fuchs *et al*. 2011).

Aerobic microorganisms use an array of different peripheral pathways to funnel the great diversity of aromatic compounds into only a few central intermediates. The main intermediates are e.g. catechol (1,2-dihydroxybenzene) or protocatechuate (3,4-dihydroxybenzoate), which are "activated" and prepared for oxidative ring-cleavage by dioxygenases. The ring opening can occur adjacent to the hydroxyl groups (*meta*-position, extradiol) or between them (*ortho*-position, intradiol) (Fig. 2A). The ring cleavage products are then converted into central metabolic intermediates by β-oxidation reactions (for reviews see Pérez-Pantoja *et al*. 2010; Fuchs *et al*. 2011).

Methylated catchols, from e.g. the aerobic degradation of *p*-xylene or 4-methylbenzoate, are mainly channeled through the *meta*-cleavage pathway, to avoid the possible formation of dead-end products in the *ortho*-cleavage pathway (Pérez-Pantoja *et al*. 2010). Alternative aerobic pathways are used for the degradation of e.g. gentisate, homogentisate, hydroxybenzoquinol, homoprotocatechuate and gallate. These pathways operate according to the same biochemical principles with respect to O_2-dependent ring activation, oxygenolytic ring cleavage and subsequent β-oxidation (for an overview see Pérez-Pantoja *et al*. 2010).

The ability to use aromatic compounds as substrate under oxic conditions is widespread among bacteria. Members of the Gram-positive Firmicutes (e.g. *Bacillus*) and Acinetobacteria (e.g. *Arthrobacter*, *Corynebacterium*, *Norcadia*), as well as members of the alpha- (e.g. *Rhizobium*, *Bradyrhizobium*), beta- (e.g. *Alcaligenes*, *Comamonas*) and gamma-subgroup (e.g. *Pseudomonas*, *Acinetobacter*) of *Proteobacteria* possess this capacity (for an overview see Prince *et al*. 2010).

Table 1 Free energy of the complete oxidation of benzoate coupled to various electron acceptors.

Reaction(s):			Free energy change ($\Delta G^{0'}$) [kJ mol^{-1}][a]
$C_7H_5O_2^-$ + 7.5 O_2 + 4 H_2O	→	7 HCO_3^- + 6 H^+	–3169
$C_7H_5O_2^-$ + 6 NO_3^- + H_2O	→	7 HCO_3^- + 3 N_2	–2973
$C_7H_5O_2^-$ + 15 MnO_2 + 24 H^+	→	7 HCO_3^- + 15 Mn^{2+} + 11 H_2O	–1968
$C_7H_5O_2^-$ + 30 $Fe(OH)_3$ + 23 HCO_3^- + 24 H^+	→	30 $FeCO_3$ + 71 H_2O	–1531
$C_7H_5O_2^-$ + 3.75 SO_4^{2-} + 1.5 H^+ + 4 H_2O	→	7 HCO_3^- + 3.75 H_2S	–182

[a] Calculation of free energy ($\Delta G^{0'}$) is based on standard values (Widdel and Musat 2010a).

3. Anaerobic degradation of monocyclic aromatic compounds

Due to the lack of or rapid depletion of molecular oxygen, many habitats (e.g. freshwater and marine sediments, groundwater aquifers or deep soil layers) are dominated by anoxic conditions. Therefore, anaerobic bacteria have to employ different, O_2-independent biochemical strategies to degrade aromatic compounds. Like aerobic strategies, the anaerobic ones also involve multiple diverse peripheral (upper) pathways, channeling the great structural diversity of aromatic compounds into only a few central (lower) pathways. A prominent example is the central anaerobic benzoyl-CoA pathway (for a review see Fuchs *et al*. 2011). Here, the CoA-thioester moiety of benzoyl-CoA facilitates the reductive dearomatization of the aromatic ring (Buckel and Keese 1995; Boll *et al*. 2000b; Möbitz and Boll 2002; Boll 2005a). Further central pathways involve the key intermediates resorcinol (1,3-dihydroxybenzene) and phloroglucinol (1,3,5-trihydroxybenzene). The hydroxyl groups *meta* positioned to each other, facilitate non-aromatic mesomeric structures, which can be easily reduced by conventional ferredoxin- or NAD(P)H-dependent reductases (for an overview see Schink *et al*. 2000; Philipp and Schink 2012).

3.1 Organisms

The energy yield from the degradation of aromatic compounds depends not only on their "energy content", but also on the electron acceptor used by microorganisms for

growth. Next to oxygen, most energy is generated with a coupling to nitrate reduction (Table 1). Other alternative electron acceptors used by microorganisms are manganese (IV), ferrous iron and sulfate, which are, however, less thermodynamically favorable electron acceptors as compared to nitrate. The smallest energy yield is obtained with sulfate (Table 1), resulting also in low growth rates (Widdel *et al*. 2010).

Most studies on anaerobic degradation of monocyclic aromatic hydrocarbons were conducted with nitrate- or sulfate-reducing bacteria isolated from marine or fresh water sediments. The majority of these strains phylogenetically affiliate with the *Proteobacteria* and denitrifiers in most cases with the *"Aromatoleum"/Azoarcus/Thauera*-cluster within *Betaproteobacteria* (Widdel *et al*. 2010). But also *Magnetospirillum* species (*Alphaproteobacteria*) degrading aromatic compounds under nitrate-reducing conditions have been described (Shinoda *et al*. 2000, 2005). Most of the 71 known anaerobic aromatic hydrocarbon-degrading bacteria can utilize toluene (66 isolates) as carbon and energy source (Widdel *et al*. 2010 and references within; Alain *et al*. 2012). Other BTEX compounds less frequently support anaerobic growth. Only 22 isolates are described to utilize *m*-xylene, five ethylbenzene, two *o*-xylene (Widdel *et al*. 2010 and references within; Alain *et al*. 2012) and only one *p*-xylene (Higashioka *et al*. 2012). Apparently, none of these isolates, including the *p*-xylene-degrading sulfate-reducer strain PP31, have been described to degrade 4-methylbenzoate, the key-substrate of the present thesis.

3.2 Anaerobic peripheral degradation pathways

Facultative anaerobic bacteria have evolved a range of conspicuous reactions for (i) O_2-independent initial activation of monocyclic aromatic hydrocarbons, (ii) conversion of the initial intermediate to only a very few central intermediates (mostly benzoyl-CoA) and (iii) reductive dearomatization and hydrolytic ring cleavage.

Addition to fumarate. The first step in the anaerobic degradation of toluene is the addition to fumarate yielding (*R*)-benzylsuccinate (Fig. 3). This reaction was originally demonstrated in *Thauera aromatica* K172 (Biegert *et al*. 1996) and subsequently confirmed for all known anaerobic toluene degraders (for reviews see Boll *et al*. 2002; Heider 2007). Moreover, the addition to fumarate is now regarded as a common principal for the activation of diverse recalcitrant compounds: xylenes

(Krieger *et al*. 1999; Morasch *et al*. 2004; Morasch and Meckenstock 2005; Rotaru *et al*. 2010), ethylbenzene (Kniemeyer *et al*. 2003), cresols (Müller *et al*. 1999; Müller *et al*. 2001), methylnaphthalene (Annweiler *et al*. 2000; Musat *et al*. 2009), cyclohexane (Musat *et al*. 2010) and *n*-alkanes (Rabus *et al*. 2001).

The biochemical and mechanistic understanding is most advanced in case of the toluene-activating, glycyl-radical bearing benzylsuccinate synthase (BssABC). The latter requires the activase BssD (Leuthner *et al*. 1998). The product (*R*)-benzylsuccinate is subsequently converted into benzoyl-CoA and succinyl-CoA by a modified β-oxidation reaction sequence (Fig. 3) (Leuthner and Heider 2000; Leutwein and Heider 2001, 2002). The liberated succinyl-CoA can be regenerated to fumarate. The genes coding for the benzylsuccinate synthase (*bss*) and the proteins of the subsequent β-oxidation (*bbs*) are usually organized in two distinct neighboring clusters (Kube *et al*. 2004).

Oxygen-independent hydroxylation. An oxygen-independent hydroxylation at the methyl group of *p*-cresol was originally observed under aerobic (Hopper 1978) and later also under anaerobic (nitrate-reducing) conditions (Bossert and Young 1986; Hopper *et al*. 1991; Rudolphi *et al*. 1991). This reaction is catalyzed by the *p*-cresol methyl-hydroxylase (PchCF) and produces 4-hydroxybenzyl alcohol, which is further oxidized by the 4-hydroxybenzaldehyde dehydrogenase (PchA) to 4-hydroxybenzoate (Fig. 3).

Another more recently discovered oxygen-independent hydroxylation occurs with ethylbenzene in the denitrifying strains EbN1 and EB1 (Fig. 3) (Ball *et al*. 1996; Rabus and Heider 1998; Kniemeyer and Heider 2001a; Johnson *et al*. 2001). The highly oxygen sensitive periplasmic ethylbenzene dehydrogenase (EbdABC) of strain EbN1 belongs to the family of molybdenum cofactor containing dimethylsulfoxide reductases (Kniemeyer and Heider 2001a; Kloer *et al*. 2006). Further degradation of the product 1-(*S*)-phenylethanol to acetophenone is catalyzed by the 1-(*S*)-phenylethanol dehydrogenase (Ped) (Kniemeyer and Heider 2001b; Höffken *et al*. 2006). In strain EbN1, the gene for the latter one co-localizes with those of ethylbenzene dehydrogenase, whereas the genes for conversion of acetophenone to benzoylacetyl-CoA are forming a disparate cluster (Rabus *et al*. 2002). Thiolytic cleavage of benzoylacetyl-CoA yields acetyl-CoA and benzoyl-CoA (Fig. 3). The latter one can be degraded through the central anaerobic benzoyl-CoA pathway.

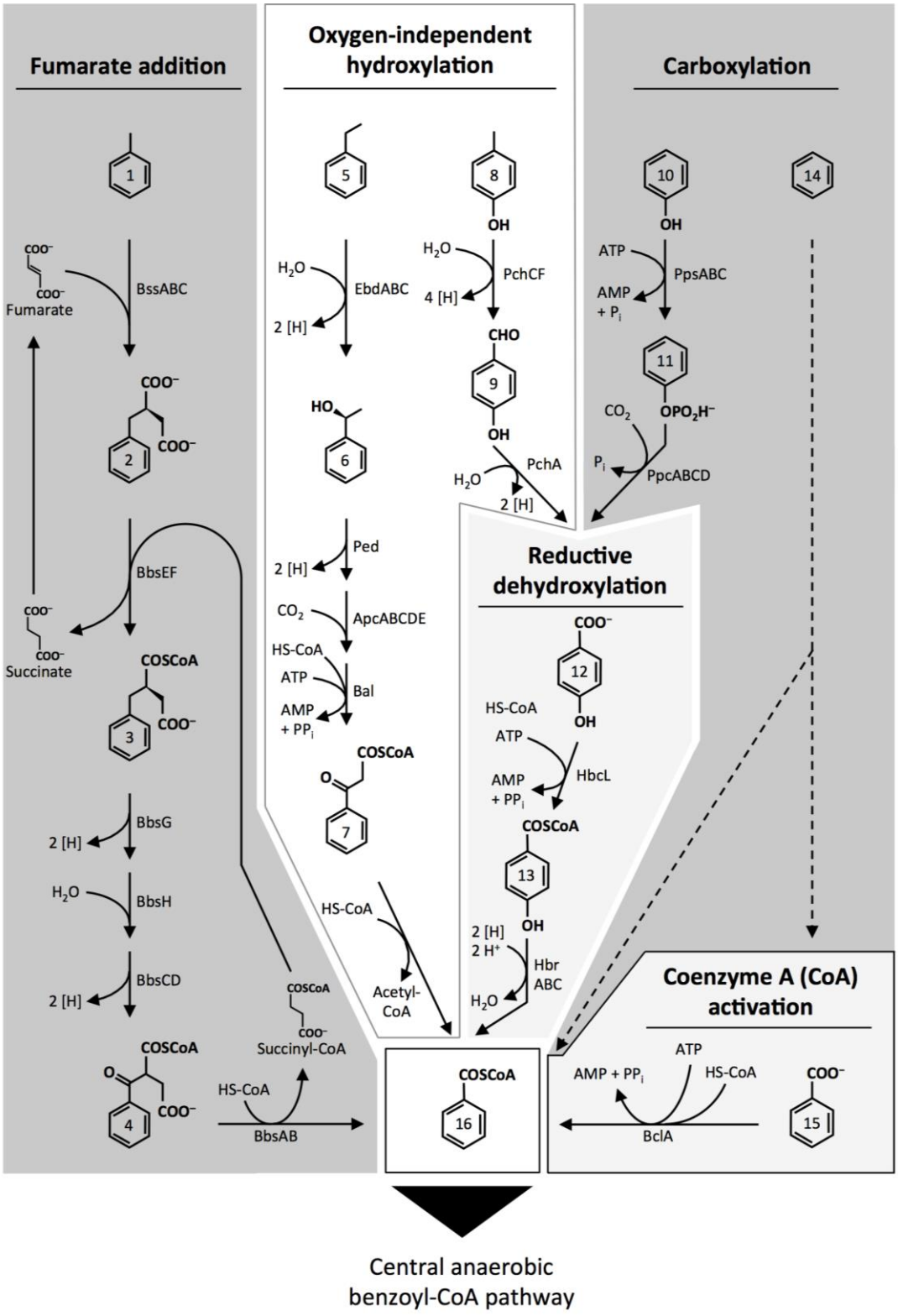

Fig. 3 Anaerobic peripheral pathways for the activation and degradation of aromatic compounds. The pathways involve examples of fumarate addition, oxygen-independent hydroxylation, carboxylation, reductive dehydroxylation and coenzyme A (CoA) activation of benzoate. All compounds given are channeled into the central anaerobic benzoyl-CoA pathway (for further information see chapter 3.2). The dashed line, associated with the activation of benzene indicates, that the biochemistry is not understood yet. Compound names: (1) toluene, (2) (*R*)-benzylsuccinate, (3) (*R*)-benzylsuccinyl-CoA, (4) benzoylsuccinyl-CoA, (5) ethylbenzene (6) 1-(*S*)-phenylethanol, (7) benzoylacetyl-CoA, (8) *p*-cresol, (9) 4-hydroxybenzaldehyde (10) phenol, (11) phenylphosphate, (12) 4-hydroxybenzoate, (13) 4-

hydroxybenzoyl-CoA, (14) benzene, (15) benzoate, (16) benzoyl-CoA. Enzyme abbreviations (in alphabetic order): (ApcABCDE) acetophenone carboxylase, (Bal) benzoylacetate-CoA ligase, (BbsAB) benzoylsuccinyl-CoA thiolase, (BbsCD) 2-[hydroxy-(phenyl)methyl]-succinyl-CoA dehydrogenase, (BbsEF) succinyl-CoA:(*R*)-benzylsuccinate CoA-transferase, (BbsG) (*R*)-benzylsuccinyl-CoA dehydrogenase, (BbsH) phenylitaconyl-CoA hydratase, (BclA) benzoate CoA-ligase, (BssABC) benzylsuccinate synthase, (EbdABC) ethylbenzene dehydrogenase, (HbcL) 4-hydroxybenzoate CoA-ligase, (HbrABC) 4-hydroxybenzoyl-CoA reductase, (PchA) 4-hydroxybenzaldehyde dehydrogenase, (PchCF) *p*-cresol methyl-hydroxylase, (Ped) 1-(*S*)-phenylethanol dehydrogenase, (PpcABCD) phenylphosphate carboxylase, (PpsABC) phenylphosphate synthetase.

Carboxylation. In *T. aromatica* K172 carboxylation of phenol to 4-hydroxybenzoate (biological Kolbe-Schmitt carboxylation) requires initial ATP-dependent activation to phenylphosphate (Fig. 3) (Breinig *et al*. 2000). The involved enzymes are phenylphosphate synthase (PpsABC) (Schmeling *et al*. 2004) and phenylphosphate carboxylase (PpcABCD) (Schühle and Fuchs 2004). Notably, obligate anaerobes (sulfate-reducing and iron-reducing bacteria bacteria) use the same ATP-dependent pathway (Schleinitz *et al*. 2009; Ahn *et al*. 2009), whereas fermenting bacteria use an ATP-independent carboxylase (Zhang and Wiegel 1994; He and Wiegel 1995; Huang *et al*. 1999). Notably, the aforementioned conversion of acetophenone to benzoylacetate in strain EbN1 involves a novel type of ATP-dependent carboxylase (ApcABCDE) targeting the methyl-group of the molecule (Kühner *et al*. 2005; Jobst *et al*. 2010). A carboxylation as initial activation step is also speculated about for the anaerobic degradation of benzene (Fig. 3) (Kunapuli *et al*. 2008; Abu Laban *et al*. 2010; Holmes *et al*. 2011).

Coenzyme A (CoA) activation. The ATP-dependent CoA-thioester formation catalyzed by AMP-dependent synthetases/ligases (e.g. benzoate-CoA ligase) (Altenschmidt *et al*. 1991) is considered a pivotal activation step to facilitate the subsequent reduction catalyzed by benzoyl-CoA reductase and 4-hydroxybenzoyl-CoA reductase (Buckel and Keese 1995; Boll *et al*. 2000b; Möbitz and Boll 2002; Boll 2005a). An alternative ATP-independent mode of CoA-activation has been described for benzylsuccinate, which receives a CoA via a CoA-transferase (BbsEF) from succinyl-CoA (Fig. 3) (Leutwein and Heider 2001).

Reductive dehydroxylation. Prior to the entrance into the central anaerobic benzoyl-CoA pathway, some aromatic compounds are reductively liberated from substituents. The best-described example is the 4-hydroxybenzoyl-CoA reductase from *T. aromatica* K172, which catalyzes the reductive removal of the *para*-hydroxyl group

to form benzoyl-CoA (Fig. 3) (for a review see Boll 2005b). The HbrABC enzyme contains FeS clusters, FAD and molybdenum cofactors (Brackmann and Fuchs 1993; Breese and Fuchs 1998) and receives the two electrons for the reductive removal from reduced ferredoxin.

3.3 The central anaerobic benzoyl-CoA pathway

Benzoyl-CoA represents the central intermediate of the anaerobic degradation of many aromatic compounds (Fig. 3) and is metabolized via the central anaerobic benzoyl-CoA pathway (Fig. 4). The pathway and involved enzymes are best understood in *T. aromatica* K172. The key-reaction and bottleneck of this pathway is the ATP-dependent reductive dearomatization of benzoyl-CoA, catalyzed by the benzoyl-CoA reductase (BcrCBAD; class I enzyme) (Boll and Fuchs 1995; Boll 2005a, b). The product of this reaction is a conjugated cyclohexa-1,5-diene-1-carbonyl-CoA (Fig. 4A), which is further degraded via β-oxidation-like reactions, catalyzed by cyclohexa-1,5-diene-1-carboxyl-CoA hydratase (Dch) (Laempe *et al*. 1998), 6-hydroxycyclohexa-1-ene-1-carbonyl-CoA dehydrogenase (Had) (Laempe *et al*. 1999) and 6-oxocyclohex-1-ene-1-carbonyl-CoA hydrolase (Oah) (Laempe *et al*. 1999). The product, 3-hydroxypimelyl-CoA, is then oxidized via a conventional β-oxidation, including a decarboxylation step (catalyzed by glutaryl-CoA dehydrogenase, GcdH), to three acetyl-CoA and CO_2 (Fig. 4A) (Härtel *et al*. 1993). Interestingly, *"A. aromaticum"* EbN1 uses a specific pathway for the anaerobic degradation of 3-hydroxybenzoate, involving a paralogous 3-hydroxybenzoyl-CoA reductase (HbrCBDA) as well as a dedicated set of enzymes for the modified β-oxidation and ring opening (Wöhlbrand *et al*. 2007). In contrast, the conventional class I BCR catalyzes the dearomatization of 3-hydroxybenzoate in *T. aromatica* K172 (Laempe *et al*. 2001). In known aromatic compound-degrading bacteria, the genes encoding the benzoyl-CoA reductase and the enzymes of the modified β-oxidation and ring opening are usually arranged in a single cluster (Fig. 4B), however the content of the cluster and overall gene arrangements differ between organism (for an overview see Carmona *et al*. 2009).

In contrast to facultative anaerobes, energy-limited obligate anaerobes, e.g. *Geobacter metallireducens*, use an ATP-independent novel type of benzoyl-CoA reductase (class II enzyme) (Kung *et al*. 2009, 2010).

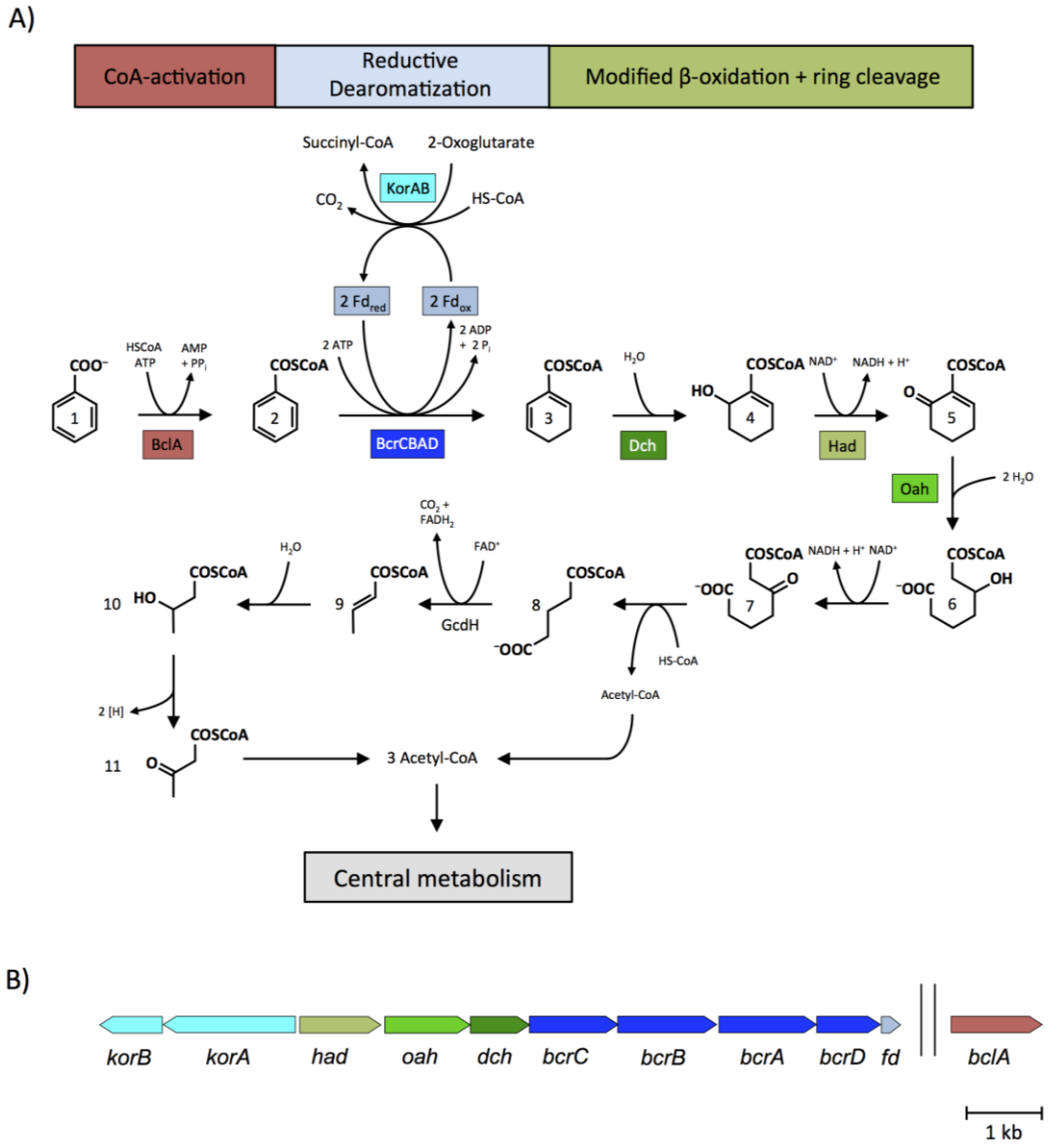

Fig. 4 Reaction sequences and genes of the central anaerobic benzoyl-CoA pathway from *T. aromatica* K172, yielding three molecules acetyl-CoA and one molecule CO_2. A) The benzoyl-CoA pathway is divided into ATP-dependent CoA-activation and reductive dearomatization, followed by a modified β-oxidation and ring cleavage. The ring is hydrolytically opened and the product can be degraded via conventional β-oxidation to acetyl-CoA, which can enter the central metabolism via the citric acid cycle. B) Genes coding for the upper part of the benzoyl-CoA pathway. Except the benzoate CoA-ligase (BclA), the genes are organized in one cluster. Enzyme names: (BclA) benzoate CoA-ligase, (BcrCBDA) class I benzoyl-CoA reductase, (Fd) ferredoxin, (KorAB) 2-oxoglutarate ferredoxin oxidoreductase, (Dch) dienoyl-CoA hydratase, (Had) 6-hydroxydienoyl-CoA dehydrogenase, (Oah) 6-oxodienoyl-CoA hydratase, (GcdH) glutaryl-CoA dehydrogenase. Compound names: 1) benzoate, 2) benzoyl-CoA, 3) cyclohexa-1,5-diene-1-carbonyl-CoA, 4) 6-hydroxy-cyclo-hex-1-ene-1-carbonyl-CoA, 5) 6-oxocyclohex-1-ene-1-carbonyl-CoA, 6) 3-hydroxypimelyl-CoA, 7) 3-oxopimelyl-CoA, 8) glutaryl-CoA, 9) crotonyl-CoA, 10) 3-hydroxybutyryl-CoA, 11) acetoacetyl-CoA.

3.3.1 Transcriptional regulation of the central anaerobic benzoyl-CoA pathway

The transcriptional regulation of the anaerobic central benzoyl-CoA pathway have thus far been studied with phototrophic *R. palustris* CGA009 and denitrifying *Azoarcus* sp. CIB. In *R. palustris* CGA009 the *bad* operon encodes BCR (*badDEFG*), benzoate-CoA ligase (*badA*) and ferredoxin (*badB*) (Egland *et al*. 1997). The MarR-type regulator BadR, is encoded upstream of the *badDEFG* genes. BadR interacts with the *badD* promoter and activates transcription of the *bad* genes with benzoyl-CoA presumably serving as inducer molecule (Egland and Harwood 1999). In addition, the Rrf2-type regulator BadM was found to repress the *badD* promoter (Peres and Harwood 2006). Finally, the Fnr-type transcriptional regulator AadR activates expression of the *badDEFG* genes under anoxic conditions (Egland and Harwood 1999). However, the exact interplay of these three regulators in an antagonistic or amplifying mode is presently unclear.

In *Azoarcus* sp. CIB the polycistronic expression of the *bzd* genes, encoding BCR (*bzdNOPQ*) and benzoate-CoA ligase (*bzdA*), is under control of the P_N promoter (López Barragán *et al*. 2004). The BzdR repressor protein binds to three different operator regions within the P_N promoter, all of which contain repetitions of TGCA (López Barragán *et al*. 2005). Similar to *R. palustris* CGA009, the P_N promoter of strain CIB can also be activated by an Fnr-type regulator (AcpR) in response to anoxia (Durante-Rodríguez *et al*. 2006). Interestingly, studies with *T. aromatica* K172, *Magnetospirillum* sp. TS-6 and *"A. aromaticum"* EbN1 showed formation of anaerobic benzoyl-CoA pathway constituents (e.g. BclA, BcrB) also in benzoate-utilizing cells under oxic condition (Heider *et al*. 1998; Shinoda *et al*. 2005; Kawaguchi *et al*. 2006; Wöhlbrand *et al*. 2007). This was unexpected, as characterized BCRs are sensitive to oxygen (Boll *et al*. 2000a). One may speculate that operability of anaerobic pathways at a low, basic level may increase responsiveness to changing conditions. E.g. *Magnetospirillum* spp. thrive in the oxic/anoxic transition zone of sediments (Blakemore *et al*. 1979; Spormann and Wolfe 1984).

The transcriptional control of the protein constituents of the lower benzoyl-CoA pathway (degradation of aliphatic intermediates) is mostly unknown. In *R. palustris* CGA009, the corresponding *pimFABCDE* genes form an operon, which is induced during anaerobic growth with benzoate (or pimelate) (Harrison and Harwood 2005). So far, only the regulation of the glutaryl-CoA dehydrogenase (GcdH) of *Azoarcus* sp. CIB

has been studied in some detail. The LysR-type activator GcdR controls the expression of the *gcdH* gene in response to glutarate or glutaconate (or their CoA derivatives) (Blázquez *et al*. 2008).

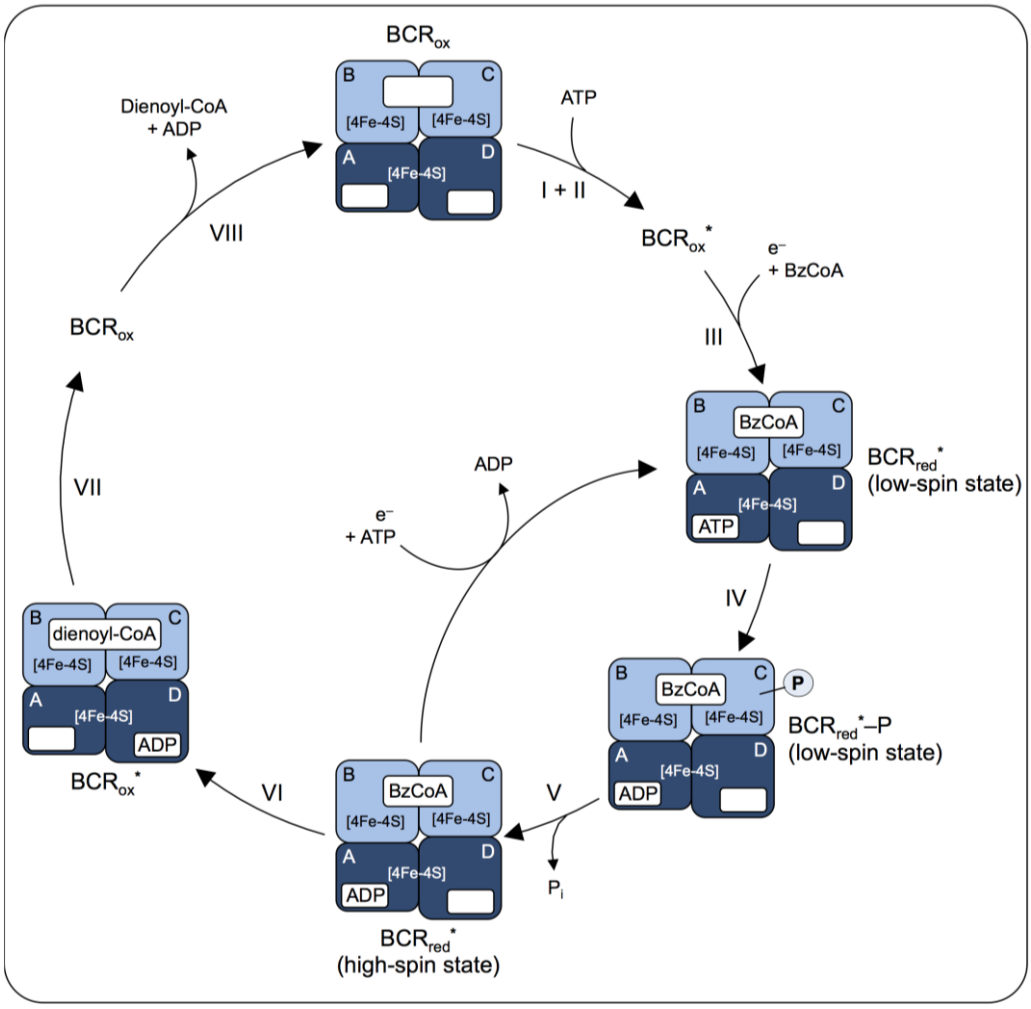

Fig. 5 Catalytic cycle of benzoyl-CoA reductase from *Thauera aromatica* K172. The defined steps (I-VIII) are indicated. Steps IV to V have to run twice. BCR, Benzoyl-CoA reductase; BzCoA, Benzoyl-CoA. The scheme is modified from (Boll 2005a, b).

3.3.2 Key-enzyme benzoyl-CoA-reductase

The key-reaction of the central anaerobic benzoyl-CoA pathway is the two electron Birch-like reductive dearomatization of benzoyl-CoA (Fig. 5A), which is catalyzed by class I and II BCRs (Boll 2005b; Kung *et al*. 2009). The strongly negative redox

potential (–1.9 V) for the first electron transfer to the aromatic ring is particularly difficult to overcome under physiological conditions (Boll *et al*. 2000b). Class I BCRs of facultative anaerobes cope with this challenge by means of stoichiometric hydrolysis of ATP. Oxygen inactivation of BCR from *T*. *aromatica* K172 is correlated to irreversible conversion of [4Fe-4S] clusters to [3Fe-4S] (Boll et al. 2000a). In contrast, class II BCRs of fermentative and strictly anaerobic bacteria are ATP-independent (Löffler *et al*. 2011), employing a yet unclear strategy to overcome the negative redox potential (Kung *et al*. 2009, 2010). Recently, a class III aryl-carboxyl-CoA reductase was discovered, which catalyzes the ATP-independent four-electron reduction of 2-naphthoyl-CoA to 5,6,7,8-tetrahydro-2-naphthoyl-CoA (THNCoA) in the sulfate-reducing enrichment culture N47 (Eberlein *et al*. 2013a, b). In contrast to class I and II BCRs, this enzyme is oxygen-tolerant.

3.3.3 Molecular composition and enzymatic principle of class I BCR

Class I BCRs can be divided into a *Thauera*- (found in *Thauera, Rhodopseudomonas* and *Magnetospirillum*) and an *Azoarcus*-subtype (found in *Azoarcus* and *"Aromatoleum"*) (Boll 2005a). The heterotetrameric BcrCBAD from *T*. *aromatica* K172 has a modular composition (Boll and Fuchs 1995; Boll 2005b) (Fig. 5B). The [4Fe-4S] cluster of the AD-module receives electrons from reduced ferredoxin (Boll and Fuchs 1998), which was beforehand activated by hydrolysis of bound ATP (Boll 2005a). The BC-module is considered to carry the active site for dearomatization of benzoyl-CoA. While, no typical CoA binding motifs have been identified, coordination sites for two additional [4Fe-4S] clusters are present (Breese *et al*. 1998; Boll 2005a, b). So far no crystal structure of class I BCRs is available. However, the phylogenetic relationship of class I BCRs with 2-hydroxyacyl-CoA dehydratases, involved in the equally difficult dehydratation of 2-hydroxy acids during the fermentation of amino acids, suggests a similar mechanism (for an overview see Buckel *et al*. 2004).

Based on studies with the enzyme of *T*. *aromatica* K172, a catalytic cycle for the ATP-dependent benzoyl-CoA reduction has been proposed (Möbitz *et al*. 2004; Boll 2005a) (Fig. 5B). Prerequisite for the reductive dearomatization by BCR is the formation of benzoyl-CoA, as the CoA-activation lowers the required potential of the first electron transfer from –3 V to –1.9 V (Boll *et al*. 2000b). Further essential

components for the two electron transfer by BCR are stoichiometric ATP-hydrolysis (Boll *et al*. 1997) and provision of low potential electrons by reduced ferredoxin (Boll and Fuchs 1998). Initially, the conformation of BCR changes upon binding of the first ATP to the AD-module (step I and II). Only in this state (BCR_{OX}^{*}) the substrate benzoyl-CoA can bind to the BC-module and one electron reduction of the enzyme shifts two [4Fe-4S] clusters to a low-spin state (step III). The hydrolysis of ATP to ADP then yields an energy-rich enzyme-phosphate linkage (step IV), the hydrolysis of which shifts one low-spin [4Fe-4S] cluster to a high-spin state (step V). This step is referred to as the electron activation step. The "activated" electron is considered to be transferred to the active site through the [4Fe-4S] clusters. After exchange of ADP with ATP and another electron transfer, steps IV-V are repeated to yield the enzyme-bound product dienoyl-CoA (step VI). Finally, release of dienoyl-CoA and ADP regenerates BCR_{OX} (VII and VIII) and a new catalytic cycle can begin.

The first electron transfer is probably facilitated by a simultaneous protonation at the *para*-position of the aromatic ring, which would avoid formation of a true radical intermediate (Möbitz and Boll 2002). This assumed protonation could explain, why the BCR from *T*. *aromatica* K172 accepts a variety of *ortho*- and *meta*-substituted but not *para*-substituted benzoyl-CoA analogues (Möbitz and Boll 2002).

3.4 Anaerobic degradation of *para*-alkylated aromatic compounds

The complete anaerobic oxidation (to CO_2) of *para*-alkylated polar monoaromatic compounds is to date only known for phenolic compounds such as *p*-cresol and *p*-ethylphenol. Conversion of *p*-cresol to 4-hydroxybenzoate may involve the hydroxylation of the *para*-methyl group (see chapter 3.2) or the addition to fumarate (Müller *et al*. 2001). *p*-Ethylphenol is initially hydroxylated and subsequently converted to 4-hydroxybenzoyl-CoA (Fig. 6A) (Wöhlbrand *et al*. 2008).

In contrast, the anaerobic degradation of *para*-alkylated monoaromatic hydrocarbons (e.g. *p*-xylene, *p*-cymene, *p*-ethyltoluene) is in large parts not understood at present (Fig. 6B). Most studies on the anaerobic degradation of *p*-xylene were based on enrichment cultures under nitrate-reducing (Haner *et al*. 1995; Rotaru *et al*. 2010) or sulfate-reducing conditions (Morasch and Meckenstock 2005; Nakagawa *et al*. 2008).

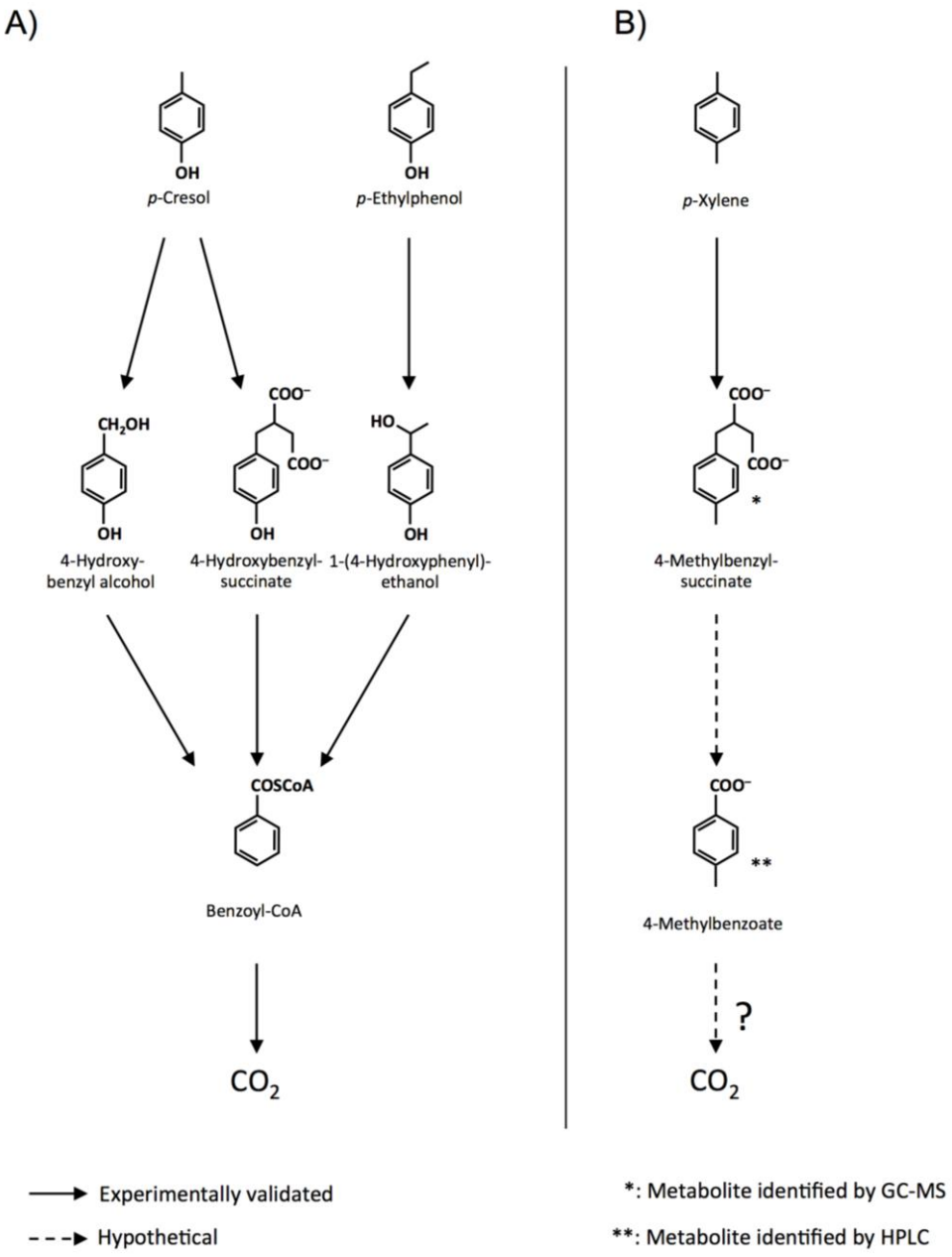

Fig. 6 Anaerobic activation and conversion of *para*-alkylated aromatic compound. A) Experimentally validated activation and conversion of *para*-alkylated phenolic compounds. B) Hypothetical activation and conversion of *p*-xylene. Metabolites identified in different studies are indicated by asterisks. For detailed information see chapter 3.4.

The poor substrate utilization of these enrichments reflects the recalcitrance of *p*-xylene. Only recently, a pure culture, strain PP31, capable of degrading *p*-xylene under sulfate-reducing conditions was reported (Higashioka *et al*. 2012). Metabolite analysis identified 4-methylbenzylsuccinate in enrichment cultures growing with *p*-xylene, indicating activation of *p*-xylene by addition of one methyl group to fumarate (Morasch

and Meckenstock 2005; Rotaru *et al*. 2010) (Fig. 6B). The 4-methyl-benzylsuccinate is most likely converted to 4-methylbenzoate via β-oxidation-like reaction sequences analogous to conversion of benzylsuccinate to benzoate during toluene degradation (see chapter 3.2). This assumption is supported by the capacity of *p*-xylene degrading, nitrate reducing enrichment cultures to also utilize 4-methylbenzoate (Haner *et al*. 1995; Rotaru *et al*. 2010). Further evidences were earlier obtained from toluene-degrading sulfate- and nitrate-reducing bacteria, which co-metabolically converted *p*-xylene to the dead-end product 4-methylbenzoate (Biegert and Fuchs 1995; Rabus and Widdel 1995a; Beller *et al*. 1996; Harms *et al*. 1999). The further degradation of 4-methylbenzoate might proceed in analogy to the known anaerobic benzoyl-CoA pathway. However, the known BCR from *T*. *aromatica* K172 cannot convert *para*-substituted benzoate analogues due to mechanistic constraints (see chapter 3.4.2). Furthermore, the tertiary ring carbon atom (binding the 4-methyl-group) would prevent one round of conventional β-oxidation upon ring reduction and cleavage (Widdel and Rabus 2001). Thus, different reactions are required for the complete oxidation to CO_2.

4. Carbon catabolite repression (CCR)

Bacteria usually use a more or less broad range of organic substrates for growth. In their natural habitats, bacteria rather face mixtures of carbon sources than single substrate species. For example soil bacteria encounter diverse plant exudates (e.g. amino acids, dicarboxylates) (Dennis *et al*. 2010) or products arising from lignocellulose degradation (e.g. glucose, phenylpropanoids) (Bugg *et al*. 2011) in their natural habitat. Substrate utilization can be simultaneous or preferential, often depending on the concentration of the individual compounds (Egli 2010). Usually, substrates allowing for higher growth rates are preferred and repress the utilization of less favored ones (Görke and Stülke 2008). As a result batch cultures often display a diauxic growth behavior, characterized by two active growth phases, separated by a lag phase lying in between. The regulatory process behind preferential substrate utilization is termed carbon catabolite repression (CCR). Several studies with bacteria showed, that the preferential utilization of carbon sources is a common principle and that often glucose is the preferred carbon source (Görke and Stülke 2008). However, mechanistic insights have been achieved mainly with *Escherichia coli*, *Bacillus subtilis* and the aerobic aromatic compound degrader

Pseudomonas putida. These organisms employ different strategies to control and optimize the utilization of carbon sources. Thus, substrate preferences and underlying regulatory mechanisms in anaerobic aromatic compound degrading bacteria are essentially unexplored.

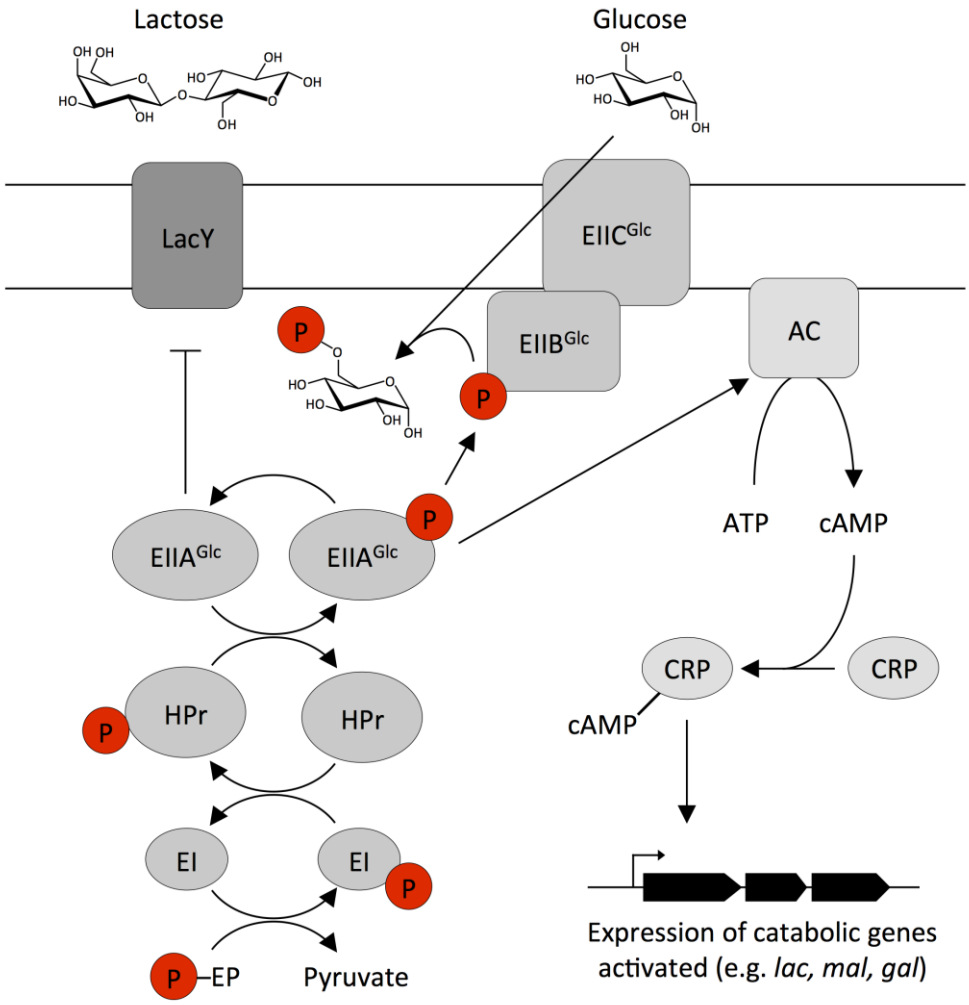

Fig. 6 Involvement of the phosphoenolpyruvate:carbohydrate phosphotransferase system (PTS) in the glucose-mediated carbon catabolite repression (CCR) of lactose in *E. coli*. In the presence of glucose, the $EIIA^{Glc}$ component is mostly present in the dephosphorylated form, which impedes the uptake of lactose by inhibiting lactose permease (LacY). In the absence of glucose the phosphoryl group remains bound to the $EIIA^{Glc}$ component. In the phosphorylated state, $EIIA^{Glc}$ activates the membrane-bound adenylate cyclase (AC). This results in the formation of cAMP, which forms a complex with the catabolite receptor protein (CRP) leading to the activation of expression of CRP controlled operons. The model is adapted from (Görke and Stülke 2008).

4.1 Preferential utilization of carbohydrates in *E. coli*

The best-described example of CCR is the glucose-lactose diauxie in *E. coli*, which was first described by Jacques Monod (1942). The key determinants of CCR in E. coli are the phosphoenolpyruvate (PEP):carbohydrate phosphotransferase system (PTS) and the global regulator Crp (Fig. 6) (reviewed in Görke and Stülke 2008). The phosphorylation state of the $EIIA^{Glc}$ component of the PTS system depends on the availability of glucose (phosphate acceptor) and PEP (phosphate donor). In the presence of glucose, the phosphate from $EIIA^{Glc}$~P is rapidly transferred to the incoming sugar. As a consequence, the non-phosphorylated form dominates the cellular pool of $EIIA^{Glc}$. The latter can bind and repress the lactose permease (LacY), resulting in blockage of lactose uptake (Fig. 6). When lactose is not taken up anymore, the inducer of the *lac* operon (allolactose) cannot be formed. This inducer exclusion represents a common principle to prevent gene expression for non-preferred substrates. When glucose is absent, the equilibrium of $EIIA^{Glc}$/$EIIA^{Glc}$~P is shifted to the phosphorylated form of $EIIA^{Glc}$, whereby inducer exclusion is revoked. Moreover, the abundant $EIIA^{Glc}$~P activates the membrane bound adenylate cyclase, which then forms cAMP (Fig. 6). The latter binds to Crp and this complex activate expression of catabolic genes for the alternative carbon source lactose.

4.2 Carbon catabolite repression in aerobic aromatic compound degradation

Comparative genomic analysis indicated, that the classical PTS system for sugar transport and phosphorylation is restricted to *Enterobacteriaceae*, *Vibrionales* and *Firmicutes* (Cases *et al*. 2007). An alternative PTS phosphoryl transfer chain was termed PTS^{Ntr} and speculated to be involved in the crosstalk between C and N regulatory circuits (Rabus *et al*. 1999; Velazquez *et al*. 2007). Remarkably, the PTS^{Ntr} system of *P. putida* participates in the glucose-mediated repression of genes required for the aerobic toluene and xylene degradation (Rojo 2010 and references therein).

In several *Pseudomonas* spp. the succinate-mediated CCR of aerobic aromatic compound degradation involves the RNA-binding global regulator protein Crc. Binding of Crc to the 5´-region of the target mRNA impedes the formation of an active translation initiation complex. In *P. putida*, Crc modulates the synthesis of at least 134 gene products, most of which are related to transport and assimilation of amino acids or

sugars; apparently Crc contributes to metabolic optimization (Moreno *et al*. 2009). In addition, Crc in *P*. *putida* indirectly controls the expression of genes for the aerobic catabolism of aromatic compounds and alkanes, by binding to the mRNA of the transcriptional activators AlkS (alkanes) and BenR (aromatic compounds) (Rojo 2010). In contrast, the Crc homolog from *Acinetobacter baylyi* seems to directly target the polycistronic *pca-qui* transcript, involved in aerobic degradation of protocatechuate and quinate (Zimmermann *et al*. 2009).

4.3 Carbon catabolite repression under conditions of carbon limitation

It is commonly perceived that preference of a "better" carbon source would increase the fitness of an organism by avoiding wasteful synthesis of catabolic enzymes for the utilization of low quality substrates (Magasanik 1976; Kalisky *et al*. 2007). However, the low concentration (10 – 100 µg l^{-1}) and the complexity of available organic carbon in most natural environments (Münster 1993; Egli 1995) would render simultaneous utilization of carbon sources beneficial.

According to present knowledge, simultaneous consumption of several carbon sources under conditions of slow growth and carbon limitation should be widespread among microorganism (reviewed in Egli 1995, 2010). Even substrates known to cause strong diauxie, e.g. glucose overriding lactose in *E*. *coli* or succinate preferred over toluene in *P*. *putida* (Rüegg *et al*. 2007), are simultaneously consumed with the non-preferred co-substrate at low to moderate dilution rates in carbon-limited chemostats. This is apparently caused by a de-repression of uptake and catabolism for alternative substrates (reviewed in Egli 1995). Benzoate-limited anoxic chemostats of *"A*. *aromaticum"* EbN1 adjusted to low growth rates revealed de-repression of uptake systems and catabolic pathways for a variety of aromatic compounds despite their absence from the feed medium (Trautwein *et al*. 2012b). This indicated that simultaneous consumption under carbon limitation occurs also in anaerobic aromatic compound degrading bacteria.

The simultaneous utilization of several carbon sources in free-living bacteria challenge the role of a regulatory mechanism such as carbon catabolite repression. However, selective utilization of carbon sources can be an advantage under "feast"

condition (e.g. in wastewaters, contaminated sites or around collapsing algal blooms) and should allow fast and optimal growth.

5 Aim of this study

Previous studies demonstrated the particular recalcitrance of *p*-alkylated monoaromatic hydrocarbons, e.g. *p*-xylene, under anoxic conditions. They also indicated that the degradation of the presumptive *p*-alkylbenzoate intermediates, e.g. 4-methylbenzoate, represents the most challenging part. The recent isolation of denitrifying strain pMbN1 growing anaerobically with 4-methylbenzoate allowed the present study to investigate the degradation of this model *para*-alkylated aromatic compound. The first objective was to characterize strain pMbN1 in terms of degradation balance for 4-methylbenzoate, aerobic and anaerobic substrate range, determination of physiological parameters (e.g. doubling-time, temperature optimum) and phylogenetic affiliation. For the second part, a genome shotgun sequence dataset of strain pMbN1 should be used to elucidate the anaerobic degradation pathways of 4-methylbenzoate and benzoate by integrating genomic, proteomic, enzymatic and metabolite analysis. The third objective was concerned with the effects of additional aromatic and aliphatic growth substrates on the anaerobic degradation of 4-methylbenzoate and benzoate. This should involve on the one hand the determination of the growth physiology of strain pMbN1 with various substrate mixtures. On the other hand, the expression of relevant substrate-specific genes should be profiled on the mRNA and protein level.

B RESULTS AND DISCUSSION

This section provides a discussion of selected results from the original research articles. Further details may be found in Part II (Publications).

1. Isolation and characterization of strain pMbN1

The availability of strain pMbN1 was the essential basis to study the mechanistically challenging anaerobic degradation of 4-methylbenzoate. For details refer to section 3.4.

1.1 Isolation of strain pMbN1

The primary enrichment culture of bacteria utilizing 4-methylbenzoate under nitrate-reducing conditions was obtained from a mud mixture collected from ditches and the Weser River in Bremen (Germany). Further transfers yielded a sediment-free enrichment, which was dominated by spirillum-shaped bacteria and showed a doubling time of 10 h, similar to earlier enrichment cultures with 4-methylbenzoate (Harms *et al*. 1999). The final isolation was achieved by anoxic agar-dilution series, as described elsewhere (Widdel and Bak 1992). The isolate of a pure culture with the best growth performance was chosen for further analysis and designated pMbN1.

1.2 Phylogenetic affiliation of strain pMbN1

Most isolated facultative anaerobic bacteria degrading aromatic compounds affiliate with the *"Aromatoleum"*/*Azoarcus*/*Thauera* cluster within the *Betaproteobacteria* (Widdel and Rabus 2001). Analysis of the 16S rRNA gene sequence revealed the affiliation of strain pMbN1 with the genus *Magnetospirillum* within the *Alphaproteobacteria* and showed highest sequence identity with *Magnetospirillum* sp. AMB-1 (Fig. 8). *Magnetospirillum* spp. capable of degrading aromatic compounds under nitrate-reducing conditions (e.g. strains BM1232 and TS-6) have been previously described (Shinoda *et al*. 2005), thus strain pMbN1 extends the knowledge of anaerobic aromatic compound degrading *Alphaproteobacteria*.

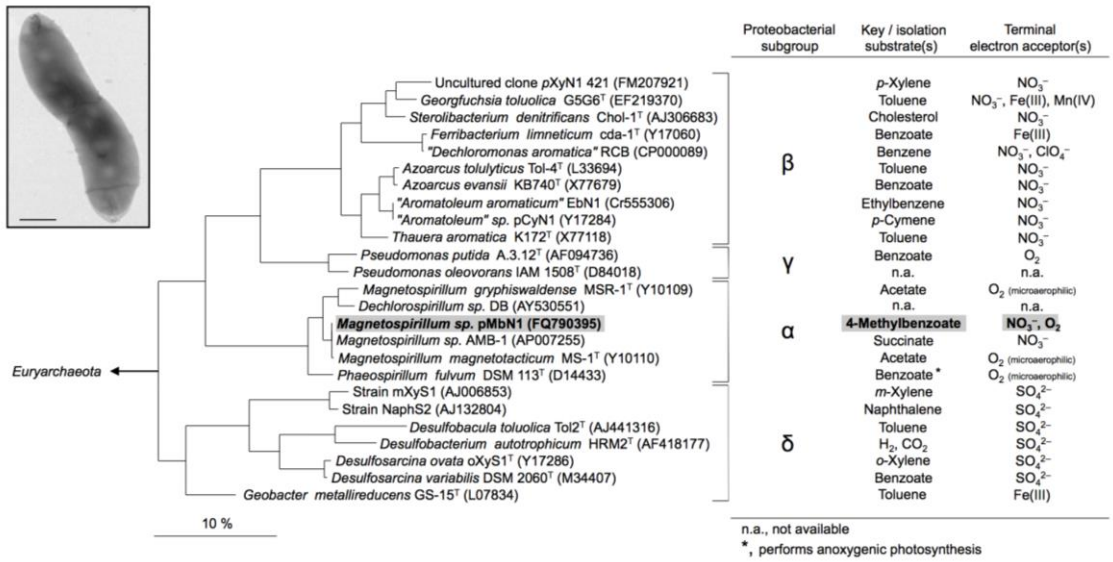

Fig. 8 Electron micrograph and phylogenetic affiliation of strain pMbN1. The electron micrograph was taken of negatively stained cells and the scale bar in the image represents 0.5 µm. The phylogenetic tree was constructed using 16S rRNA gene sequence from strain pMbN1 and selected members of the *Proteobacteria*. The scale bar below the tree represents 10% sequence divergence. For further details see Part II. The graphics were modified from the original article "Lahme *et al*. (2012a) Anaerobic degradation of 4-methylbenzoate by a newly isolated denitrifying bacterium, strain pMbN1" (Part II), which also includes the references for the displayed table.

1.3 Physiological characteristics of strain pMbN1

Table 2 summarizes the main physiological characteristics of strain pMbN1. The helical shape (Fig. 8) and cellular dimensions of strain pMbN1 are similar to other members of the *Magnetospirillum* genus (Maratea and Blakemore 1981; Schleifer *et al*. 1991; Shinoda *et al*. 2000; Thrash *et al*. 2010). However, strain pMbN1 did not show magentotaxis during microaerobic growth (Schüler and Baeuerlein 1998; D. Schüler, personal communication). Strain pMbN1 is capable of aerobic and also anaerobic (denitrification) growth (Table 2). In contrast to other denitrifying aromatic compound degraders (Rabus and Widdel 1995b; Harms *et al*. 1999), no intermediary accumulation of NO_2^- was observed. The denitrification phenotype and non-magnetotactic behavior of strain pMbN1 agree with those observed for the aromatic compound-degrading *Magnetospirillum* sp. CC-26 (Shinoda *et al*. 2000).

Strain pMbN1 utilizes 13 aromatic (non-hydrocarbon) and 13 aliphatic compounds coupled to nitrate reduction and only five different aromatic compounds under oxic conditions (Lahme *et al*. 2012a). This may indicate a specialization of strain

pMbN1 to utilize aromatic compounds preferentially under anoxic conditions. In agreement, the denitrification phenotype of strain pMbN1 may be advantageous in habitats experiencing longer periods of anoxia (Liu *et al*. 2013).

Table 2 Physiological characteristics of strain pMbN1. Parameters were determined during anaerobic growth with either 4-methylbenzoate or benzoate. Magnetotaxis was tested under microoxic conditions. The data was compiled from "Lahme *et al*. (2012a) Anaerobic degradation of 4-methylbenzoate by a newly isolated denitrifying bacterium, strain pMbN1" (Part II).

	Cells grown with	
Physiological characteristics	4-Methylbenzoate	Benzoate
Doubling time (t_d)	9.5 h	6.1 h
Maximal specific growth rate (μ_{max})	0.10 h^{-1}	0.16 h^{-1}
Electron acceptors	NO_3^-, O_2	NO_3^-, O_2
NO_2^- accumulation during anaerobic growth	–	–
N_2O accumulation during anaerobic growth	(+)[a]	–
N_2 formation during anaerobic growth	+	+
NH_4^+ accumulation during anaerobic growth	–	–
Cell length	1.8 – 3.6 µm	ND
Cell width	0.6 – 0.8 µm	ND
Temperature range	11.9 – 37.2 °C	ND
Temperature optimum	34.0 – 35.7 °C	ND
pH range	6.8 – 8.0	ND
pH optimum	7.3 – 7.7	ND
Motility	+	+
Magnetotaxis	–	ND

[a] Accumulation of N_2O was observed at the end of growth.
ND not determined

1.4 Strain pMbN1 oxidizes 4-methylbenzoate completely to CO_2

So far, strain pMbN1 is the only known isolate capable of utilizing 4-methylbenzoate as sole source of carbon and energy for anaerobic growth. The mechanistic challenges exerted by the tertiary carbon carrying *para*-methyl substituent (see chapter 3.4) on the terminal oxidation to CO_2 necessitated the quantitative determination of the growth balance. Around 80% of electrons dissimilated from 4-methylbenzoate were consumed by reduction of nitrate (Lahme *et al*. 2012a). This agreed well with a complete oxidation

of 4-methylbenzoate to CO_2 coupled to denitrification, as described by the following stoichiometric equation:

$$C_8H_7O_2^- + 7.2\ NO_3^- + 0.4\ H_2O + 0.2\ H^+ \rightarrow 8\ HCO_3^- + 3.6\ N_2$$

$$\Delta G^{\circ\prime} = -3{,}583\ kJ \cdot (mol\ 4\text{-methylbenzoate})^{-1}$$

The molar growth yields observed during anaerobic growth with benzoate ($Y_{X/S}$ = 66 g mol^{-1}; $Y_{X/C}$ = 10 g mol^{-1}) and 4-methylbenzoate ($Y_{X/S}$ = 65 g mol^{-1}; $Y_{X/C}$ = 9 g mol^{-1}) are in good agreement with previously calculated yields from facultative denitrifying bacteria utilizing ethylbenzene ($Y_{X/S}$ = 80 g mol^{-1}; $Y_{X/C}$ = 10 g mol^{-1}) (Rabus and Widdel 1995b) or *p*-cymene ($Y_{X/S}$ = 58 g mol^{-1}; $Y_{X/C}$ = 6 g mol^{-1}) (Harms *et al*. 1999). The higher growth rate (Table 2) and higher growth yield per mol carbon during anaerobic growth with benzoate, suggests a higher efficiency of this pathway compared to the utilization of 4-methylbenzoate in strain pMbN1.

2. Anaerobic degradation of 4-methylbenzoate and benzoate via two distinct yet analogous pathways

The general recalcitrance of 4-methylbenzoate together with obvious challenges of the *para*-methyl-group for reductive dearomatization by known class I-BCRs and for the β-oxidation following ring cleavage motivated the search for a distinct pathway for anaerobic 4-methylbenzoate degradation.

2.1 Physiological evidences for different catabolic pathways for the anaerobic degradation of benzoate and 4-methylbenzoate

First evidence for the occurrence of different anaerobic degradation pathways for benzoate and 4-methylbenzoate in strain pMbN1 were obtained from whole cell adaptation experiments. All investigated adaptation conditions led to immediate consumption of the adaptation substrate (Fig. 9A, B). In contrast, 4-methylbenzoate-adapted cells displayed an about five hour-long lag phase prior to the onset of benzoate utilization (Fig. 9A). *Vice versa* the beginning of 4-methylbenzoate utilization in benzoate-adapted cells also fell short of about five hours compared to that of benzoate

(Fig. 9B). This indicates that strain pMbN1 specifically induces the capacities to utilize the respective aromatic substrates.

2.2 Anaerobic degradation of 4-methylbenzoate involves a specific set of proteins

Differential protein profiles (2D-DIGE) revealed the formation of a set of proteins that showed abundance increases specifically during anaerobic growth with 4-methylbenzoate (Fig. 9C). Interestingly, this set contained four proteins (MbrBCAD) that are homologous to the subunits of class I BCR. These proteins were absent in benzoate-adapted cells (Fig. 9C) and are proposed to function as a specific 4-methylbenzoyl-CoA reductase, dearomatizing 4- methylbenzoyl-CoA to the corresponding dienoyl-CoA (Fig. 10A). In agreement with such a function, metabolite analysis of anaerobic 4-methylbenzoate-adapted cells revealed the formation of a 4-methylcyclohexadienecarboxylate, which could not be detected in benzoate- or succinate-adapted cells (Lahme *et al*. 2012b). In addition to MbrBCAD, another 4-methylbenzoate-specific set of proteins was detected (Fig. 9C). They were termed Dch-2, Oah-2 and Had-2 due to their homology with Dch, Oah and Had from *T*. *aromatica* K172 (see Fig. 4 for enzyme names). These enzymes are supposed to constitute a modified β-oxidation reaction sequence converting 4-methyldienoyl-CoA to 3-hydroxy-5-methylpimelyl-CoA (Fig. 10A). The latter can undergo one round of conventional β-oxidation to yield 3-methylglutaryl-CoA. Possible enzyme candidates could be Orf440 (predicted 3-hydroxyacyl-CoA dehydrogenase) and Orf430 (predicted acetyl-CoA acetyltransferase). Indeed, metabolite analysis revealed the specific formation of 3-methylglutarate (Lahme *et al*. 2012b), indicating that the *para*-methyl group is conserved during dearomatization and ring fission (Fig. 10A). The tertiary carbon atom of 3-methylglutaryl-CoA cannot be overcome by conventional β-oxidation. Thus, subsequent degradation most likely proceeds by reactions similar to the leucine/isovalerate pathway (Fig. 10A). Proposed enzymes for this reactions are Orf530, Orf790 (both predicted acyl-CoA dehydrogenase), Orf420 (predicted enoyl-CoA hydratase) and Orf460 (predicted 3-hydroxy-3-methylglutaryl-CoA lyase). Analysis of transcript levels of *orf460* in 4-methylbenzoate-, benzoate- and succinate-adapted cells by reverse transcription real-time PCR (RTqPCR), revealed an about 27-fold higher abundance in 4-methylbenzoate-adapted cells compared to succinate and benzoate-

adapted cells (Table 3). The above described proteins and genes are proposed to constitute a specific pathway for the anaerobic degradation of 4-methylbenzoate (Fig. 10A). The involved genes form two distinct clusters on a single contig (~142 kb) assembled from a genomic shotgun sequence database. The two gene clusters are separated by ~27 kb (Fig. 10B). This arrangement is distinct from genes required for anaerobic degradation of benzoate to 3-hydroxypimelyl-CoA, which form a single cluster in strain pMbN1 (Lahme *et al*. 2012b), *T*. *aromatica* K172 (Breese *et al*. 1998) and *Magnetospirillum* spp. (López Barragán *et al*. 2004b; Shinoda *et al*. 2005). Further information on the genes constituting these clusters can be found in the original publication in Part II.

Surprisingly, proteins constituting the classic anaerobic benzoyl-CoA pathway in strain pMbN1 (BcrBCA, Dch-1 and Oah-1) were also detected in 4-methylbenzoate-adapted cells, although with lower abundances (Fig. 9C). This suggests a less specific regulation of the anaerobic benzoyl-CoA pathway compared to the 4-methylbenzoyl-CoA pathway. Such a relaxed stringency may be advantageous considering the great variety of aromatic compounds, which are channeled into the benzoyl-CoA pathway. At least 11 of the aromatic growth substrate of strain pMbN1 are known to be degraded via the anaerobic benzoyl-CoA pathway in other denitrifiers, such as *"Aromatoleum aromaticum"* EbN1 (Wöhlbrand *et al*. 2007; Carmona *et al*. 2009; Trautwein *et al*. 2012b).

Table 3 Fold change in transcript abundance determined by reverse transcription real-time PCR (RTqPCR) of *orf460* (predicted 3-hydroxy-3-methylglutaryl-CoA lyase) in cells anaerobically adapted to 4-methylbenzoate, benzoate or succinate. Succinate served as the reference state.

Gene name	Putative function of gene product	Fold change in transcript abundance[a, b]	
		Benzoate *vs*. succinate	4-Methylbenzoate *vs*. succinate
orf460	3-Hydroxy-3-methylglutaryl-CoA lyase	**–2 ± 1**	**27 ± 3**

[a] Samples for transcript analysis were collected during active growth (OD_{660} ~ 0.2) with benzoate, succinate or 4-methylbenzoate as substrate.

[b] Primerpair used resulting in a 182 bp product: HMG 497F (5´-TTGGCGGATTTCGGCTGCTACG-3´), HMG 678R (5´- CCAAGATCCAATGCGGCGAGAATA-3´)

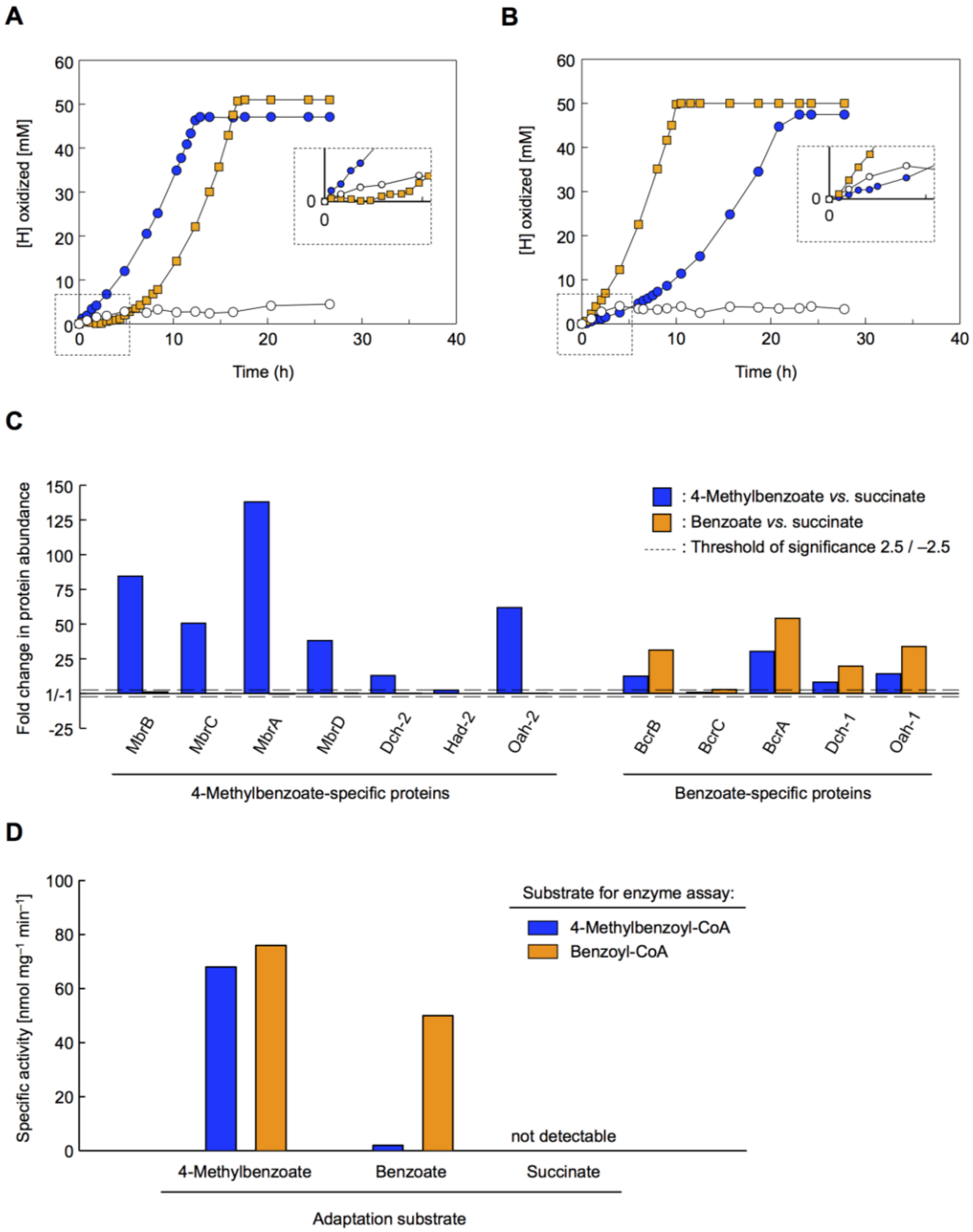

Fig. 9 Anaerobic degradation of aromatic compounds by substrate-adapted cell suspensions of strain pMbN1. A) Cells were adapted to 4-methylbenzoate. B) Cells were adapted to benzoate. Substrate-adapted cells were shifted to either benzoate (orange-filled squares) or 4-methylbenzoate (blue-filled circles). Controls (open circles) did not contain any organic substrate. C) Substrate-dependent fold changes in abundance of proteins related to the degradation of 4-methylbenzoate and benzoate respectively, as determined by 2D-DIGE. D) Enzyme activity with the substrates 4-methylbenzoyl-CoA or benzoyl-CoA in crude extracts from pMbN1 cells adapted to 4-methylbenzoate, benzoate or succinate, respectively. For further information see "Lahme *et al*. (2012b) Anaerobic degradation of 4-methylbenzoate via a specific 4-methylbenzoyl-CoA pathway" (Part II). The graphics A, B and C were modified from this original article.

2.3 Involvement of a novel 4-methylbenzoyl-CoA reductase

In agreement, with the above-proposed function of MbrBCAD, only extracts from anaerobic 4-methylbenzoate-adapted cells displayed high activity for the conversion of 4-methylbenzoyl-CoA to 4-methyldienoyl-CoA (Fig. 9D). In contrast, extracts from anaerobic, benzoate-adapted cells showed high activity only with benzoyl-CoA and those from anaerobic succinate-adapted cells lacked activity for both substrates (Fig. 9D). The combination of proteomic and enzymatic data indicates the requirement of MbrBCAD for efficient 4-methylbenzoyl-CoA dearomatization. The similarly high activity of extracts from 4-methylbenzoate-adapted cells for both aromatic CoA-esters (Fig. 9D), suggests that MbrBCAD could also dearomatize benzoyl-CoA. In agreement, BCR from *T. aromatica* can convert a variety of *ortho*- and *meta*-substituted benzoyl-CoA derivatives with different rates (Boll and Fuchs 1995; Möbitz and Boll 2002). Furthermore, the BCR activity of benzoate-adapted cells of strain pMbN1 is in good agreement with activities reported for other denitrifying aromatic compound degraders (Heider *et al*. 1998; Trautwein *et al*. 2012a; Juárez *et al*. 2013).

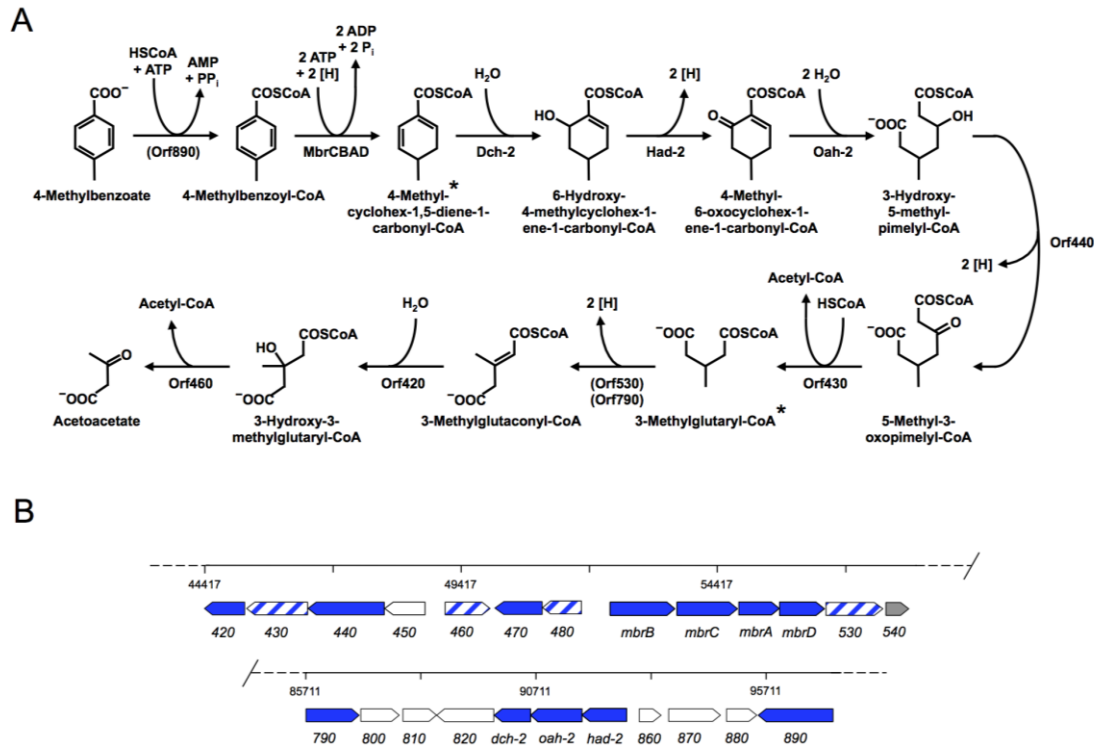

Fig. 10 A) Proposed pathway for the anaerobic degradation of 4-methylbenzoate in strain pMbN1. Acetoacetate at the end of the pathway is split into two molecules acetyl-CoA, which are oxidized to CO_2

via the citric acid cycle or fuel anaplerotic resp. anabolic functions. Metabolites marked with an asterisk were identified by GC-MS in cells adapted to 4-methylbenzoate. B) Organization of genes proposed to be involved in the pathway. The scale bar indicates the nucleotide position of the gene on the assembled contig. Products of genes identified on 2D-DIGE gels are marked in blue (assigned function) or grey (unassigned function). Genes with proposed function in the pathway but not identified by proteomics are hatched. Products of genes marked in white have also not been identified by proteomics. For further information see "Lahme *et al*. (2012b) Anaerobic degradation of 4-methylbenzoate via a specific 4-methylbenzoyl-CoA pathway" (Part II). The graphics were modified from this original article.

The subunits of the classic benzoyl-CoA reductase (BcrBCDA) of strain pMbN1 show higher protein sequence identities with the *Thauera*- than with the *"Aromatoleum"*/*Azoarcus*-subclass of BCRs (Fig. 11), similar to other *Magentospirillum* spp. (López Barragán *et al*. 2004b; Shinoda *et al*. 2005). In contrast, the MbrBCDA proteins differ markedly from the subunits of conventional class I BCRs and form new subgroups in the *Thauera*-subclass or in case of MbrB even outside of the subclasses (Fig. 11).

Despite the low sequence identities of Mbr subunits with those of Bcr from *T. aromatica* K172 (Fig. 11), the Mbr subunits contain the expected functional domains and amino acid residues for coordinating the electron transferring FeS-clusters as well as for ATP binding and hydrolysis (Hans *et al*. 1999; Locher *et al*. 2001; Boll 2005b). The assignment of the functional domains suggests a similar mechanism as proposed for BCR from *T. aromatica* K172.

2.4 Methylbenzoyl-CoA reductases beyond strain pMbN1

The use of specific reductases for the conversion of methyl-substituted and unsubstituted benzoyl-CoA seems to be not exclusive for strain pMbN1. Recently, a pathway for the anaerobic degradation of 3-methylbenzoate was described in *Azoarcus* sp. CIB, which involves another novel class I BCR for the conversion of 3-methylbenzoyl-CoA to 3-methyldienoyl-CoA (Juárez *et al*. 2013). In analogy to the 4-methylbenzoate-specific gene expression in strain pMbN1, the gene cluster for 3-methylbenzoate degradation in strain CIB was only expressed in the presence of 3-methylbenzoate, but not with benzoate (Juárez *et al*. 2013). Interestingly, the subunits of 3-methylbenzoyl-CoA reductase from strain CIB and 4-methylbenzoyl-CoA reductase from strain pMbN1 show high sequence identities (52–78%), which may point towards a common evolutionary origin of methylbenzoyl-CoA reductases (Juárez *et al*. 2013).

Strain pMbN1 is also capable of anaerobic growth with 3-methylbenzoate (Lahme *et al*. 2012a), but it is presently unclear whether its degradation involves the benzoate, 4-methylbenzoate or an hitherto unknown gene cluster(s).

The apparent ability of methylbenzoyl-CoA reductases to also convert benzoyl-CoA, raises the question of the evolutionary advantage to maintain two reductases. BCR from *T*. *aromatica* K172 can convert also different *ortho*- and *meta*-substituted benzoyl-CoA analogues, but not *para*-substituted ones (Boll and Fuchs 1995; Möbitz and Boll 2002). This may indicate that methylbenzoyl-CoA reductases could have a different substrate spectrum with respect to ring substitutions, thus increasing the aromatic substrate range of strains pMbN1 and CIB.

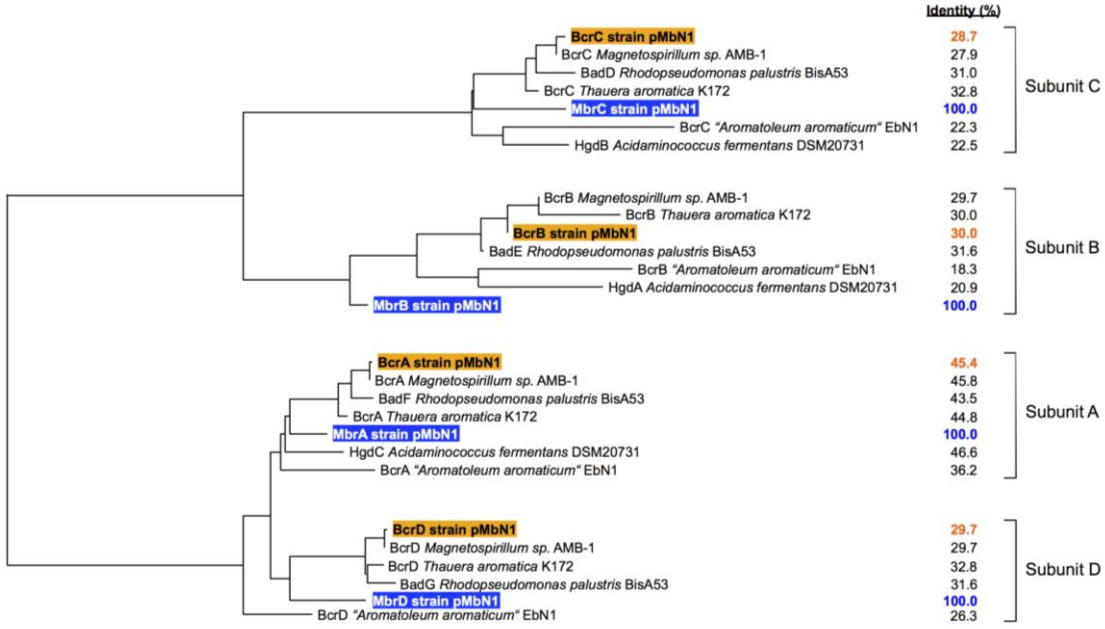

Fig. 11 Protein sequence relationships of (4-methyl-)benzoyl-CoA reductases. Subunits from strain pMbN1 are marked in bold and highlighted either in blue (4-methylbenzoyl-CoA reductase) or orange (benzoyl-CoA reductase). The protein sequence identities are shown on the right side and subunits of 4-methylbenzoyl-CoA reductase from strain pMbN1 were set to 100%. Bcr and Bad, benzoyl-CoA reductase; Mbr, 4-methylbenzoyl-CoA reductase; Hgd, 2-hydroxyglutaryl-CoA dehydratase. For further information see original article: "Lahme *et al*. (2012b) Anaerobic degradation of 4-methylbenzoate via a specific 4-methylbenzoyl-CoA pathway" (Part II). The graphic was modified from the original article.

3. Anaerobic growth of strain pMbN1 with mixtures of aromatic and aliphatic acids

In their natural habitat bacteria encounter complex substrate mixtures rather than single carbon sources (Münster 1993; Egli 1995). Investigated aerobic (*Pseudomonas putida*, *Acinetobacter baylyi*) and anaerobic (*Azoarcus* sp. CIB) aromatic compound degraders prefer easily degradable low-molecular weight organic acids (e.g. succinate) over aromatic compounds (e.g. benzoate) (Morales *et al*. 2004; López Barragán *et al*. 2004a; Fischer *et al*. 2008). In contrast, from mixtures of aromatic compounds (containing e.g. phenol or phthalate together with benzoate), benzoate is often preferred under oxic (e.g. in *Ralstonia eutropha* (Ampe *et al*. 1998), *Acinetobacter* spp. (Mazzoli *et al*. 2007; Zhan *et al*. 2009) and *Rhodococcus* sp. DK17 (Choi *et al*. 2007)), or anoxic conditions (e.g. *Thauera aromatica* AR-1 (Philipp and Schink 2000)). The discovery of two highly similar anaerobic pathways for the degradation of 4-methylbenzoate and benzoate in strain pMbN1 raised the question about differential operability under mixed substrate conditions.

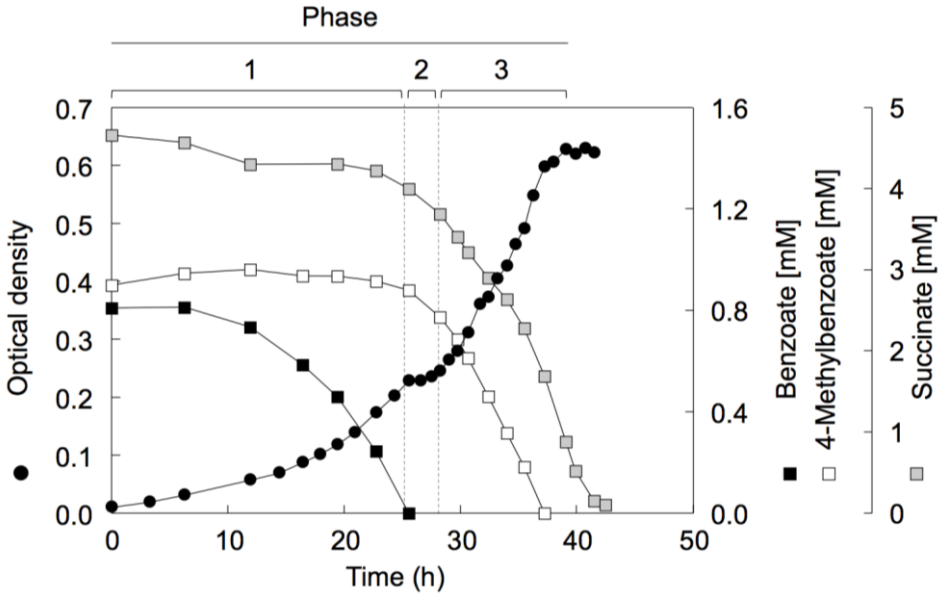

Fig. 12 Anaerobic growth of strain pMbN1 with a ternary substrate mixture consisting of benzoate, 4-methylbenzoate and succinate. The cells had been anaerobically adapted to 4-methylbenzoate. Dashed lines indicate the different phases of diauxic growth. Modified from the manuscript: "Lahme *et al*., *Magnetospirillum* sp. pMbN1 prefers benzoate from binary and ternary substrate mixtures during anaerobic growth" (Part II).

3.1 Benzoate is preferred over aliphatic acids and 4-methylbenzoate

Anaerobically 4-methylbenzoate-adapted cells of strain pMbN1 were incubated with a ternary substrate mixture composed of 4-methylbenzoate (1 mM), benzoate (1 mM) and succinate (5 mM). Under this conditions diauxic growth behavior was observed with a preferential utilization of benzoate during the first active growth phase (Fig. 12, Table 4). Interestingly, ~15% of the supplied succinate were already consumed during the benzoate utilization phase (phase 1) prior to the diauxic lag phase (phase 2), whereas the levels of 4-methylbenzoate remained constant throughout phase 1 (Fig. 12). The bulk of succinate and 4-methylbenzoate were then simultaneously and completely consumed in the second active growth phase (phase 3).

Benzoate was also the preferred substrate in binary mixtures with fumarate, L-malate, pyruvate or oxaloacetate. All four different binary mixtures resulted in diauxic growth (Table 5). This suggests that strain pMbN1 generally prefers benzoate over C_4-dicarboxylic and β-keto acids. However, this behavior was restricted to benzoate, since either other aromatic co-substrates (4-methylbenzoate, phenylacetate) and succinate were co-utilized or succinate was even preferred over 4-hydroxybenzoate (Table 5). In contrast, phenylacetate was also preferred over 4-methylbenzoate (Table 5), contrasting the above-described preference of benzoate (but not phenylacetate or 4-hydroxybenzoate) over succinate. This may indicate that aromatic substrates channeled into the anaerobic benzoyl-CoA pathway are preferred over 4-methylbenzoate in strain pMbN1.

Surprisingly, the length of phase 2 (intermediary lag phase) observed with the ternary mixture was about 50% reduced (Table 4) compared to the binary mixture of 4-methylbenzoate and benzoate (Table 5). This may be interpreted as the concomitant succinate degradation to energetically compensate for the extensive synthesis of enzymes required for degradation of 4-methylbenzoate (Fig. 10). A positive effect of succinate on the time requirement for induction of aerobic benzene degradation has been reported previously with *Ralstonia pickettii* PKO1 (Bucheli-Witschel *et al*. 2009).

The substrate preference observed for the different substrate mixtures did not correlate with growth rates, i.e. benzoate (μ_{max} varied between 0.15–0.25 h^{-1} depending on the adaptation substrate see Table 5) was preferred over substrates supporting higher (fumarate; $\mu_{max} = 0.27\ h^{-1}$, L-malate; $\mu_{max} = 0.33\ h^{-1}$) or similar (succinate; $\mu_{max} = 0.21\ h^{-1}$, 4-methylbenzoate; $\mu_{max} = 0.16\ h^{-1}$) maximum specific growth rates (Table 5). This

differs from the general view that substrates allowing higher growth rates are preferentially utilized (Harder and Dijkhuizen 1982; Görke and Stülke 2008) and suggests that other factors influence substrate preferences in bacteria as well.

Table 4 Anaerobic growth behavior of strain pMbN1 with a ternary substrate mixture. Taken from the manuscript: "Lahme *et al*., *Magnetospirillum* sp. pMbN1 prefers benzoate from binary and ternary substrate mixtures during anaerobic growth" (Part II).

Substrate[a]			Results for[b]: Phase 1		Phase 2	Phase 3	
S_{Ad}	S_{Co1}	S_{Co2}	OD_{max}	μ_{max} (h^{-1})	Duration of diauxic lag phase (h)	OD_{max}	μ_{max} (h^{-1})
4-Methylbenzoate (1 mM)	**Benzoate (1 mM)**	Succinate (5 mM)	0.23	0.16	1.8 ± 0.1	0.62	0.31

[a] Preferentially utilized substrate is highlighted in boldface. Applied substrate concentrations are indicated in parentheses. S_{Ad}, adaptation substrate; S_{Co1}, first co-substrate; S_{Co2}, second co-substrate.
[b] Values for maximal optical density (OD_{max}) and maximum specific growth rates (μ_{max}) are based on three biological replicates yielding standard deviation of <5%. μ_{max} was calculated from the slope of the active growth phase (m): $\mu_{max} = m \cdot 1/\Delta OD$. The duration of the diauxic lag phase (phase 2) is indicated with standard deviation.

3.2 Effects of aliphatic and aromatic acids on the anaerobic benzoate utilization

Abundance changes of selected transcripts (10 time points) and proteins (5 time points) related to the uptake and degradation of benzoate, 4-methylbenzoate and succinate were analyzed across phases 1–3 of a culture grown with the ternary substrate mixture. Succinate-grown cells harvested at half maximal OD served as reference state (Fig. 13). In addition, abundances of respective transcripts and proteins were also determined in cells grown (until half maximal OD) with 4-methylbenzoate or benzoate supplied as single substrate.

The preferential utilization of benzoate during phase 1 (Fig. 12) coincided with an increase in abundance (up to 24.3-fold) for proteins and transcripts (up to 84-fold) related to the anaerobic benzoyl-CoA pathway (BclA, BcrCBDA, Dch-1, Had-1, and Oah-1) (Fig. 13). Transcript levels of benzoate-CoA ligase (*bclA*) and the C-subunit of benzoyl-CoA reductase (*bcrC*) decreased immediately (within 30 min) upon benzoate depletion (Fig. 13), agreeing with the requirement of benzoate for the synthesis of

benzoyl-CoA reductase and benzoate-CoA ligase in *Magnetospirillum* spp. (López Barragán *et al*. 2004b; Shinoda *et al*. 2005; Kawaguchi *et al*. 2006). However, in agreement with previous findings (Lahme *et al*. 2012b), partial induction of the anaerobic benzoyl-CoA pathway by 4-methylbenzoate was also observed in 4-methylbenzoate-adapted cells and in phase 3 (after benzoate depletion) of the present growth experiment with the ternary substrate mixture (Fig. 13).

Table 5 Anaerobic growth of strain pMbN1 with binary mixtures of aliphatic and aromatic acids. Taken from the manuscript: "Lahme *et al*., *Magnetospirillum* sp. pMbN1 prefers benzoate from binary and ternary substrate mixtures during anaerobic growth" (Part II).

		OD_{max}, μ_{max} (h^{-1}) and duration of diauxic lag phase (h)[b]				
		Single substrates		Mixtures of S_{Ad} and S_{Co}		
Adaptation substrate[a] (S_{Ad})	Co-substrate[a] (S_{Co})	S_{Ad}	S_{Co}	Phase 1[c]	Phase 2[c]	Phase 3[c]
Succinate-adapted cells						
Succinate	**Benzoate**	0.38	0.18	0.30		0.42
(5 mM)	**(1 mM)**	0.21 h^{-1}	0.21 h^{-1}	0.19 h^{-1}	2.1 ± 0.1	0.28 h^{-1}
Succinate	4-Methylbenzoate	0.38	0.15	0.61		
(5 mM)	(1 mM)	0.21 h^{-1}	0.13 h^{-1}	0.24 h^{-1}	None	None
Succinate	4-Hydroxybenzoate	0.36	ND	0.43		0.51
(5 mM)	(1 mM)	0.20 h^{-1}		0.27 h^{-1}	3.3 ± 0.1	0.50 h^{-1}
Succinate	Phenylacetate	0.36	0.19	0.66		
(5 mM)	(1 mM)	0.20 h^{-1}	0.16 h^{-1}	0.25 h^{-1}	None	None
4-Methylbenzoate-adapted cells						
4-Methylbenzoate	**Benzoate**	0.27	0.28	0.27		0.41
(1.5 mM)	**(1.5 mM)**	0.16 h^{-1}	0.15 h^{-1}	0.29 h^{-1}	4.0 ± 0.0	0.29 h^{-1}
4-Methylbenzoate	**Phenylacetate**	0.27	0.32	0.30		0.45
(1.5 mM)	**(1.5 mM)**	0.16 h^{-1}	0.16 h^{-1}	0.29 h^{-1}	5.0 ± 0.0	0.28 h^{-1}
Cells adapted to other aliphatic acids						
Pyruvate	**Benzoate**	0.34	0.18	0.30		0.54
(6 mM)	**(1 mM)**	0.18 h^{-1}	0.21 h^{-1}	0.17 h^{-1}	1.3 ± 0.3	0.37 h^{-1}
Fumarate	**Benzoate**	0.37	0.19	0.30		0.43
(5 mM)	**(1 mM)**	0.27 h^{-1}	0.17 h^{-1}	0.17 h^{-1}	2.3 ± 0.0	0.29 h^{-1}
L-Malate	**Benzoate**	0.37	0.16	0.31		0.49
(5 mM)	**(1 mM)**	0.33 h^{-1}	0.25 h^{-1}	0.44 h^{-1}	2.6 ± 0.1	0.28 h^{-1}
Oxaloacetate	**Benzoate**	0.27	0.17	0.31		0.42
(6 mM)	**(1 mM)**	0.20 h^{-1}	0.23 h^{-1}	0.19 h^{-1}	1.0 ± 0.0	0.34 h^{-1}

[a] Preferentially utilized substrates are highlighted in boldface. Applied substrate concentrations are indicated in parentheses.

[b] Values for maximal optical density (OD_{max}) and maximum specific growth rates (μ_{max}) are based on at least two replicate cultures (three for each substrate mixture) yielding standard deviation of <5%. The μ_{max} was calculated from the slope of the active growth phase
(m): $\mu_{max} = m \cdot 1/\Delta OD$. If present, the duration of the diauxic lag phase (phase 2) is indicated with standard deviation.

[c] In the case of diauxic growth, phase 1 corresponds to the first active growth phase. In the case of monophasic growth, phase 1 corresponds to the only observed active growth phase. If present, phase 3 corresponds to a second active growth phase, with phase 2 representing the diauxic lag phase between phase 1 and 3.
ND, not determined.

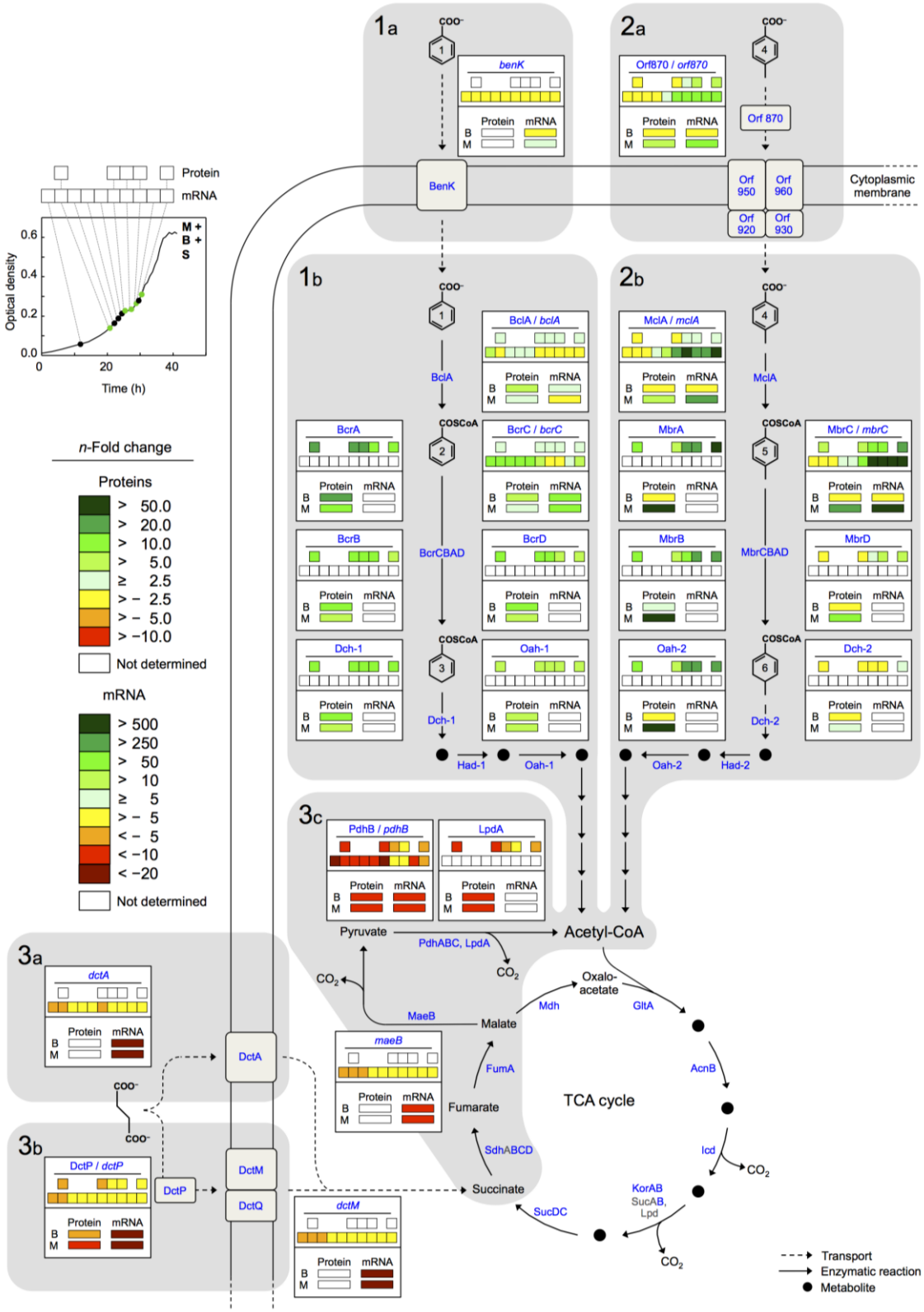

Fig. 13 Time-resolved changes in the abundance of proteins and transcripts (compared to succinate-adapted cells) related to uptake and catabolism of (1) benzoate, (2) 4-methylbenzoate and (3) succinate during anaerobic growth of 4-methylbenzoate-adapted cells with a ternary substrate mixture or single substrates (S, succinate; B, benzoate; M, 4-methylbenzoate). Sampling points for proteomic and transcript analyses are indicated in the growth curve (top left). Identified proteins are highlighted in blue. Modified from the manuscript: "Lahme *et al*., *Magnetospirillum* sp. pMbN1 prefers benzoate from binary and ternary substrate mixtures during anaerobic growth" (Part II).

A homolog of the benzoate/H^+ symporter BenK (Collier *et al*. 1997) was detected in benzoate- or 4-methylbenzoate- and in all three phases of the ternary substrate mixture experiment, but was not detectable in succinate-grown cells (Table 6). Despite increased abundance in benzoate-grown cells the levels of *benK* transcript across phases 1–3 were comparable to succinate-grown cells (Fig. 13).

3.3 Effects of benzoate on the anaerobic 4-methylbenzoate utilization

Despite the non-utilization of 4-methylbenzoate in phase 1 of the ternary substrate mixture experiment, some proteins of the anaerobic 4-methylbenzoyl-CoA pathway (MbrCBA and Oah-2) were already present during benzoate utilization (Fig. 13). However, decreased transcript levels of *mclA* and *mbrC* during phase 1 suggest rather slow degradation of remaining proteins from the 4-methylbenzoate-adaptation process than new synthesis. Upon benzoate depletion, protein and transcript abundances increased during phase 2 and reached highest levels (up to 60.1-fold for proteins and 1046-fold for transcripts) in phase 3, accompanied by the consumption of 4-methylbenzoate (Fig. 12). The decreased level of proteins and transcripts in benzoate-adapted cells suggests a tight control of the anaerobic 4-methylbenzoyl-CoA pathway in strain pMbN1 (Fig. 13). Based on the molecular data and genetic localization, the uptake of 4-methylbenzoate is predicted to be mediated by an ABC transporter (formed by Orf920, 930, 950, 960, and the periplasmic solute-binding protein Orf870) and transcript and protein levels of Orf870 showed similar changes (Fig. 13). Furthermore, the membrane-associated components Orf920–960 were identified only in the membrane protein-enriched fraction of phases 2/3 and in cells grown solely with 4-methylbenzoate (Table 6).

The strong increase of *mclA*, *mbrC* and *orf870* transcripts immediately (30 min) after benzoate depletion (Fig. 13), suggests that benzoate mediates a strong repression of gene expression related to uptake and catabolism of 4-methylbenzoate in phase 1. The low protein abundances of Orf870 and MclA during benzoate utilization might result in reduced uptake of 4-methylbenzoate and conversion into 4-methylbenzoyl-CoA. Activities of regulators for anaerobic aromatic compound degradation pathways (involving CoA thioesters) are often controlled by the acyl-CoA derivatives rather than the free acids (López Barragán *et al*. 2005; Fuchs *et al*. 2011). Thus, the reduced uptake

of 4-methylbenzoate and formation of 4-methylbenzoyl-CoA might prevent inducer formation in strain pMbN1. Expression of genes for 4-methylbenzoate uptake and degradation could be controlled by the putative TetR-type repressor Orf880 encoded directly downstream of *orf870* encoding the ABC-type periplasmic-binding protein in *cis* and directly upstream of *mclA* (encoding 4-methylbenzoate-CoA ligase) in *trans*. A TetR-type transcriptional regulator (MbdR) was recently proposed to control gene expression for anaerobic 3-methylbenzoate uptake and catabolism in *Azoarcus* sp. CIB (Juárez *et al*. 2013).

Table 6 Scores (Mascot) for selected proteins identified in the membrane protein-enriched fraction. Modified from the manuscript: "Lahme *et al*., *Magnetospirillum* sp. pMbN1 prefers benzoate from binary and ternary substrate mixtures during anaerobic growth" (Part II).

		Single substrates			Ternary substrate mixture (M + B + S)[a]		
Protein	Predicted function	B	M	S	Phase 1	Phase 2	Phase 3
Proposed benzoate uptake							
BenK	Benzoate/ H^+ symporter	476	277		304	325	301
Proposed 4-methylbenzoate uptake							
Orf960	ABC-type transport system, permease component						100
Orf950	ABC-type transport system, permease component		74			62	185
Orf930	ABC-type transport system, ATPase component		115				58
Orf920	ABC-type transport system, ATPase component		153			46	133
Proposed C_4-dicarboxylate uptake							
DctQ	TRAP-type C_4-dicarboxylate transport system	82		252		129	157
DctM	TRAP-type C_4-dicarboxylate transport system			98	63		
DctA	C_4-dicarboxylate/ Na^+ symporter			254	65	113	137

Mascot score: ND | > 20 | > 200 | > 500 | > 1000

[a] Abbreviation: M, 4-methylbenzoate; B, benzoate; S, succinate. For details on the respective phases see Fig. 13.
ND: not detected

3.4 Effects of aromatic acids on C_4-dicarboxylate utilization

A homology search in the genomic shotgun sequence database of strain pMbN1 revealed genes for two putative succinate uptake systems, a C_4-dicarboxylate TRAP transporter (DctPQM) and a C_4-dicarboxylate symporter (DctA). In addition, strain pMbN1 requires the malic enzyme (MaeB) and the pyruvate dehydrogenase (PdhABC, LpdA) to replenish acetyl-CoA during growth with succinate (Fig. 13). Proteins and transcripts involved in uptake and catabolism of succinate showed significantly decreased abundances in phase 1 during growth with the ternary substrate mixture (Fig. 13). The strongest decrease in protein (up to –11.8-fold) and transcript (up to –19-fold) abundances was observed for the β-subunit of the pyruvate dehydrogenase E1 component (PdhB) and levels were comparable to growth with benzoate or 4-methylbenzoate supplied as single substrates (Fig. 13). In contrast, transcript levels of *dctPM*, *dctA* and *maeB* were already increased during phase 1 (ternary substrate mixture) compared to growth with benzoate or 4-methylbenzoate (single substrates) (Fig. 13). Moreover, DctP, DctM and DctA proteins were already present in phase 1 (Fig. 13, Table 6), indicating the ability of cells to transport succinate. This agrees with the partial depletion of succinate from the culture medium already during phase 1 (Fig. 12) and suggests that the presence of succinate partially induced expression of genes involved in uptake (*dctPM, dctA*) and conversion of malate to pyruvate (*maeB*) (Fig. 13).

Bacteria containing several dicarboxylate transport systems usually control their formation in response to certain growth condition (e.g. aerobic/anaerobic) and property or quantity of the transported solute (for a review see Janausch *et al*. 2002). E.g. DctA of *E. coli* is only synthesized under oxic conditions (Davies *et al*. 1999). Surprisingly, strain pMbN1 forms not only DctA under nitrate-reducing conditions, but apparently employs two different dicarboxylate transport systems during growth with succinate. The similar dynamics of *dctA* and *dctPM* transcripts throughout phases 1–3 (Fig. 13) is suggestive of a coordinated control of dicarboxylate uptake in strain pMbN1. In *P. aeruginosa* PAO1 gene expression for the transporters DctA and DctPQM is controlled by the two-component regulatory system DctBD and reversely modulated by the RNA-binding protein Crc (Valentini *et al*. 2011; Valentini and Lapouge 2013). While the strategy of strain pMbN1 to control formation of DctA and DctPQM on the transcriptional level is presently unknown, homologs of DctBD and DctSR controlling

gene expression for C_4-dicarboxylate transporters in other bacteria (reviewed in Janausch *et al*. 2002) are encoded in the shotgun genome sequence of strain pMbN1.

Unlike *maeB*, *dctPM* and *dctA*, transcript levels of *pdhB* were not affected by the additional presence of succinate in phase 1 (compared to single substrates; Fig. 13). In agreement, pyruvate dehydrogenase gene expression in *Sinorhizobium meliloti* (Cabanes *et al*. 2000) and *E. coli* (Quail *et al*. 1994; Quail and Guest 1995) requires intracellular pyruvate as inducer rather than succinate. Thus, the strongly reduced *pdhB* levels might have resulted from decreased succinate uptake and pyruvate formation in the presence of benzoate. Pyruvate is not a substrate of known DctPQM and DctA transporters (Shaw *et al*. 1991; Walmsley *et al*. 1992; Youn *et al*. 2009) and the preference of benzoate over pyruvate (Table 5) may indicate a direct benzoate-mediated repression of pyruvate dehydrogenase gene expression. Overall, the results indicate that the observed diauxie may be caused by a synergistic effect of reducing succinate uptake (*dctA*, *dctPM*) and acetyl-CoA forming reactions (*maeB*, *pdhB*).

3.5 Possible regulatory mechanism of benzoate-mediated repression

The molecular mechanism causing the observed benzoate-mediated repression of 4-methylbenzoate and C_4-dicarboxylate utilization in strain pMbN1 are presently unknown, but several possibilities can be envisioned.

Global regulators (e.g. Crp and Crc) are often involved in CCR observed in other bacteria and can affect expression of several hundred genes (reviewed in Görke and Stülke 2008; Rojo 2010). Repression of 4-methylbenzoate utilization could also involve a dually functional regulator, as observed for BenR in *P. putida*. In the presence of benzoate, BenR activates expression of genes for aerobic benzoate degradation and simultaneously represses the gene expression of the 4-hydroxybenzoate permease (PcaK) (Cowles *et al*. 2000). Another possibility could be an antagonistic binding of benzoate and 4-methylbenzoate to the same regulator (e.g. Orf880), similar to regulators of aerobic phenol degradation in *Ac. calcoaceticus* (Zhan *et al*. 2009) and 4-hydroxybenzoate in *Cupriavidus necator* (Donoso *et al*. 2011). Moreover, repression of C_4-dicarboxylate uptake might result from direct or indirect interference with the signal transduction of possible two-component regulatory systems (e.g. DctSR and/or DctBD).

3.6 Possible advantages of the observed substrate utilization phenotypes of strain pMbN1

Recently, a similar growth phenotype with a preference of benzoate over succinate was observed for the denitrifying aromatic compound degrader *"A. aromatoleum"* EbN1 (Trautwein *et al*. 2012a). However, strain EbN1 possesses only the DctPQM system for the uptake of succinate and benzoate was proposed to negatively control DctSR-mediated expression of *dctPQM* (Trautwein *et al*. 2012a). DctP level in strain EbN1 displayed a larger decrease during the benzoate utilization phase compared to strain pMbN1 and external succinate concentration remained constant during the first diauxic growth phase (Trautwein *et al*. 2012a). *Magnetospirillum* spp. are proposed to use the rather complex ethylmalonyl-CoA pathway to replenish TCA cycle intermediates drained by biosynthetic reactions (Erb *et al*. 2009). Strain EbN1 on the other hand fuels the TCA cycle through the glyoxylate shunt (Rabus *et al*. 2005), consisting of only two reactions. Therefore, partial consumption of succinate during phase 1 (Fig. 12) might be beneficial for strain pMbN1 to refill TCA cycle intermediates and thereby avoiding the need for anaplerotic reactions. Furthermore, the nature and concentration of aromatic compounds seems to be very well distinguished by strain pMbN1 and enzymes for the degradation of 4-methylbenzoate are only formed in the presence of 4-methylbenzoate. On the other hand, some genes for anaerobic benzoate degradation are co-induced by 4-methylbenzoate and this relaxed regulatory specificity might reflect the higher probability of strain pMbN1 to encounter substrates of the anaerobic benzoyl-CoA pathway in its natural habitat. Different reasons may exist why strain pMbN1 does not utilize substrates of the anaerobic benzoyl-CoA pathway concomitantly with 4-methylbenzoate. Possible explanations might be the higher efficiency of the benzoyl-CoA pathway in terms of growth yield and growth rate (Lahme *et al*. 2012a) and requirement of the same co-factors (CoA, reduced ferredoxin, ATP) for both pathways. In addition, benzoyl-CoA and 4-methylbenzoyl-CoA may compete for the active center of the classic benzoyl-CoA reductase (BcrCBAD) when consumed simultaneously, which could lead to a decreased rate of benzoyl-CoA dearomatization in the presence of 4-methylbenzoyl-CoA. Indeed, *para*-substituted benzoyl-CoA analogs (e.g. 4-fluoro-, 4-hydroxybenzoyl-CoA) are not substrates of BCR from *T. aromatica* (Boll and Fuchs 1995) and 4-fluorobenzoyl-CoA was shown to be a competitive inhibitor of BCR (Möbitz and Boll 2002; Boll 2005).

3.7 Substrate preferences as organism-specific features

So far, information on substrate preferences of anaerobic aromatic compound degrading bacteria was only available for *Betaproteobacteria* (*Thauera*, *Azoarcus* and *"Aromatoleum"* spp.) (Philipp and Schink 2000; López Barragán *et al*. 2004a; Trautwein *et al*. 2012a; Juárez *et al*. 2013). This is the first study investigating substrate preferences in an anaerobic aromatic compound degrading alphaproteobacterium and insights obtained by Trautwein and colleagues (2012a) allows the comparison between different subclasses of *Proteobacteria* isolated from similar habitats.

Despite distant phylogenetic relation of strain pMbN1 and EbN1, both strains preferred benzoate over C_4-dicarboxylates, which was not observed in close relatives of strain EbN1 (*"Aromatoleum"* strains HxN1 and ToN1, *T*. *aromatica* K172) (Trautwein *et al*. 2012a). Interestingly, preferences of strain EbN1 and pMbN1 differed with other co-substrates, e.g. strain EbN1 preferred phenylacetate over succinate, co-utilized 4-hydroxybenzoate with succinate or preferred oxaloacetate over benzoate (Trautwein *et al*. 2012a). The differences and similarities in substrate preferences and utilization patterns among close and distant relatives isolated from similar habitats suggests a different evolution of regulatory networks and may reflect the spatiotemporal availability of substrates each strain has encountered in its natural habitat (Cases and de Lorenzo 2005). The observed phenotypic plasticity is not exclusive for anaerobic aromatic compounds degrading *"Aromatoleum"* spp. (Rabus and Widdel 1995b; Ehrenreich *et al*. 2000; Trautwein *et al*. 2012a) and has been also reported for aerobic aromatic compound degrading *Pseudomonas* spp. (Heinaru *et al*. 2001). Therefore, substrate preferences should be assessed individually for aromatic compound degrading bacteria even among close relatives.

4. Outlook

Most studies on anaerobic aromatic compound degradation were performed with members of the *Betaproteobacteria*. The present study expands the knowledge to anaerobic aromatic compound degrading *Alphaproteobacteria* and may help to identify differences and similarities among different genera in their anabolic and catabolic pathways and their regulation. The identification of the anaerobic 4-methylbenzoyl-CoA pathway in strain pMbN1 represents a solution for the fate of 4-methylbenzoate in

the anaerobic degradation of *p*-xylene. However, strain pMbN1 is unable to grow with *p*-xylene, thus it would be necessary to prove the operation of the anaerobic 4-methylbenzoyl-CoA pathway in anaerobic *p*-xylene degrading bacteria. In this context the recent discovery of the 3-methylbenzoyl-CoA pathway in *Azoarcus* sp. CIB (capable of anaerobic growth with *m*-xylene) (Juárez *et al*. 2013) supports the functioning of the 4-methylbenzoyl-CoA pathway in anaerobic *p*-xylene degrading bacteria. The recent isolation of the sulfate-reducing bacterium strain PP31, capable of anaerobic *p*-xylene degradation (Higashioka *et al*. 2012), enables the study of the entire anaerobic degradation pathway of *p*-xylene. Furthermore, aromatic compound degrading sulfate-reducing bacteria (and other strictly anaerobic bacteria) are using usually ATP-independent class II BCR to dearomatize the aromatic ring of benzoyl-CoA, due to energetic constraints (Boll 2005b; Kung *et al*. 2009; Löffler *et al*. 2011). Thus, one may speculate that an ATP-independent 4-methylbenzoyl-CoA reductase may exist in *p*-xylene (or 4-methylbenzoate) degrading strict anaerobes.

The present studies with strain pMbN1 indicate that the *para*-methyl group is retained during and beyond dearomatization of the aromatic ring. However, detection of 4-methylbenzoate and terephthalate during the anaerobic degradation of *p*-xylene in methanogenic enrichment cultures and in a gas-condensate contaminated aquifer suggest the possibility of another pathway for 4-methylbenzoate degradation (Elshahed *et al*. 2001). In this pathway the *para*-methyl group is suggested to be oxidized to form terephthalate, which is then decarboxylated to benzoate or benzoyl-CoA (Kleerebezem *et al*. 1999b; Elshahed *et al*. 2001).

The apparent ability of 4-methylbenzoyl-CoA reductase to also convert benzoyl-CoA (Lahme *et al*. 2012b) raises the question for the necessity to sustain two reductases in strain pMbN1. One possibility might be the ability to deal with different ring substitutions, e.g. benzoyl-CoA from *T*. *aromatica* can use a variety of different benzoyl-CoA analogous (Boll and Fuchs 1995). Thus, purification of both reductases and their biochemical characterization and comparison could yield valuable information on this point. Employing two reductases may increase the substrate spectrum of strain pMbN1 and/or increase the efficiency of the respective catabolism by using evolutionary optimized enzymes.

Gene expression of the anaerobic 4-methylbenzoyl-CoA pathway in strain pMbN1 seems to be strictly controlled and repressed in the presence of benzoate. The identification of specific regulators and factors responsible for the tight control may

help to understand the ability of strain pMbN1 to distinguish between structurally highly similar molecules (e.g. benzoate and 4-methylbenzoate). Furthermore, molecular mechanism of carbon catabolite repression have so far only been addressed in aerobic aromatic compound degraders (e.g. *P*. *putida*), but not in their anaerobic counterparts. Despite similar growth phenotypes of strain EbN1 and pMbN1 with a mixture of benzoate and succinate, there might be difference in their underlying control mechanisms.

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Part II: Publications

A MAIN RESEARCH ARTICLES

1

Anaerobic Degradation of 4-Methylbenzoate by a Newly Isolated Denitrifying Bacterium, Strain pMbN1

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Anaerobic Degradation of 4-Methylbenzoate by a Newly Isolated Denitrifying Bacterium, Strain pMbN1

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A novel alphaproteobacterium isolated from freshwater sediments, strain pMbN1, degrades 4-methylbenzoate to CO_2 under nitrate-reducing conditions. While strain pMbN1 utilizes several benzoate derivatives and other polar aromatic compounds, it cannot degrade *p*-xylene or other hydrocarbons. Based on 16S rRNA gene sequence analysis, strain pMbN1 is affiliated with the genus *Magnetospirillum*.

Aromatic compounds are structurally diverse and abundant in nature, ranging from alkylbenzenes and other crude oil components to lignin monomers and amino acids. Under anoxic conditions, which prevail in many habitats, most monoaromatic compounds are channeled via compound-specific reaction sequences into the central anaerobic benzoyl-coenzyme A (CoA) pathway for further degradation (5, 10). Among alkylbenzenes, *p*-xylene seems to be particularly difficult to degrade under anoxic conditions; for example, enrichment cultures of nitrate- (31) and sulfate-reducing bacteria (29, 44) with crude oil as the only source of organic carbon readily consumed *o*- and *m*-alkyltoluenes, but not the *p*-alkylated isomers. Attempts to isolate anaerobic bacteria growing with *p*-xylene as the sole source of carbon and energy have thus far not transcended the level of enrichment cultures (12,

TABLE 1 Anaerobically alkylbenzene- and alkyl benzoate-degrading proteobacteria[a]

Organism	Subgroup of *Proteobacteria*	Substrate for anaerobic growth		Cometabolic conversion of *p*-xylene to 4-methylbenzoate[d]	Reference(s)
		Alkylbenzene[b]	Benzoate/alkylbenzoate[c]		
Nitrate-reducing bacteria					
Thauera aromatica K172	*Beta-*	Toluene	Benzoate	+	2, 4
"*Aromatoleum aromaticum*" EbN1	*Beta-*	Toluene, ethylbenzene	Benzoate	ND	28
"*Aromatoleum*" sp. pCyN1	*Beta-*	Toluene, *p*-cymene, *p*-ethyltoluene	Benzoate, 4-isopropylbenzoate, 4-ethylbenzoate	ND	13
Magnetospirillum sp. pMbN1	*Alpha-*		Benzoate, 3-methylbenzoate, 4-methylbenzoate	ND	This study
Sulfate-reducing bacteria					
Desulfobacula toluolica strain Tol2	*Delta-*	Toluene	Benzoate	+	27, 30
Strain oXyS1	*Delta-*	Toluene, *o*-xylene	Benzoate, 2-methylbenzoate	ND	14
Strain mXyS1	*Delta-*	Toluene, *m*-xylene	Benzoate, 3-methylbenzoate	ND	14
Iron-reducing bacterium					
Geobacter metallireducens GS15	*Delta-*	Toluene	Benzoate	ND	20
Phototrophic bacterium					
Blastochloris sulfoviridis ToP1	*Alpha-*	Toluene	Benzoate	ND	48

[a] For a detailed overview of anaerobically alkylbenzene-degrading bacteria, see reference 43.
[b] None of the listed strains is capable of anaerobic degradation of *p*-xylene.
[c] Except for *Magnetospirillum* sp. pMbN1, none of the listed strains is capable of anaerobically degrading 4-methylbenzoate.
[d] Toluene-utilizing cells cometabolically convert *p*-xylene into 4-methylbenzoate as a dead-end product. +, positive for conversion; ND, not determined.

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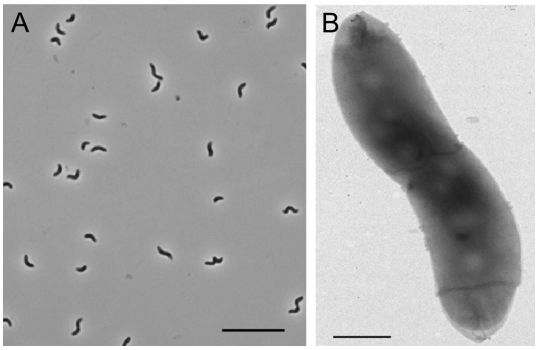

FIG 1 Micrographs of strain pMbN1. (A) Phase contrast micrograph. Bar, 10 μm. (B) Electron micrograph of negatively stained cell. Bar, 0.5 μm.

25, 32, 46). The recalcitrance of *p*-xylene is unlikely to result from difficulties with initial substrate activation, since early experiments with anaerobically toluene-degrading bacteria demonstrated *p*-xylene conversion to 4-methylbenzoate as a dead-end metabolite (4, 27) and analysis of *p*-xylene-consuming enrichment cultures revealed formation of (4-methylbenzyl)succinate as the initial metabolite (3, 25, 32). Thus, the subsequent degradation of 4-methylbenzoate appears to be the bottleneck in anaerobic *p*-xylene degradation, agreeing with the inability of presently known alkylbenzene-degrading (facultatively) anaerobic isolates to utilize 4-methylbenzoate (Table 1) (for an overview, see reference 42). Moreover, studies with wastewater-treating anaerobic bioreactors demonstrated the markedly longer time requirement for 4-methylbenzoate degradation than that for benzoate and phthalate isomers (16).

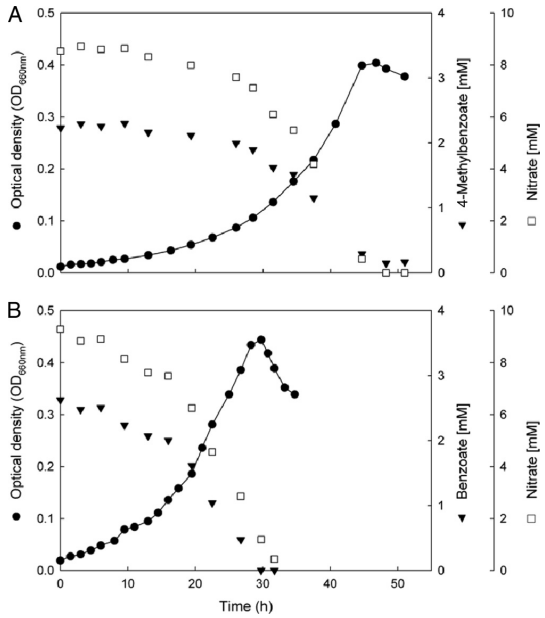

FIG 2 Anaerobic growth of strain pMbN1 with benzoates under nitrate-reducing conditions. (A) With 4-methylbenzoate. (B) With benzoate. Symbols are as indicated along the *y* axes. No intermediate formation of nitrite could be detected.

TABLE 2 Anaerobic and aerobic growth tests of denitrifying strain pMbN1 with different aromatic and nonaromatic compounds[a]

Compound tested (concn)[b]	Growth[c]	
	Anaerobic	Aerobic
Aromatic compounds		
4-Methylbenzoate (1, 2.5)	+	+
3-Methylbenzoate (1, 2)	+	−
Benzoate (1, 4)	+	+
2-Aminobenzoate (1, 2)	+	−
4-Hydroxybenzoate (1, 4)	+	−
Phenylacetate (1, 4)	+	−
Cinnamate (2)	+	+
Hydrocinnamate (2)	+	+
p-Coumarate (2)	+	+
Phenol (0.5, 2)	+	−
p-Cresol (0.5, 2)	+	−
Benzyl alcohol (0.5, 2)	+	−
Benzaldehyde (0.5, 2)	+	−
Nonaromatic compounds		
Acetate (1, 5)	+	+
Propionate (1, 4)	+	+
Butyrate (1, 4)	+	+
Isobutyrate (1, 4)	−	+
Adipate (1, 5)	+	+
Lactate (5, 10)	+	+
Malate (1, 5)	+	+
Pyruvate (1, 5)	+	+
Succinate (1, 5)	+	+
Ethanol (1, 5)	+	+
Complex media		
Yeast extract (0.5% [wt/vol])	+	+
Peptone (0.5% [wt/vol])	−	−
Casamino acids (0.5, 2% [wt/vol])	−	−

[a] Further compounds tested but not utilized by strain pMbN1 under nitrate-reducing or oxic conditions are as follows (concentrations given in percent volume/volume refer to dilutions of poorly water soluble compounds in heptamethylnonane as inert carrier phase): toluene (2%), 4-ethyltoluene (2%), ethylbenzene (2%), propylbenzene (2%), *m*-xylene (2%), *p*-xylene (1%), 2-methylbenzoate (1, 2 mM), 4-ethylbenzoate (1, 2 mM), 4-propylbenzoate (0.5, 2 mM), 4-isopropylbenzoate (1, 2 mM), 4-butylbenzoate (0.5, 2 mM), 4-*tert*-butylbenzoate (0.5, 2 mM), terephthalate (0.5, 2 mM), resorcinol (1 mM), *o*-cresol (0.5, 2 mM), *m*-cresol (0.5, 2 mM), 1-phenylpropanol (0.5, 2 mM), (*R*)-1-phenylethanol (0.5, 2 mM), (*S*)-1-phenylethanol (0.5, 2 mM), acetophenone (1%), propiophenone (1%), *p*-cymene (2, 5%), α-phellandrene (1, 2%), α-terpinene (1, 2%), limonene (1, 2%), cyclohexane carboxylate (0.5, 2 mM), cyclohexanol (1, 2%), cyclohexane-1,2-diol (1, 5 mM), *n*-hexane (2%), glucose (1, 5 mM), fructose (1, 5 mM), all 20 amino acids (0.5, 2 mM), ascorbate (4 mM), formate (10, 20 mM), acetone (0.5, 2 mM).

[b] Each compound was tested at the concentrations shown in parentheses and given in mM unless otherwise indicated.

[c] +, optical density at 660 nm of >0.1; −, no growth.

In the present study, enrichment of bacteria degrading 4-methylbenzoate under nitrate-reducing conditions was attempted in defined mineral medium (28) at 28°C in 250-ml bottles containing 200 ml of mineral medium, 15 ml of mud mixture (collected from ditches and the Weser River in Bremen, Germany), 1.5 mM 4-methylbenzoate, and 5 mM $NaNO_3$. Nitrate consumption was monitored by high-pressure liquid chromatog-

TABLE 3 Quantification of 4-methylbenzoate (substrate) and nitrate consumption and of produced biomass by strain pMbN1[a]

Experiment	Amt of substrate added (mmol)	Amt of substrate disappeared[b] (mmol)	Amt of nitrate disappeared (mmol)	Amt of cell dry mass formed[c] (mg)	Amt of substrate dissimilated[d] (mmol)	Amt of electrons from substrate dissimilated[e] (mmol)	Amt of electrons consumed by nitrate reduction[f] (mmol)
Cells with limiting amt of substrate	0.34 (0.05)	0.34 (0.13)	1.4 (0.4)	21 (3)	0.25 (0.06)	9.0 (2.0)	7.0 (1.8)
Cells with excess amt of substrate	0.89 (0.07)	0.83 (0.17)	3.4 (0.2)	56 (6)	0.57 (0.05)	20.7 (2.0)	17.1 (1.2)
Cells without substrate (control)	0.00[g]	0[g]	0.0[g]	0.0[g]			0.0[g]
Sterile medium without cells (control)	0.83[g]	0[g]	0.0[g]				0.0[g]

[a] Incubation experiments were carried out in anoxic flat glass bottles with a culture volume of 400 ml containing 3.4 mmol nitrate. Numbers in parentheses represent the standard deviation calculated from triplicate experiments.
[b] Difference between substrate added and substrate recovered at the end of incubation in the culture supernatant.
[c] The amount of cell dry mass added with the inoculum has been subtracted.
[d] Differences between substrate dissimilated and substrate assimilated. The assimilated amount of 4-methylbenzoate was calculated according to the following equation: $17\ C_8H_7O_2^- + 8\ HCO_3^- + 25\ H^+ + 50\ H_2O \rightarrow 36\ C_4H_7O_3$. Thus, 1 mg of cell dry mass requires 0.00458 mmol 4-methylbenzoate.
[e] Thirty-six moles of electrons are derived from 1 mol of 4-methylbenzoate if oxidized to CO_2.
[f] Electrons consumed = 5 × (nitrate added − nitrate remaining). Nitrite has not been observed in detectable quantities during growth.
[g] No detectable deviations in duplicate experiments.

raphy (HPLC), and nitrate was several times replenished upon depletion. The initial enrichment culture grew within 14 days. The sediment-free subcultures obtained after four transfers displayed a doubling time of 10 h, resembling those of earlier reported enrichments with 4-methylbenzoate and nitrate (13). After several passages, the sediment-free enrichment cultures were dominated by spirillum-shaped cells. Isolation of 4-methylbenzoate-degrading nitrate-reducing bacteria was then attempted with anoxic agar-dilution series (41). Whitish colonies were retrieved by means of finely-drawn Pasteur pipettes and transferred to liquid media. The best-growing strain was chosen for further analysis and designated pMbN1. For further cultivation of strain pMbN1 under nitrate-reducing conditions, the reductant ascorbate (4 mM) was routinely added to the mineral medium. All of the following growth experiments were conducted in triplicate. Additional information on materials and methods is provided in the supplemental material.

The isolate strain pMbN1 had a spirillum-like shape (Fig. 1) with dimensions of 1.8 to 3.6 μm by 0.6 to 0.8 μm. Cells stained as Gram-negative and were motile. However, they did not display a magnetotactic response during microaerobic growth (33; D. Schüler, personal communication). Strain pMbN1 did not grow on solid media (rich or mineral) under oxic or nitrate-reducing conditions; anaerobic incubation of plates was carried out at 28°C in jars under an N_2 atmosphere as recently described (45).

The temperature range of anaerobic growth of strain pMbN1 with 4-methylbenzoate was tested in a temperature gradient block. The observed temperature range of growth was 11.9 to 37.2°C, with an optimum range of 26.2 to 35.7°C (determined by means of maximal growth rates [μ_{max}]; see the Arrhenius plot in Fig. S1A in the supplemental material). All subsequent cultures were incubated at 28°C. The pH range of anaerobic growth with 4-methylbenzoate was pH 6.8 to pH 8.0, with an optimum around pH 7.3 to pH 7.7 (determined using μ_{max}; see Fig. S1B in the supplemental material).

Approximate doubling times of strain pMbN1 during anaerobic growth with 4-methylbenzoate and benzoate were 9.5 h (specific μ_{max}, 0.10 h^{-1}) and 6.1 h (specific μ_{max}, 0.16 h^{-1}), respectively (Fig. 2).

In addition to 4-methylbenzoate, a wide range of other benzoate derivatives, phenylpropanoids, and aliphatic carboxylates were anaerobically utilized by strain pMbN1. All compounds tested for supporting aerobic or anaerobic growth are indicated together with the applied concentrations in Table 2. Remarkably, strain pMbN1 could not grow anaerobically with other 4-alkylbenzoates, *p*-xylene, or other hydrocarbons, indicating a specialization for benzoate derivatives and simple aliphatic carboxylates. Although strain pMbN1 is facultatively aerobic, it could degrade only a few aromatic compounds under oxic conditions. The nutritional specialization of strain pMbN1 is further underpinned by its inability to utilize amino acids or carbohydrates (Table 2) and by the lack of lithoautotrophic growth with H_2 (10% [vol/vol] in gas headspace) as the electron donor and nitrate (10 mM), chlorate (5 and 10 mM), or perchlorate (5 and 10 mM) as the electron acceptor.

During growth with 4-methylbenzoate under nitrate (NO_3^-)-reducing conditions, strain pMbN1 did not intermediately excrete nitrite (NO_2^-), as do other aromatic compound-degrading denitrifiers, such as "*Aromatoleum aromaticum*" EbN1 (28). At the end of nitrate-limited growth (10 mM NO_3^- consumed), no accumulation of ammonium (NH_4^+) was observed, but formation of 1.3 mM dinitrogen monoxide (N_2O) and 2.4 mM dinitrogen (N_2) was detected. This suggests that strain pMbN1 reduces nitrate via denitrification. NO_3^- and NO_2^- were quantified using an HPLC system equipped with an anion exchange column and an UV detector as described previously (28). NH_4^+ was spectrophotometrically measured using the indophenol method (23). Formation of N_2O and N_2 was determined by gas chromatography as recently described (47).

The biochemical challenges imposed by the *para*-methyl group of 4-methylbenzoate on the dearomatization of this compound as well as on the subsequent reaction sequence (ring cleavage and β-oxidation) raised a question about the capacity of strain pMbN1 to anaerobically oxidize 4-methylbenzoate completely to CO_2. Thus, the degradation of this compound coupled to denitrification was balanced (Table 3) using cultures (400 ml) of strain pMbN1 provided with limiting (0.34 mmol) or excess (0.89 mmol) amounts of 4-methylbenzoate relative to the added

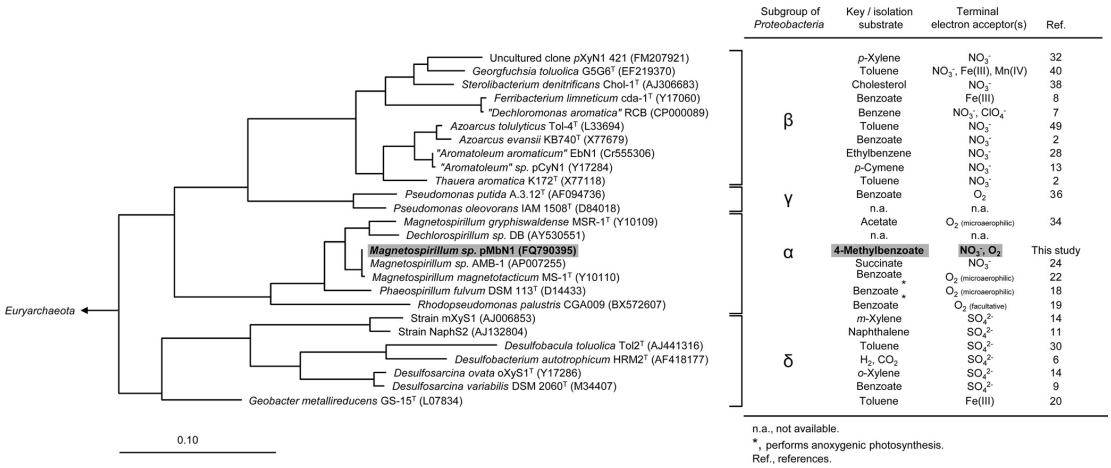

FIG 3 Phylogenetic tree of 16S rRNA gene sequence from strain pMbN1. The cluster shown represents selected members of the *Proteobacteria*. Scale bar, 10% sequence divergence.

amount of electron acceptor (3.4 mmol nitrate). Determination of the growth balance was based on quantifying consumption of 4-methylbenzoate and nitrate (by HPLC as described above), as well as on formation of biomass when cultures reached the stationary growth phase. Determination of the dry mass of cells involved a washing step with Tris buffer (100 mM Tris-HCl, 5 mM $MgCl_2$, pH 7.5) and drying at 80°C to constant weight. The addition of limiting amounts of 4-methylbenzoate resulted in its complete depletion, while about half of the provided nitrate was recovered. In the experiment with excess amounts of 4-methylbenzoate, 7.2% (±0.6%) of the organic substrate was recovered, while the added nitrate was completely consumed. The average molar growth yield of strain pMbN1 determined from these two organic substrate conditions was 65 g of dry mass per mol 4-methylbenzoate. The amount of electrons formed from dissimilation of 4-methylbenzoate was close to the amount of electrons consumed by nitrate reduction, in agreement with the following stoichiometric equation for complete substrate oxidation:

$$C_8H_7O_2^- + 7.2\ NO_3^- + 0.4\ H_2O + 0.2\ H^+ \rightarrow 8\ HCO_3^- + 3.6\ N_2$$

$$\Delta G^{\circ\prime} = -3{,}583\ \text{kJ/mol 4-methylbenzoate}$$

Calculation of free energy ($\Delta G^{\circ\prime}$) is based on standard values (37, 39). The dissimilated amount of 4-methylbenzoate was about 20% higher than required for nitrate reduction, as has previously also been observed for the anaerobic oxidation of ethylbenzene and *p*-cymene in the denitrifying *Aromatoleum* strains EbN1 and pCyN1, respectively (13, 28), assumed to result from partial conversion of the substrate to unknown organic compounds.

The G+C content of 65.9 mol% was inferred from a merged draft genome sequence of strain pMbN1 (5 Mbp, 251 contigs; M. Kube and R. Reinhardt, personal communication). The 16S rRNA gene sequence was retrieved and finished from the genomic shotgun database using the BLASTN (1) and RNAmmer (17) programs. Phylogenetic analysis using the Silva database (26) and the ARB software package (21) revealed affiliation of strain pMbN1 with the genus *Magnetospirillum* of the *Alphaproteobacteria* (34) (Fig. 3). Thus, strain pMbN1 is phylogenetically distinct from the *Aromatoleum*/*Azoarcus*/*Thauera* cluster within the *Betaproteobacteria*, which comprises the majority of currently known aromatic compound-degrading denitrifiers (15, 43). Some members of the genus *Magnetospirillum* were previously shown to degrade various aromatic compounds under nitrate-reducing conditions; growth tests with the isomers of methylbenzoate were not reported (35). Among members of the related genus *Phaeospirillum*, utilization of aromatic compounds seems to be less well studied (18).

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Supplementary material

FIG. S1. Temperature and pH range for growth of strain pMbN1 with 4-methylbenzoate under nitrate-reducing conditions. (A) Arrhenius plot of temperature range determined from incubation experiments in a temperature gradient block. (B) pH dependent anaerobic growth rates.

2

Anaerobic degradation of 4-methylbenzoate via a specific 4-methylbenzoyl-CoA pathway

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Anaerobic degradation of 4-methylbenzoate via a specific 4-methylbenzoyl-CoA pathway

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Summary

The pathway for anaerobic degradation of 4-methylbenzoate was studied in the denitrifying alphaproteobacterium *Magnetospirillum* sp. strain pMbN1. Adaptation studies with whole cells indicated substrate-dependent induction of the capacity to degrade 4-methylbenzoate. Differential protein profiling (2D-DIGE) of 4-methylbenzoate- in comparison with benzoate- or succinate-adapted cells revealed the specific abundance increase of substrate-specific protein sets. Their coding genes form distinct clusters on the genome, two of which were assigned to 4-methylbenzoate and one to benzoate degradation. The predicted functions of the gene products agree with a specific 4-methylbenzoyl-CoA degradation pathway in addition to and analogous to the known anaerobic benzoyl-CoA degradation pathway. *In vitro* benzoyl-CoA and 4-methylbenzoyl-CoA reductase activities revealed the electron donor and ATP-dependent formation of the corresponding conjugated cyclic dienoyl-CoA/4-methyl-dienoyl-CoA products. The 4-methylbenzoyl-CoA reductase activity was induced in the presence of 4-methylbenzoate. In accordance, metabolite analysis of cultures grown with 4-methylbenzoate tentatively identified 4-methylcyclohex-1,5-diene-1-carboxylate. The 4-methylbenzoate induced genes were assigned to code for the putative 4-methylbenzoyl-CoA reductase; their products display pronounced sequence disparity from the conventional class I benzoyl-CoA reductase, which does not accept substituents at the *para*-position. Identification of 3-methylglutarate together with the formation of specific proteins for ring cleavage and β-oxidation in 4-methylbenzoate-adapted cells suggest conservation of the methyl group along the specific 4-methylbenzoyl-CoA degradation pathway.

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Introduction

Aromatic compounds represent a structurally diverse and globally widespread class of organic substances. They range from alkylbenzenes and other crude oil components to lignin monomers, secondary plant metabolites and amino acids. Many aromatic compounds can be utilized by aerobic or anaerobic bacteria. Under anoxic conditions most mono-aromatic compounds are channelled without O_2 involvement via a multitude of compound-specific reaction sequences into the central anaerobic benzoyl-CoA pathway for further degradation (Harwood *et al.*, 1999; Boll, 2005; Fuchs, 2008; Carmona *et al.*, 2009; Fuchs *et al.*, 2011).

Among alkyltoluenes and -benzoates, *p*-xylene and 4-methylbenzoate were found to be particularly recalcitrant. Denitrifying (Rabus *et al.*, 1999) and sulfate-reducing (Rabus *et al.*, 1996; Wilkes *et al.*, 2000) enrichment cultures growing with crude oil readily consumed *o*- and *m*-alkyltoluenes, but not the *p*-alkylated isomers. Co-metabolic conversion of *p*-xylene to 4-methylbenzoate by anaerobic toluene degraders (Biegert and Fuchs, 1995; Rabus and Widdel, 1995a) as well as detection of (4-methylbenzyl)succinate in *p*-xylene-utilizing enrichment cultures (Beller *et al.*, 1996; Morasch and Meckenstock, 2005; Rotaru *et al.*, 2010) indicated that the observed recalcitrance of *p*-xylene did not arise from hindrance(s) regarding the initial activation and/or the peripheral pathway, but rather from subsequent degradation of the 4-methylbenzoate intermediate. This

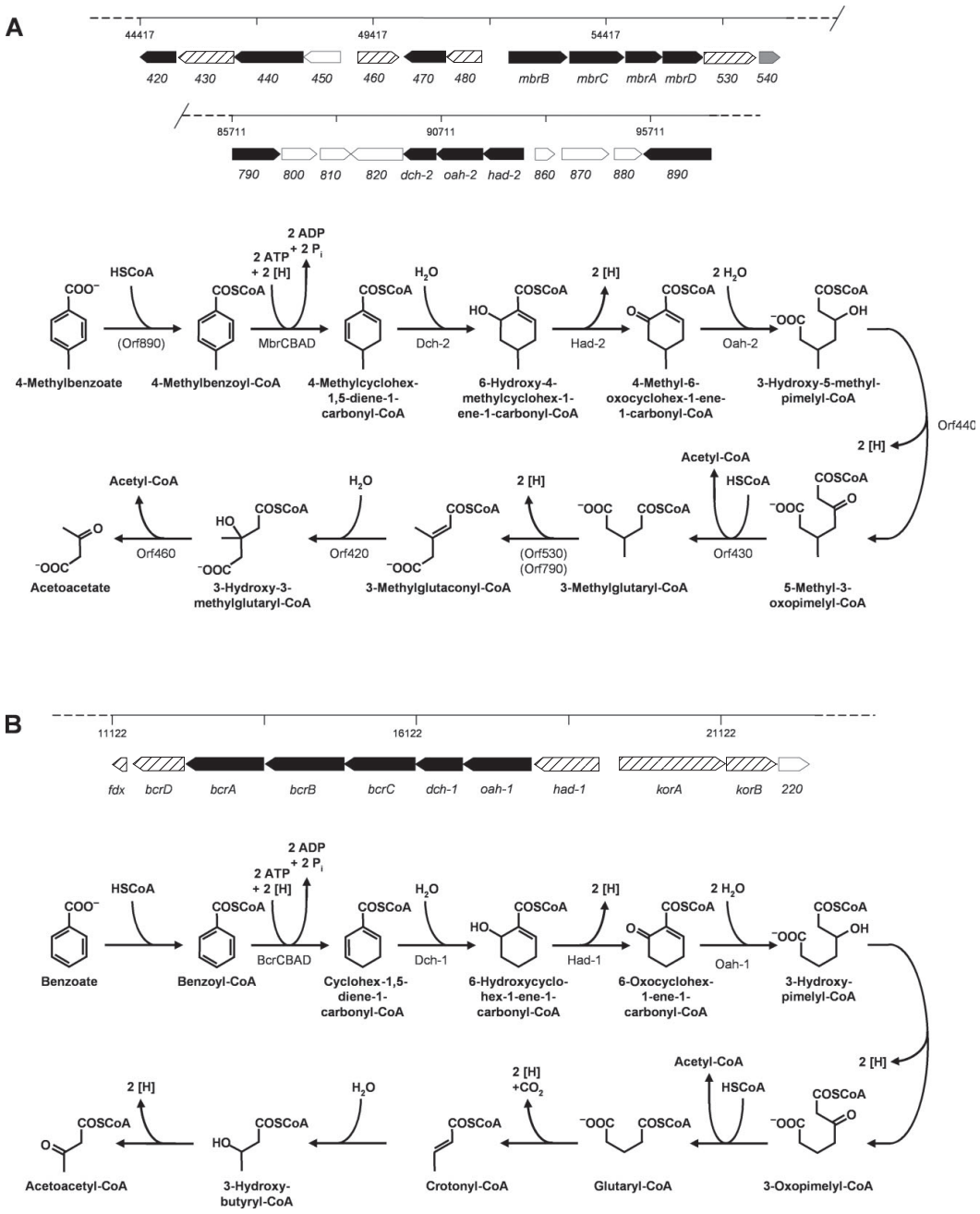
agrees with the inability of presently known anaerobic alkylbenzene degraders to grow with 4-methylbenzoate (e.g. Lovley *et al*., 1993; Rabus *et al*., 1993; Anders *et al*., 1995; Rabus and Widdel, 1995b; Harms *et al*., 1999a,b).

The biochemistry of anaerobic benzoate degradation is best understood in the facultatively anaerobic, denitrifying betaproteobacteria *Thauera aromatica* K172 and *Azoarcus evansii* KB740 (Anders *et al*., 1995) and the phototrophic alphaproteobacterium *Rhodopseudomonas palustris* (Gibson and Harwood, 2002). The degradation pathway can be divided into four major parts (Fig. 1). (i) Benzoate CoA-ligase activates benzoate to the CoA-thioester at the expense of ATP hydrolysis. Next to benzoate, the enzyme from *T. aromatica* K172 also converts 2-amino- and 2-, 3- and 4-fluorobenzoate; however, an activation of monomethylbenzoate isomers was not reported (Schühle *et al*., 2003). Similar properties were reported for benzoate CoA-ligase from *Azoarcus* sp. CIB (López Barragán *et al*., 2004a) and *Magnetospirillum* sp. strain TS-6 (Kawaguchi *et al*., 2006). (ii) The key enzyme of the pathway, benzoyl-CoA reductase, catalyses the ferredoxin-dependent reduction of benzoyl-CoA to cyclohex-1,5-diene-1-carbonyl-CoA (dienoyl-CoA), requiring hydrolysis of 2 MgATP to 2 MgADP for transfer of electrons (at −1.9 V) to the aromatic ring (Boll and Fuchs, 1995; 1998; Boll *et al*., 1997; 2000; Unciuleac and Boll, 2001). There are two classes of benzoyl-CoA reductases: class I enzymes are FeS enzymes, which couple benzoyl-CoA reduction to a stoichiometric ATP hydrolysis and are used by facultative anaerobes. The recently identified class II benzoyl-CoA reductases are W-cofactor containing enzymes that are ATP-independent and occur in strict anaerobes (Kung *et al*., 2009). Notably, an ATP-consuming class I benzoyl-CoA reductase and not the class II type was recently detected in the archaeon *Ferroglobus placidus* (Holmes *et al*., 2012). Based on kinetic and spectroscopic studies with *T. aromatica* K172, the dearomatization of benzoyl-CoA by benzoyl-CoA reductase was proposed to proceed via a sequential transfer of electrons and protons analogous to the chemical Birch reduction (Boll *et al*., 2001; Möbitz and Boll, 2002). The first electron transfer is considered rate-limiting, yielding a radical anion intermediate with a high electron density in the *para*-position. The subsequent protonation at this position should drive the unfavourable electron transfer forward. As a consequence the benzoyl-CoA reductase does not accept any analogue with a substituent at the *para*-position. A *para*-methyl substituent may represent a dual challenge for benzoyl-CoA reductase, due to its inductive effect on the radical intermediate and space filling property at the initial protonation site. (iii) A modified β-oxidation reaction sequence produces an intermediate suitable for ring cleavage. In a first step, a specific dienoyl-CoA hydratase (Dch) adds water to dienoyl-CoA (Laempe *et al*., 1998). Then, a 6-hydroxycyclohex-1-ene-1-carbonyl-CoA dehydrogenase (Had) oxidizes the secondary alcohol generating 6-oxocyclohex-1-ene-1-carbonyl-CoA, which is then hydrolytically cleaved by 6-oxocyclohex-1-ene-1-carbonyl-CoA hydrolase (Oah) to 3-hydroxypimelyl-CoA (Laempe *et al*., 1999). Notably, a variant of this reaction sequence leading to pimelyl-CoA exists in the photosynthetic bacterium *Rhodopseudomonas palustris* (Laempe *et al*., 1998; Pelletier and Harwood, 1998; 2000). (iv) 3-Hydroxypimelyl-CoA is degraded by further conventional β-oxidation-like reactions via glutaryl-CoA and crotonyl-CoA to 3 acetyl-CoA and 1 CO_2 (Harwood *et al*., 1999). The genes for the anaerobic benzoyl-CoA pathway in facultative anaerobes are highly similar with respect to sequence and genomic organization (Fig. 1B), e.g. *T. aromatica* K172 (Breese *et al*., 1998), *R. palustris* (Egland *et al*., 1997), *Azoarcus* sp. strain CIB (López Barragán *et al*., 2004a), *Magnetospirillum* strains (López Barragán *et al*., 2004b; Shinoda *et al*., 2005) and '*Aromatoleum aromaticum*' EbN1 (Rabus *et al*., 2005).

Considering the mechanistic constraints of conventional Bcr towards conversion of 4-methylbenzoate, two scenarios for its degradation may be envisioned. (i) The *para*-methyl group is initially oxidized to a carboxyl group, which is subsequently removed. The inability of strain pMbN1 to grow with terephthalate (Lahme *et al*., 2012) would disagree with such a scenario, if this incapacity were not due to lack of substrate uptake. (ii) The *para*-methyl group can be handled by a novel type of Bcr and is removed after dearomatization or retained along the complete degradation pathway.

Here, we report on a specific 4-methylbenzoyl-CoA pathway, containing a novel class I Bcr and retaining the

Fig. 1. Likely anaerobic degradation pathways and related, identified gene clusters in denitrifying strain pMbN1.
A. For 4-methylbenzoate.
B. For benzoate; as originally proposed for *T. aromatica* K172 (e.g. Breese *et al*., 1998) and subsequently also shown for other anaerobic degraders (for overview see, e.g. Carmona *et al*., 2009).
Acetoacetate (pathway A), or acetoacetyl-CoA (pathway B) are both further converted to two acetyl-CoA, which are terminally oxidized to CO_2 via the TCA-cycle. Enzyme names and predicted functions of the indicated gene products are provided in Table 1. Scale models for gene organization (contig c393 in A; contig c375 in B), with the scale bar indicating nucleotide positions on the assembled contigs rather than chromosomal location. Products of genes identified on 2D-DIGE gels (Table 1 and Fig. 4) are marked in black (assigned function) or grey (unassigned function). Genes with proposed function in the degradation pathway, the products of which were not identified by proteomics are striped. Products of genes marked in white have also not been identified by proteomics.



para-methyl group, in the denitrifying *Magnetospirillum* sp. strain pMbN1. This alphaproteobacterium was isolated from freshwater sediment and completely oxidizes 4-methylbenzoate as well as benzoate to CO_2 under anoxic conditions (Lahme *et al*., 2012).

Results and discussion

Cultures of strain pMbN1 adapted to anaerobic growth with 4-methylbenzoate, benzoate or succinate were used to detect substrate-specific metabolites, proteins and enzyme activities, in order to elucidate the pathway for anaerobic degradation of 4-methylbenzoate and to identify the respective genes.

Adaptation studies with cell suspensions

Initial hints for differences in the anaerobic utilization of benzoate and 4-methylbenzoate were obtained from whole-cell adaptation experiments (Fig. 2). Under all three adaptation conditions (succinate, benzoate and 4-methylbenzoate), the respective adaptation substrate was utilized without noticeable delay. Irrespective of the adaptation substrate, however, pronounced differences in the time-courses of benzoate and 4-methylbenzoate utilization were observed. In succinate-adapted cells (Fig. 2A), the benzoate utilization started after about 5 h of incubation, while the onset of 4-methylbenzoate utilization required more than 10 h. In benzoate-adapted cells (Fig. 2B), utilization of 4-methylbenzoate lagged about 5 h behind that of benzoate. Finally, in 4-methylbenzoate-adapted cells (Fig. 2C), the lag period for benzoate utilization also lasted for about 5 h. Triplicate cultivation experiments for all tested conditions yielded highly reproducible results (Fig. S1). Thus, the induction of the capacities for anaerobic utilization of benzoate and 4-methylbenzoate, respectively, appear to be substrate-specifically regulated.

Metabolite formation during anaerobic growth with 4-methylbenzoate

In 4-methylbenzoate-adapted cells, two metabolites were detected by gas chromatographic-mass spectrometric (GC-MS) analysis that did not occur in benzoate- or succinate-adapted cells (Fig. S2). The first metabolite was tentatively identified based on interpretation of the mass spectrometric fragmentation pattern, which is fully in agreement with the structure of a 4-methylcyclohexadiene-carboxylic acid methyl ester as illustrated in Fig. 3A. The locations of the double bonds in the six-membered ring cannot be deduced from the mass spectrum, but is suggested in analogy to the product of benzoyl-CoA reductase (Boll *et al*., 2000). The second metabolite was identified as 3-methylglutarate by comparison with an authentic standard (Fig. 3B). These metabolites agree with the pathway proposed in Fig. 1A. The identification of 3-methylglutarate provides strong evidence that the *para*-methyl group of 4-methylbenzoate is conserved beyond

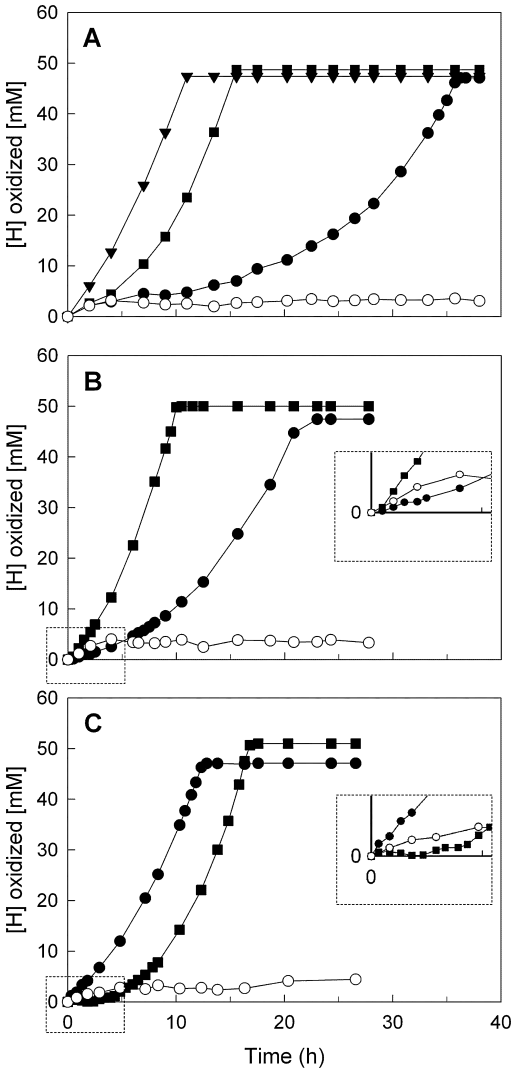


Fig. 2. Anaerobic degradation of aromatic compounds by substrate-adapted cell suspensions of strain pMbN1.
A. Cells were adapted to succinate.
B. Cells were adapted to benzoate.
C. Cells were adapted to 4-methylbenzoate.
Substrate-adapted cells were shifted to either succinate (▼), benzoate (■) or 4-methylbenzoate (●). Controls (○) did not contain any organic substrate. Replicate experiments are shown in Fig. S1.

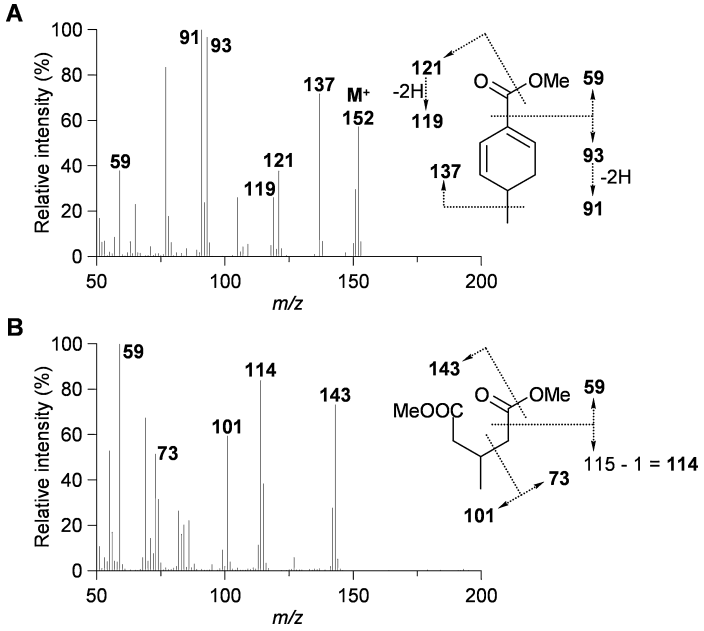


Fig. 3. Mass spectra of metabolites identified from 4-methylbenzoate-utilizing, denitrifying cultures of strain pMbN1.
A. 4-Methylcyclohexadienecarboxylic acid methyl ester.
B. 3-Methylglutaric acid dimethyl ester.
GC-MS analysis was performed after derivatization with diazomethane. The origin of key fragment ions is illustrated. In the mass spectrum of 3-methylglutaric acid dimethyl ester, the molecular ion is absent (molecular mass 174 amu).

the level of ring cleavage. Moreover, the 4-methylbenzoate-specific formation of both metabolites is congruent with the whole-cell adaptation experiments in that the capacity for 4-methylbenzoate degradation is induced by the substrate.

Proteomic signatures of 4-methylbenzoate- versus benzoate-adapted cells

The proteomic signatures of 4-methylbenzoate- and benzoate-adapted cells of strain pMbN1 were revealed by two-dimensional difference gel electrophoresis (2D-DIGE), with succinate-adapted cells serving as reference state. In total, 124 protein spots displayed significant abundance differences (*n*-fold change of $> |2.5|$). Of these, 57 were identified by mass spectrometry and shown to represent 31 different protein species (Table S1). Finally, 17 of the identified proteins could be assigned to the anaerobic degradation pathways for 4-methylbenzoate and benzoate respectively (Table 1, Figs 1 and 4).

4-Methylbenzoate-adapted cells contained a set of proteins that was specifically upregulated (~10- to ~130-fold; absent in benzoate-adapted cells) and highly abundant (in total ~15.3% of 2DE-resolvable proteome). Four of these proteins, termed MbrBCAD, represent homologues of conventional class I Bcr and are proposed to function as 4-methylbenzoyl-CoA reductase, dearomatizing 4-methylbenzoate to 4-methylcyclohex-1,5-diene-1-carbonyl-CoA (4-methyldienoyl-CoA). Subsequently, identified Dch-2, Had-2 and Oah-2 proteins (homologues of dienoyl-CoA hydratase, 6-hydroxycyclohex-1-ene-1-carbonyl-CoA dehydrogenase and 6-oxocyclohex-1-ene-1-carbonyl-CoA hydrolase from *T. aromatica* K172) could convert 4-methyldienoyl-CoA to 3-hydroxy-5-methylpimelyl-CoA. Further degradation to 3-methylglutaryl-CoA could be accomplished via conventional β-oxidation by Pmbn1w00440 (identified by proteomics; predicted 3-hydroxyacyl-CoA dehydrogenase) and Pmbn1w00430 (predicted acetyl-CoA acetyltransferase). Finally, 3-methylglutaryl-CoA, which contains a tertiary carbon atom impeding conventional β-oxidation, could be further degraded by a reaction sequence analogous to the leucine/isovalerate pathway yielding acetoacetate. Enzyme candidates for this reaction sequence could be Pmbn1w00530, Pmbn1w00790 (both predicted acyl-CoA dehydrogenase), Pmbn1w00420 (predicted enoyl-CoA hydratase) and Pmbn1w00460 (predicted hydroxymethylglutaryl-CoA lyase). Taken together, these proteins could be assumed to constitute a specific 4-methylbenzoyl-CoA pathway capable of dealing with the *para*-methyl group.

Benzoate-adapted cells showed highest abundances of BcrBCA (conventional class I benzoyl-CoA reductase), Dch-1 and Oah-1 (both involved in β-oxidation of the dienoyl-CoA), which together accounted for ~6.7% of the 2DE-resolvable proteome. Surprisingly, these proteins could also be detected in 4-methylbenzoate-adapted

Table 1. Proteins likely involved in anaerobic degradation of 4-methylbenzoate and benzoate in denitrifying strain pMbN1.

			Protein identification	
Gene identifier[a]	Protein name	Predicted function	Mascot score (spot No.)	Peptide counts
420		Enoyl-CoA hydratase	221	23
430		Acetyl-CoA acetyltransferase	n.d.	n.d.
440		3-Hydroxyacyl-CoA dehydrogenase	248	28
450		Enoyl-CoA hydratase	n.d.	n.d.
460		Hydroxymethylglutaryl-CoA lyase	n.d.	n.d.
470		Electron transfer flavoprotein, alpha subunit	142	15
480		Electron transfer flavoprotein, beta subunit	n.d.	n.d.
490	MbrB	4-Methylbenzoyl-CoA reductase, subunit B	327 (I), 287 (II)	34 (I), 32 (II)
500	MbrC	4-Methylbenzoyl-CoA reductase, subunit C	152	19
510	MbrA	4-Methylbenzoyl-CoA reductase, subunit A	188 (I), 163 (II), 53 (III)	19 (I), 18 (II), 9 (III)
520	MbrD	4-Methylbenzoyl-CoA reductase, subunit D	100 (I), 176 (II), 122 (III), 149 (IV)	14 (I), 20 (II), 16 (III), 18 (IV)
530		Acyl-CoA dehydrogenase	n.d.	n.d.
540		Uncharacterized conserved protein	118	14
790		Acyl-CoA dehydrogenase domain protein	231	26
800		MaoC-like dehydratase	n.d.	n.d.
810		Enoyl-CoA hydratase	n.d.	n.d.
820		Acetyl-CoA hydrolase/transferase	n.d.	n.d.
830	Dch-2	4-Methylcyclohex-1,5-diene-1-carbonyl-CoA hydratase	73	10
840	Oah-2	4-Methyl-6-oxocyclohex-1-ene-1-carbonyl-CoA hydrolase	263 (I), 313 (II)	28 (I), 31 (II)
850	Had-2	6-Hydroxy-4-methylcyclohex-1-ene-1-carbonyl-CoA dehydrogenase	154	18
860		Acyl dehydratase	n.d.	n.d.
870		ABC-type branched-chain amino acid transport systems periplasmic component	n.d.	n.d.
880		TetR-family transcriptional regulator	n.d.	n.d.
890		AMP-dependent synthetase and ligase	192	28
120	Fdx	Ferredoxin	n.d.	n.d.
130	BcrD	Benzoyl-CoA reductase, subunit D	n.d.	n.d.
140	BcrA	Benzoyl-CoA reductase, subunit A	1501	22
150	BcrB	Benzoyl-CoA reductase, subunit B	1089 (I), 848 (II)	19 (I), 16 (II)
160	BcrC	Benzoyl-CoA reductase, subunit C	1138	21
170	Dch-1	Cyclohex-1,5-diene-1-carbonyl-CoA hydratase	348	6
180	Oah-1	6-Oxocyclohex-1-ene-1-carbonyl-CoA hydrolase	1313 (I), 1562 (II)	25 (I), 25 (II)
190	Had-1	6-Hydroxycyclohex-1-ene-1-carbonyl-CoA dehydrogenase	n.d.	n.d.
200	Kor-A	2-Oxoglutarate:ferredoxin oxidoreductase, alpha subunit	n.d.	n.d.
210	Kor-B	2-Oxoglutarate:ferredoxin oxidoreductase, beta subunit	n.d.	n.d.
220		Uncharacterized conserved protein	n.d.	n.d.

a. The three-digit number has to substitute the triple X at the end of the identifier pmbn1w00XXX.
n.d., not determined.

cells, even though at lower abundance (~2.7% of the 2DE-resolvable proteome) (Fig. 4), suggesting that the conventional anaerobic benzoyl-CoA pathway is regulated with a somewhat relaxed substrate specificity.

Gene clusters for anaerobic degradation of benzoate and 4-methylbenzoate

Gene clusters for the anaerobic degradation of benzoate and 4-methylbenzoate were identified by proteomic-directed assembly (Table S2) of reads from a genomic shotgun sequence database of strain pMbN1, yielding two contigs (131 091 bp and 142 219 bp) of finished quality. The two gene clusters (13 757 bp and 11 496 bp) assigned to anaerobic degradation of 4-methylbenzoate to acetoacetyl-CoA are present on the larger contig, where they are separated by 27 537 bp (Fig. 1A). Genes for the anaerobic degradation of benzoate to 3-hydroxypimelyl-CoA cluster in a single operon-like structure (11 424 bp) (Fig. 1B); the gene organization is similar to that previously reported for the denitrifying betaproteobacteria *T. aromatica* K172 (Breese *et al.*, 1998), *Azoarcus* sp. CIB (López Barragán *et al.*, 2004a), *Magnetospirillum* strains (López Barragán *et al.*, 2004b; Shinoda *et al.*, 2005) and '*Aromatoleum aromatoleum*' EbN1 (Rabus *et al.*, 2005). The predicted functions of the genes constituting these three clusters are listed in Table 1 (InterPro/COG references and relevant BLAST hits are presented in Table S3).

In addition to overall sequence similarity with benzoyl-CoA reductase subunits from *T. aromatica* K172, those of the proposed 4-methylbenzoyl-CoA reductase from strain pMbN1 contained the following expected functional domains (Fig. S3): the MbrAD proteins share the same amino acids with BcrAD (*T. aromatica* K172) and HgdC (*Acidaminococcus fermentans*) required for binding and hydrolysis of ATP as well as the conserved Cys residues for interfacial coordination of the electron transferring [4Fe-4S] cluster (Hans *et al.*, 1999; Locher *et al.*, 2001).

The two subunits of the electron transfer flavoprotein (Etf), Pmbn1w00470 (abundantly formed) and Pmbn1w00480 (not detected by proteomics) encoded within the *mbrBCAD*-containing gene cluster may be involved in β-oxidation reactions, by serving as intermediary electron shuttles between an acyl-CoA dehydrogenase and NAD^+ (for overview on Etfs in anaerobic bacteria see Herrmann *et al.*, 2008).

Activity measurement of 4-methylbenzoyl-CoA reductase

Specific activities of presumptive 4-methylbenzoyl-CoA reductase and conventional class I benzoyl-CoA reductase were determined in cell extracts of substrate-adapted cells, using synthesized 4-methylbenzoyl-CoA and benzoyl-CoA as substrates (Fig. 5).

Benzoate-adapted cells showed high activity (50 nmol mg protein^{-1} min^{-1}) with benzoyl-CoA, but only marginal activity (2 nmol mg protein^{-1} min^{-1}) with 4-methylbenzoyl-CoA. This agrees with earlier observations that purified benzoyl-CoA reductase from *T. aromatica* K172 did not use 4-methylbenzoyl-CoA as substrate (M. Boll, unpubl. data). In contrast, 4-methylbenzoate-adapted cells showed similarly high activities with 4-methylbenzoyl-CoA (68 nmol mg protein^{-1} min^{-1}) and benzoyl-CoA (76 nmol mg protein^{-1} min^{-1}). As expected, succinate-adapted cells had no activity for reducing either of the two provided aromatic CoA-thioesters.

The presented proteomic data showed the presence of both conventional Bcr and novel Mbr in 4-methylbenzoate-grown cells (Fig. 4C), agreeing with the observed reduction activities for both CoA-thioesters under this adaptation condition. Conversely, the formation of the novel proteins (MbrBCAD, Dch-2, Had-2 and Oah-2) assigned to the 4-methylbenzoyl-CoA pathway exclusively in 4-methylbenzoate-adapted cells (Fig. 4D) is in accord with the marginal activity of extracts from benzoate-adapted cells for 4-methylbenzoyl-CoA.

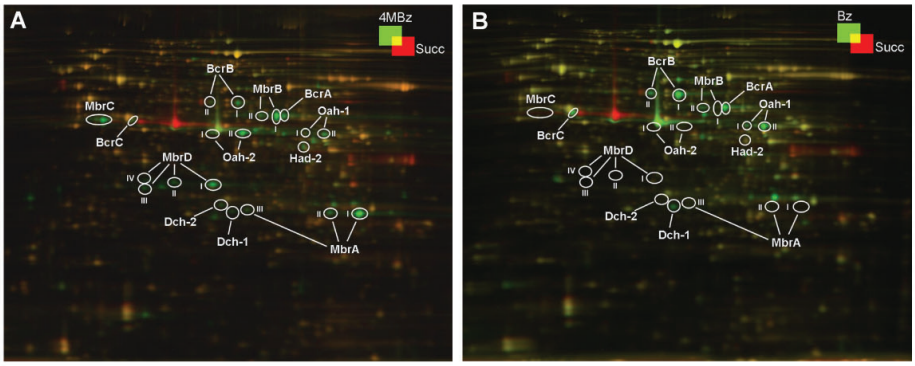
Phylogenetic relationships

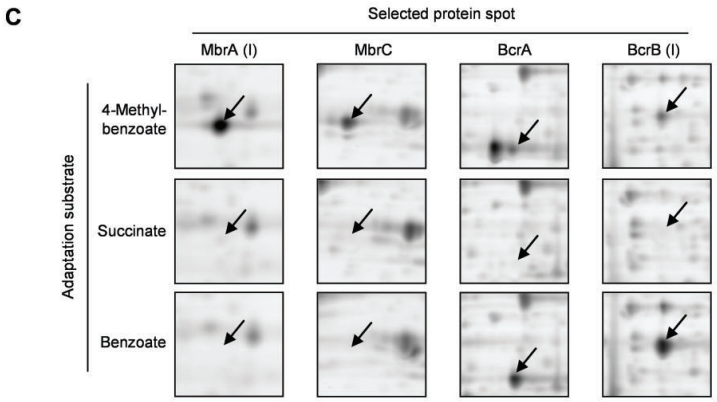
A phylogenetic tree of Bcr and Mbr proteins from strain pMbN1 is shown in Fig. 6 (alignments are shown in Fig. S3). The Bcr proteins affiliate more closely with the *Thauera* subclass of Bcrs than with the '*Aromatoleum*'/*Azoarcus* subclass, as has previously been reported for Bcrs of other *Magnetospirillum* spp. (López Barragán *et al.*, 2004b; Shinoda *et al.*, 2005). In contrast, the Mbr proteins display lower and varying degrees of relatedness with the subunits of conventional class I Bcrs. Most notably, MbrB branches even outside of the 2-hydroxyglutaryl-CoA (Hgd) line, while each of the MbrACD proteins forms a new subgroup within the *Thauera*-subclass of Bcrs. MbrBC should carry the catalytic centre of 4-methylbenzoyl-CoA reductase. In contrast, the MbrAD subunits are assumed not to be involved in ring reduction, but merely in activation of electrons. Thus, the observed phylogenetic affiliations of the Mbr subunits are in accord with the ability of the Mbr enzyme to dearomatize 4-methylbenzoyl-CoA.

Dch-2, Had-2 and Oah-2 proteins (Fig. S4) show high sequence similarities with the orthologues from *T. aromatica* K172.

Conclusions

The comparative genomic, proteomic and enzymatic data presented in this study suggest that two different benzoyl-





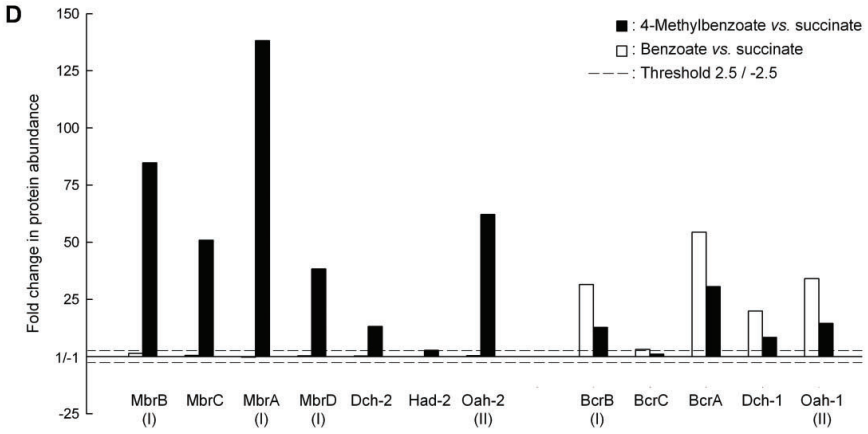


Fig. 4. Differential protein profiles of substrate-adapted cells of strain pMbN1 as revealed by two-dimensional difference gel electrophoresis (2D-DIGE).
A. Comparison of 4-methylbenzoate- with succinate-adapted cells.
B. Comparison of benzoate- with succinate-adapted cells.
C. Zoom-in images of selected proteins across the three adaptation states.
D. Substrate-dependent changes in abundance (*n*-fold) of proteins related to the degradation of 4-methylbenzoate and benzoate respectively. For isoelectric focusing a non-linear pH gradient from 3 to 10 was used. High M_r, top; low M_r, bottom. The roman numbers I–IV indicate multiple, 2DE-separated spots of a single protein species.

CoA reductases are involved in the degradation of 4-methylbenzoate (Mbr) and benzoate (Bcr). This assumption is supported by the observations that (i) genes putatively coding for Mbr were only expressed during anaerobic growth with 4-methylbenzoate but not with benzoate, and (ii) substantial Mbr activity was only present in extracts from cells grown with 4-methylbenzoate, but not with benzoate. As no other genes with similarities to benzoyl-CoA reductases were detected in the draft genome of strain pMbN1, the most likely scenario is that the 4-methylbenzoate induced gene products with homologies to known Bcrs catalyse the two-electron reduction of 4-methylbenzoyl-CoA to the corresponding cyclic 4-methyl-dienoyl-CoA. In accord, the downstream enzymes responsible for further transformation of the 4-methyldienoyl-CoA can be expected to be

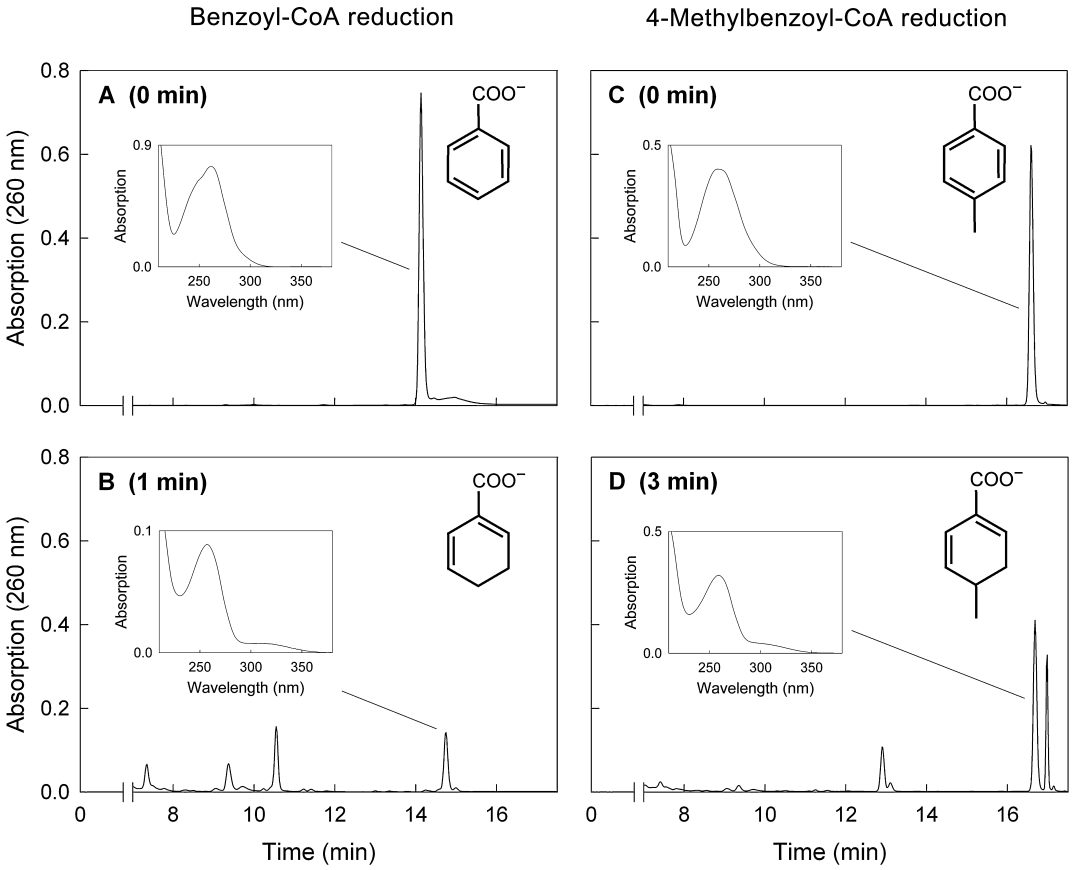


Fig. 5. HPLC analysis of benzoyl-CoA and 4-methylbenzoyl-CoA conversion by extracts of strain pMbN1 anaerobically grown with 4-methylbenzoate. The time-dependent reduction of benzoyl-CoA (A and B) and 4-methylbenzoyl-CoA (C and D) in extracts from 4-methylbenzoate-grown cells of strain pMbN1 are shown. The inserts show UV/VIS spectra of benzoyl-CoA (A) and 4-methylbenzoyl-CoA (C) at the beginning of incubation (0 min) and those of the corresponding reduced conjugated dienoyl-CoA after 1 min (B) and methyldienoyl-CoA after 3 min (D) of incubation. Further detected HPLC peaks represent intermediates of (methyl)dienoyl-CoA degradation.

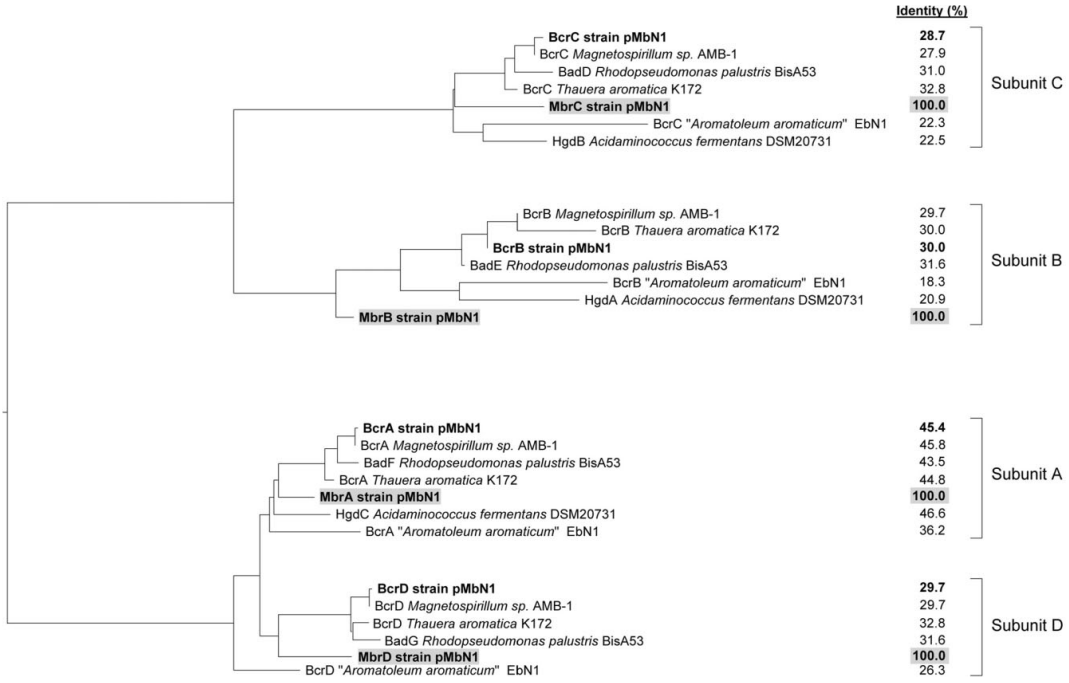


Fig. 6. Relationships of benzoyl-CoA and 4-methylbenzoyl-CoA reductase subunits from strain pMbN1 with proteins from other bacteria. Subunits of the novel enzyme type specific for 4-methylbenzoyl-CoA are marked in bold and highlighted in grey. Subunits of conventional class I benzoyl-CoA reductase are marked in bold only. For identity values (%), the subunits of 4-methylbenzoyl-CoA reductase from strain pMbN1 were set 100%. Bcr and Bad, benzoyl-CoA reductase; Mbr, 4-methylbenzoyl-CoA reductase; Hgd, 2-hydroxyglutaryl-CoA dehydratase. Accession numbers except for proteins from strain pMbN1 (from top to bottom): YP_421504.1, YP_779546.1, CAA12247.1, YP_160025.1, YP_003399483.1, YP_421503.1, CAA12248.1, YP_779545.1, YP_160026.1, YP_003399484.1, YP_421502.1, YP_779544.1, CAA12249.1, YP_003399485.1, YP_160028.1, YP_421501.1, CAA12250.1, YP_779543.1, YP_160027.1.

modified to accommodate the *para*-methyl group, as supported by the identification of homologues of other enzymes from the conventional benzoyl-CoA pathway.

In energy-limited, obligate anaerobes, a class II Bcr is employed to generate the required low redox potential electrons without the expenditure of ATP hydrolysis. It would be interesting to learn, whether 4-methylbenzoate-degrading obligate anaerobes exist, also using a specialized class II Bcr to cope with the challenges of the *para*-methyl group.

Experimental procedures

Strain and growth conditions

Strain pMbN1 was subcultured in our laboratory since its isolation (Lahme *et al*., 2012) and was cultivated in ascorbate-reduced (4 mM) and bicarbonate-buffered defined mineral medium under nitrate-reducing (10 mM) conditions and an anoxic atmosphere of N_2/CO_2 (90:10, v/v) as described previously (Rabus and Widdel, 1995b). 4-Methylbenzoate (2 mM), benzoate (2 mM) and succinate (5 mM) were added from sterile stock solutions. Growth was monitored by measuring the optical density at 660 nm in a spectrophotometer (UV-1202, Shimadzu, Kyoto, Japan).

Mass cultivation was performed to supply sufficient cell material for differential proteomic analysis. Cultures (400 ml of medium in 500 ml bottles) were inoculated with cells adapted over at least five passages to anaerobic growth with 4-methylbenzoate, benzoate or succinate. Cells were harvested at midlinear growth phase as described previously (Champion *et al*., 1999; Wöhlbrand *et al*., 2007). To account for biological variation in changes of protein abundance resolved by 2D-DIGE, parallel cultures were generated for each substrate condition.

For metabolite analysis, cultures adapted to anaerobic growth with 4-methylbenzoate, benzoate or succinate were used. Per adaptation condition at least two independent cultures were incubated until nitrate was nearly depleted, as determined by means of Merckoquant test strips (Merck, Darmstadt, Germany). Then, cultures were heat inactivated, chilled on ice, acidified to pH 1.5 and extracted with diethyl ether (three times), as described previously (Rabus *et al*., 2001).

Mass cultivations for measurement of enzymatic activities were also carried out in 400 ml culture volume (500 ml

bottles), using substrate-adapted cells as inocula. Harvesting of cells under strictly anoxic conditions was carried out as recently described (Trautwein *et al*., 2012). Per substrate condition (4-methylbenzoate, benzoate or succinate) about 3 g of wet weight were generated.

Cell suspension experiments

Cell suspensions were prepared from actively growing, substrate-adapted cultures essentially as described before (Harms *et al*., 1999a). Harvesting of cultures and preparation of cell suspensions (final OD 0.2) were performed inside an anoxic chamber under an N_2/CO_2 (90:10, v/v) atmosphere. The cell density was increased twofold by resuspending cell pellets in half of the original volume using nitrate-free mineral medium devoid of organic substrate. Aliquots of 50 ml were distributed in serum bottles (100 ml) and sealed with butyl rubber stoppers. Nitrate (10 mM), benzoate (3 mM), 4-methylbenzoate (2.5 mM) and/or succinate (10 mM) were added with N_2-flushed syringes. For each tested condition, triplicate cell suspension experiments were conducted. The cell suspensions were incubated at 28°C. Nitrate depletion was measured by HPLC analysis in samples retrieved from the cell suspensions with N_2-flushed syringes as described before (Rabus and Widdel, 1995b). Substrate consumption was indirectly determined by calculating oxidized reduction equivalents from nitrate consumed (Rabus and Widdel, 1995b).

Metabolite analysis

Dried extracts (with anhydrous Na_2SO_4) were methylated prior to GC-MS analysis using a solution of diazomethane in diethyl ether. GC-MS measurements were performed using a Trace GC-MS (Thermoelectron, Dreieich, Germany). The GC was equipped with a temperature-programmable injection system and a BPX5-fused silica capillary column (SGE; length, 50 m; inner diameter, 0.22 mm; film thickness, 0.25 μm). Helium was used as carrier gas. The GC oven temperature was programmed from 50°C (1 min isothermal) to 310°C (20 min isothermal) at a rate of 3°C min^{-1}. The MS was operated in electron impact mode at an ion source temperature of 230°C. Full-scan mass spectra were recorded over the mass range of 50–600 Da at a rate of 2.5 scans s^{-1}. 3-Methylglutarate was identified using an authentic standard purchased from Sigma-Aldrich (Taufkirchen, Germany).

Two-dimensional difference gel electrophoresis (2D-DIGE)

Cell disruption with a PlusOne sample grinding kit (GE Healthcare, Munich, Germany) and preparation of protein extracts were performed as previously described (Gade *et al*., 2003). Protein concentration was determined according to the method described by Bradford (1976).

Isoelectric focusing was carried out as described previously (Gade *et al*., 2003) using an IPGphor system (GE Healthcare) and 24 cm IPG strips with a non-linear pH gradient of 3–10 (GE Healthcare). DeStreak rehydration solution (GE Healthcare) was used to enhance resolution and reproducibility in the alkaline pH range. An EttanDalt II system (GE Healthcare) was used to carry out separation according to molecular mass in 12.5% acrylamide gels as described recently (Wöhlbrand *et al*., 2007; 2008). 2D-DIGE was essentially carried out as described by Gade and colleagues (2003). A total of 200 pmol of CyDye Fluors was used to label 50 μg of protein sample. Pre-electrophoretic labelling with different cyanine dyes allows co-separation of up to three samples in a single gel. An individual experiment in the present study contained (per gel) equal amounts of reference state, test state and internal standard. To account for biological variation, protein extracts from three independently grown cultures were prepared for each substrate condition. Three parallel gels for each protein extract were analysed to also account for technical variation. Protein extracts from succinate-adapted cells served as a reference state to differentiate between enzymes constituting the anaerobic degradation pathways for benzoate and 4-methylbenzoate respectively. The reference state was labelled with Cy5. Protein extracts from cells anaerobically grown with 4-methylbenzoate or benzoate served as test states and were each labelled with Cy3. All experiments contained the same internal standard, which was composed of equal amounts of all protein preparations of the reference and all test states and labelled with Cy2.

Two-dimensional difference gel electrophoresis gels were scanned immediately after electrophoresis with a Typhoon 9400 scanner (GE Healthcare). Analysis of cropped images was performed with the DeCyder software (version 5.0; GE Healthcare). Parameters for co-detection of protein spots were as described by Wöhlbrand and colleagues (2007). Protein spots that were defined as significantly regulated fulfilled the following criteria: an average ratio (*n*-fold change) of < -2.5 or > 2.5, an analysis of variance *P* value of < 0.05, a *t*-test value of $< 10^{-4}$, and matched in at least 18 gels. Overall, the DeCyder analysis included 24 gels (representing 72 gel images), which contained on average 1569 detected protein spots.

In order to identify differentially regulated spots by MS, separate preparative gels (300 μg protein load) were run for all test states. These gels were stained with colloidal Coomassie Brilliant Blue according to the method described by Doherty and colleagues (1998). The high reproducibility of spot patterns allowed MS-based identification of differentially regulated proteins as determined by 2D-DIGE/DeCyder. Only unambiguously matching spots were included in this analysis.

Protein identification by Edman degradation microsequencing and mass spectrometry

In a first step, 4-methylbenzoate-specific proteins resolved by 2DE were subjected to Edman degradation microsequencing (Toplab GmbH, Martinsried, Germany), essentially as described (Hunkapiller *et al*., 1983). The generated Edman sequences were used for directing gene cluster assembly from the genomic shotgun database of strain pMbN1.

For confirmation, tryptic digest of excised proteins was performed as described previously (Jenö *et al*., 1995). Peptide masses were determined by matrix-assisted laser

desorption ionization–time of flight (MALDI-TOF) MS. Protein identification and genome analysis were based on the genomic shotgun database of strain pMbN1 (M. Kube, R. Rabus and R. Reinhardt, unpublished). Peptide mass fingerprints were mapped to the *in silico* digests of the predicted proteins by using the MS digest program (Clauser *et al*., 1999).

Assembly of gene clusters

Construction of shotgun libraries, DNA sequencing, assembly of gene clusters directed by Edman sequences of 4-methylbenzoate- and benzoate-specific proteins, respectively, gene prediction and functional analysis using the HTGA system were essentially performed as described (Rabus *et al*., 2002).

Enzyme activity measurements

Extracts from substrate-adapted cells (~3 g wet weight per substrate condition: 4-methylbenzoate, benzoate and succinate) were anoxically prepared as previously described (Tschech and Fuchs, 1987). Synthesis of benzoyl-CoA and 4-methylbenzoyl-CoA was carried out as reported (Schachter and Taggart, 1953; Gross and Zenk, 1966). HPLC analysis of thioester intermediates of benzoyl- and 4-methylbenzoyl-CoA reductase were conducted as previously described (Möbitz and Boll, 2002). The technical variance of the assay is maximal 15%.

Phylogenetic analysis

Amino acid sequences were aligned using CLUSTAL W of the DNASTAR program (Lasergene, Madison, USA). Phylogenetic trees were constructed by the neighbour-joining method, using the DNASTAR program (Lasergene).

Nucleotide sequence accession numbers

The determined nucleotide sequences of strain pMbN1 for the conventional benzoyl-CoA pathway (contig c375) and the specific 4-methylbenzoyl-CoA pathway (contig c393) have been submitted in GenBank under Accession No. FQ976920 and FQ976921 respectively.

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Supporting information

Additional Supporting Information may be found in the online version of this article:

Fig. S1. Anaerobic degradation of aromatic compounds by substrate-adapted cell suspensions of strain pMbN1. For each adaptation condition (anaerobic growth with succinate, benzoate or 4-methylbenzoate) three independent cell suspension experiments (biological replicates) were conducted. Each data point shown, represents the average of two independent HPLC analysis (technical replicates).
A1–3. Biological replicates with cells adapted to succinate.
B1–3. Biological replicates with cells adapted to benzoate.
C1–3. Biological replicates with cells adapted to 4-methylbenzoate.
Substrate-adapted cells were shifted to either succinate (▼), benzoate (■) or 4-methylbenzoate (●). Controls (○) did not contain any organic substrate.
Fig. S2. Partial ion chromatograms for $m/z = 114 + 137 + 143 + 152$ for extracts of strain pMbN1 obtained upon anaerobic growth with 4-methylbenzoate (**A**), benzoate (**B**) and succinate (**C**). The selected ions are indicative for 3-methylglutaric acid dimethyl ester and tentatively identified 4-methylcyclohex-1,5-diene-1-carboxylic acid methyl ester (see the corresponding mass spectra in Fig. 4 in main paper). The absence of the corresponding peaks in traces (**B**) and (**C**) corroborates their specific formation during anaerobic growth with 4-methylbenzoate.
Fig. S3. Aligned amino acid sequences of the four subunits (A–D) constituting benzoyl-CoA or 4-methylbenzoyl-CoA reductases from different bacteria. Sequences were aligned using CLUSTAL W of the DNASTAR program (Lasergene). Conserved amino acid residues are shaded in grey, whereas amino acids with a previously proposed function in substrate conversion (Hans *et al*., 1999; Locher *et al*., 2001; Boll, 2005) are shaded in black.
Fig. S4. Relationships of proteins involved in initial β-oxidation of dienoyl-CoA and 4-methyldienoyl-CoA during anaerobic degradation of benzoate and 4-methylbenzoate in denitrifying strain pMbN1. Oah, 6-oxocyclohex-1-ene-1-carbonyl-CoA hydrolase; Dch, cyclohexa-1,5-diene-1-carbonyl-CoA hydratase; Had, 6-hydroxycyclohex-1-ene-carbonyl-CoA dehydrogenase; BadI, 2-oxocyclohexanoyl-CoA hydrolase; BadK, cyclohexenoyl-CoA hydratase; BadH, 2-hydroxycyclohexanoyl-CoA dehydrogenase. Sequences were aligned using CLUSTAL W of the DNASTAR program (Lasergene). Paralogous proteins from strain pMbN1 are shown in bold letters and those related to the degradation of 4-methylbenzoate are shaded in grey. Database accession numbers of proteins from bacteria other than strain pMbN1 are provided in Table S4.

Table S1. Identified proteins with changed abundances during anaerobic growth of strain pMbN1 with 4-methylbenzoate (4MBz) and benzoate (Bz) respectively. Cells grown with succinate (Succ) served as reference state. Fold changes in protein abundance are illustrated by colour code (see coloured scale bar). Fold changes were determined on the basis of eight 2D-DIGE gels (four each for 4MBz and Bz). The Mascot scores of proteins identified by LC-ESI-MS are marked with a grey background; the other Mascot scores are based on analysis by MALDI-TOF-MS.
Table S2. Amino acid sequences (Edman microsequencing) of substrate-specific proteins were used to select DNA sequence reads and assemble gene clusters from genomic shotgun database. The locations of the amino acid sequences in the assembled genes are marked in red.
Table S3. Functional assignment for genes proposed to be involved in anaerobic degradation of benzoate and 4-methylbenzoate in strain pMbN1.
Table S4. Database accession numbers of proteins from bacteria other than strain pMbN1 used in the phylogenetic tree shown in Fig. S4.

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3

Magnetospirillum sp. pMbN1 prefers benzoate from binary and ternary substrate mixtures during anaerobic growth

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ABSTRACT

Diauxic growth was observed in 4-methylbenzoate-adapted cells of *Magnetospirillum* sp. pMbN1 during anaerobic growth with a ternary substrate mixture of 4-methylbenzoate, benzoate and succinate. Following benzoate utilization in the first active growth phase, 4-methylbenzoate and (the bulk of) succinate were depleted simultaneously in the second active growth phase. Preference of benzoate and co-utilization of 4-methylbenzoate were also observed from binary substrate mixtures. Time-resolved transcript (11 selected) and differential protein profiles during anaerobic growth with the ternary substrate mixture revealed dynamic abundance changes for uptake and catabolism of each substrate, reflecting the order of their utilization. Apparently, simultaneous repression of 4-methylbenzoate and succinate utilization from the ternary substrate mixture resulted from (i) inhibition of 4-methylbenzoate uptake, and (ii) combined inhibition of succinate uptake (via DctPQM and DctA) and succinate conversion to acetyl-CoA (via pyruvate dehydrogenase). Despite the similar diauxic growth behavior, benzoate-mediated repression of C_4-dicarboxylate utilization in strain pMbN1 is distinct from that described in "*Aromatoleum aromaticum*" EbN1. Furthermore, strain pMbN1 displays a broader range of aliphatic acids, the anaerobic utilization of which is inhibited in the presence of benzoate.

INTRODUCTION

Aromatic compounds are widely distributed and abundant constituents of natural organic matter (1), and support as energy-rich substrates the growth of heterotrophic bacteria. In the environment aromatic compounds occur in mixtures with various and generally more easily degradable substrates (e.g. aliphatic acids, carbohydrates, amino acids), with each component mostly being present only at very low concentrations. Depending on the actual habitat, however, the concentration of aromatic compounds can be (at least transiently) higher, i.e. concentrations range from only few µg l^{-1} to several g l^{-1} (e.g., in petrochemical wastewaters) (2, 3).

In contrast to the generally observed simultaneous utilization of different substrates in environments with high substrate diversity, but very low concentrations of individual compounds (4, 5), the mechanisms of carbon catabolite repression (CCR)

govern the sequential utilization of carbon sources at higher substrate concentrations. Often substrates supporting faster growth are preferred, while utilization of subordinate ones is inhibited (6–9), thus often resulting in biphasic (diauxic) growth with two active growth phases, separated by an intermediary lag phase (6). CCR has been intensely studied for carbohydrate utilization in standard bacteria (e.g., *Escherichia coli*, *Bacillus subtilis*), which, amongst others, employ different phosphotransferase systems (PTS) for sugar uptake (7, 8). However, information on substrate preferences and CCR in non-standard environmental bacteria is limited and also expected to differ from the known concepts, as evident, e.g. from PTS-independent sugar uptake in *Pseudomonas* spp. (9), and the inability of many nutritional specialists to utilize carbohydrates. So far, only few studies investigated substrate preferences of anaerobic aromatic compound-degrading bacteria. Under nitrate-reducing conditions and growth at comparable rates, succinate-adapted *Azoarcus* sp. CIB prefers utilization of succinate from a binary mixture with benzoate (10). Most recently, the regulatory protein AccR was demonstrated to mediate this succinate-dependent CCR (11). In contrast to strain CIB, *"Aromatoleum aromaticum"* EbN1 prefers benzoate (12). In the latter, benzoate mediates repression of succinate uptake via the C_4-dicarboxylate TRAP transporter DctPQM (12).

The denitrifying alphaproteobacterium *Magnetospirillum* sp. pMbN1 is the only known isolate utilizing 4-methylbenzoate under anoxic conditions (13). 4-Methylbenzoate and benzoate are degraded anaerobically via two distinct, yet analogous pathways (14) that involve: (i) substrate-CoA ligation, (ii) reductive dearomatization of (4-methyl)benzoyl-CoA by dedicated 4-methylbenzoyl-CoA (MbrCBAD) or benzoyl-CoA (BcrCBAD) reductase, and (iii) a series of β-oxidation-like reactions to yield 3-hydroxy-5-methylpimelyl-CoA (4-methylbenzoate-derived) or 3-hydroxypimelyl-CoA (benzoate-derived). Further degradation of the latter two intermediates to acetyl-CoA then proceeds differently either via reactions analogous to the leucine/isovalerate pathway or by β-oxidation, respectively (14). In the absence of benzoate, 4-methylbenzoate-adapted cells of strain pMbN1 additionally formed proteins of the anaerobic benzoyl-CoA pathway, although at lower abundance (14). This raised the question, if both degradation pathways could operate simultaneously, or if one of the two pathways (resp. substrates) would be preferred.

With focus on (4-methyl)benzoate, this study combines physiology with differential transcript and protein analyses to comprehensively investigate the substrate preferences of strain pMbN1 from binary and (for the first time) ternary mixtures of aromatic and aliphatic substrates under anoxic conditions.

MATERIALS AND METHODS

Media and cultivation. The denitrifying bacterium *Magnetospirillum* sp. pMbN1 was cultivated in an ascorbate-containing (4 mM), bicarbonate-buffered mineral medium under nitrate-reducing (10 mM) conditions (15). Anoxic media were prepared as described previously in butyl rubber sealed 500-ml (or 250-ml) flat glass bottles, containing 400 ml (or 200 ml) of medium under an anoxic atmosphere of N_2/CO_2 (90:10, vol/vol) (15). Adaptation substrates and co-substrates were added from sterile stock solutions; their respective concentrations in the medium are provided in Tables 1 and 2. All chemicals used were of analytical grade.

Growth experiments. The growth behavior of strain pMbN1 was analyzed in the presence of various binary (Table 1; Figs. S1–S3) and one ternary (Table 2) substrate mixture(s). Cells were cultivated with each adaptation substrate for at least 5 passages before using 5% (vol/vol) of an active culture to inoculate fresh medium for growth experiments (three and two replicate cultures with each substrate mixture and single substrate, respectively). Over the time course of growth, 3 ml samples were repeatedly removed from each culture with sterile, N_2-flushed syringes. From each 3 ml sample, a 1 ml aliquot was used for monitoring the optical density (OD) at 660 nm (UV-mini 1202; Shimadzu, Duisburg, Germany), and a 2 ml aliquot was immediately filtered (nitrocellulose, pore size 0.2 µm) and stored at −20°C for subsequent determination of substrate concentrations by high-performance liquid chromatography (HPLC). Negative controls lacked the inoculum and were treated in the same way.

Mass cultivation. To obtain sufficient cell material for proteomic and targeted transcript analyses, strain pMbN1 was cultivated in 5- or 10-liter Duran bottles (Ochs, Bovenden, Germany) filled with 4 or 8 liters of anoxic medium under an N_2/CO_2 (90:10, vol/vol) atmosphere. Bottles had three ports, allowing for gassing, inoculation

or sampling, and repeated harvesting of cells during the time course of growth. Strain pMbN1 was harvested during anaerobic growth with four selected substrate mixtures: (i) During diauxic growth with succinate (adaptation substrate; 5 mM) and benzoate (co-substrate; 1 mM) in phase 1 (OD 0.17), phase 2 (OD 0.30), and phase 3 (OD 0.37) (Fig. 1A). (ii) During monophasic growth with succinate (adaptation substrate; 5 mM) and 4-methylbenzoate (co-substrate; 1 mM) at two different time points (OD 0.14 and 0.30) (Fig. 1B). (iii) During diauxic growth with 4-methylbenzoate (adaptation substrate; 1.5 mM) and benzoate (co-substrate; 1.5 mM) in phase 1 (OD 0.18), phase 2 (OD 0.24) and phase 3 (OD 0.33) (Fig. 1C). (iv) During diauxic growth with 4-methylbenzoate (adaptation substrate; 1 mM), benzoate (co-substrate; 1 mM) and succinate (co-substrate; 5 mM) in phase 1 (OD 0.14), transition into and out of phase 2 (both OD 0.23), and at the beginning (OD 0.25) and middle (OD 0.31) of phase 3 (Fig. 1D). Cells grown with single substrates, i.e. succinate (5 mM), benzoate (2 mM) or 4-methylbenzoate (2 mM), were harvested (400 ml medium in 500 ml flat glass bottles) at an OD of ~0.20. Harvesting of cells for proteomic analyses was performed as previously described (16). Resulting cell pellets were immediately frozen in liquid nitrogen and stored at −80°C. For RNA analysis, cultures with single substrates were harvested at an OD of ~0.20 and those with the ternary substrate mixture at 10 different time points during diauxic growth. 5 or 10 ml portions of culture broth were directly collected with sterile glass pipettes. For cultures growing with single substrates, one sample each was retrieved from three replicate cultures per substrate condition. In case of the experiment with the ternary substrate mixture, three parallel samples were retrieved per time point from a single large-scale culture. Samples were mixed immediately with two times the volume of RNAprotect® Bacterial Reagent (Qiagen, Hilden, Germany), incubated for 5 min at room temperature, followed by centrifugation at 4,500 g for 30 min at 4°C. Pellets were then resuspended in 0.5 ml RNAprotect® Bacterial Reagent and transferred into 2 ml microcentrifuge tubes (Eppendorf, Wesseling-Berzdorf, Germany) prior to centrifugation (20,000 g, 5 min, 4°C). The supernatants were discarded and pellets were stored at −80°C.

Chemical analysis. Substrate concentrations were analyzed with a Dionex UltiMate 3000 Rapid Separation LC (ThermoScientific GmbH, Idstein, Germany). Separation of aliphatic organic acids was performed using a Eurokat H separation column (8 by 300 mm, 10 µm; Knauer, Berlin, Germany) that was temperature

controlled at 75°C. With 5 mM H_2SO_4 as the eluent and a flow rate of 0.8 ml min^{-1}, aliphatic acids were detected using a UV detector (210 nm). The retention times (with detection limits in parentheses) of UV-detected substrates were as follows: L-malate, 7.0 min (25 µM); oxaloacetate, 7.2 min (25 µM); pyruvate, 7.2 min (25 µM); succinate, 8.4 min (25 µM); fumarate, 9.3 min (5 µM); and acetate, 11.0 min (25 µM). Aromatic acids were analyzed on the same HPLC system, but equipped with a Dionex Acclaim 120 reversed-phase separation column (2.1 by 250 mm, 10 µm; ThermoScientific GmbH) that was temperature-controlled at 25°C. Separation was achieved using a non-linear gradient from 5–90 % (vol/vol) acetonitrile as the eluent (pH adjusted to 2.8) at a flow rate of 0.5 ml min^{-1}. Retention times (detection limit and compound-specific wavelength in parentheses) of UV-detected aromatic acids were as follows: 4-hydroxybenzoate, 7.5 min (1 µM, 260 nm); benzoate, 11.8 min (1 µM, 236 nm); phenylacetate, 12.2 min (1 µM, 195 nm); 4-methylbenzoate, 14.7 min (1 µM, 236 nm). Concentrations of succinate, benzoate, 4-methylbenzoate (Fig. 1) and phenylacetate (Fig. S1) were additionally determined by HPLC as previously described (for method, retention times and detection limit see 12).

Soluble protein fraction: 2D DIGE and MALDI-TOF-MS/MS. Preparation of cell-free protein extracts and two-dimensional difference gel electrophoresis (2D DIGE) were performed as previously reported (17, 18). Cells were disrupted with the PlusOne sample grinding kit (GE Healthcare, Munich, Germany), and the protein concentration was determined as described by Bradford (19). Isoelectric focusing (IEF) was performed using the IPGphor system (GE Healthcare) and commercial 24-cm immobilized pH gradient (IPG) strips with a nonlinear pH gradient of 3 to 11 (GE Healthcare). The EttanDalt II system (GE Healthcare) was used for separation according to the molecular mass in 12.5% acrylamide gels. Pre-electrophoretic labeling with different fluorescent dyes allows co-separation of three samples in a single gel, representing the reference state (Cy5-labeled), the test state (Cy3-labeled), and the (pooled) internal standard (Cy2-labeled). Protein extracts from cells adapted to anaerobic growth with succinate served as the reference state. Individual test states were prepared from protein extracts of cells grown anaerobically with four different substrate mixtures (see above section on Mass cultivation) and with benzoate or 4-methylbenzoate as single substrates. The internal standard was composed of equal amounts of protein extracts of the reference and all 16 test states. 2D DIGE gels were

scanned immediately after electrophoresis with a Typhoon 9400 scanner (GE Healthcare). Cropped images were analyzed with the DeCyder software (version 7.0; GE Healthcare). Parameters for co-detection of spots were previously described (20). Matching of differentially abundant spots was manually controlled, which had to fulfill the following criteria: average ratio (*n*-fold change in protein abundance) of ≤ -2.5 or ≥ 2.5, analysis of variance (ANOVA) *P* value of <0.05, and *t* test value of $<10^{-4}$, and matched in at least 75% of the analyzed gels. To achieve statistical confidence, 3–4 parallel gels were included in the analysis for each tested state, resulting in 180 gel images (representing 60 gels). 1608 ± 110 spots were detected across all gels.

Protein spots displaying abundance changes were manually excised from preparative two-dimensional gel electrophoresis (2DE) gels (300 µg protein load) stained with colloidal Coomassie Brilliant Blue (cCBB) (21). The high reproducibility of spot patterns allowed mass spectrometry (MS)-based identification of differentially abundant proteins as determined by 2D DIGE/DeCyder. Tryptic digestion of excised 2DE-separated proteins was performed as previously described (22) Peptide mass analysis and tandem MS was performed with an Ultraflextreme MALDI-TOF/TOF mass spectrometer (Bruker Daltonik GmbH, Bremen, Germany) operated as described recently (23). Protein identification was performed with ProteinScape (version 3.1, Bruker Daltonik GmbH) on a Mascot server (version 2.3, Matrix Science Ltd; UK) based on a shotgun sequence dataset of strain pMbN1 (14) translated into amino acid sequences as described recently (23).

Membrane protein-enriched fraction: SDS-PAGE and LC-ESI-MS/MS. Membrane protein-enriched fractions were prepared as described previously (24) from cells adapted to single substrates (4-methylbenzoate, benzoate or succinate) and at five different time points during diauxic growth with the ternary substrate mixture (see above section on Mass cultivation), followed by separation according to molecular mass in 12.5% acrylamide gels (25 by 30 cm) by SDS-PAGE (Bio-Rad, Munich, Germany). From the ternary substrate mixture, three of the five lanes (corresponding to phase 1, 2b and 3b) (Fig. S4) were further processed for protein identification. Each sample lane was subdivided into 14 gel slices, and each slice was further cut into small pieces (~1 mm^3), prior to washing, reduction, alkylation and tryptic digestion (22). Separation of peptides was performed with a nanoLC system (Thermo Scientific GmbH), online-

coupled to an ion trap mass spectrometer (amazonETD; Bruker Daltonik GmbH) as described previously (24). Protein identification was performed as described above.

Preparation of total RNA. All chemicals used during RNA preparation were of molecular biology grade. Total RNA was extracted from stored cell pellets within two weeks after cell harvest (see above section on Mass cultivation). (i) For single substrates, RNA was extracted from three biological replicates. (ii) For the ternary substrate mixture, RNA was extracted from three subsamples per time point (technical replicates). RNA extraction was essentially performed as previously described (25) using 60°C-hot saturated, acidic phenol. The aqueous phase was transferred into a new reaction tube and treated again with hot, acidic phenol. One volume phenol/chloroform/isoamylalcohol (25:24:1) was added to the aqueous phase in 2 ml Phase Lock Gel™ tubes (5 Prime GmbH, Hamburg, Germany). Subsequently, nucleic acids were precipitated with ice-cold ethanol (96% pure) during incubation at –80°C for 30 min. After centrifugation (20,000 g, 30 min, 4°C), the pellet was washed with 1 ml ice-cold ethanol (75%, vol/vol) and centrifuged again. The resulting pellet was dried and then resuspended in RNase-free water. Each RNA preparation was subjected to DNase I (RNase-free; Qiagen, Hilden, Germany) digestion. Removal of DNA was confirmed by PCR. RNA quality was controlled by the RNA 6000 Nano assay using an Agilent 2100 Bioanalyzer (Agilent Technologies, Böblingen, Germany). RNA concentration was determined using the Quant-iT™ Ribogreen® RNA assay kit (Life Technologies GmbH, Darmstadt, Germany). Total RNA was stored in aliquots at –80°C until further analysis.

Reverse transcription (RT) real-time PCR. Gene specific primers (Table S1) were designed for 11 target genes using the DNASTAR Lasergene program (version 7.0.0; DNASTAR Inc., Madison, USA). Reverse transcription and real-time PCR detection was performed in a 20 µl one-tube reaction using the Brilliant III Ultra-Fast SYBR® Green QRT-PCR master mix (Agilent Technologies) and an IQ5 real-time PCR detection system (Bio-Rad). The one-tube RT PCR reaction was performed as follows: one cycle of reverse transcription for 10 min at 50°C, followed by one cycle of PCR initiation for 3 min at 95°C, 40 cycles of 30 s denaturation at 95°C, 30 s annealing and extension at 60°C and real-time detection for 10 s between 82–87°C (Table S1). Each reaction was performed with 5 ng of total RNA. The specificity of accumulated

products was verified by melting curve analysis, ranging from 60–95°C in 0.5°C steps. Each RNA sample was analyzed by two (ternary substrate mixture) or four (single substrates) independent PCR reactions, corresponding to six (ternary substrate mixture) and 12 (single substrates) independent PCR reactions per analyzed time point.

The present physiological experiments are characterized by changes in (substrate-dependent) growth rates as well as intermediary lag phases. Such discrepant growth behaviors are known to affect the transcript abundance of reference "housekeeping" genes (e.g. 26, 27). Therefore, we relinquished to use a reference gene for relative transcript quantification in the present study. With respect to the highly similar PCR efficiencies of each primer pair (standard deviation <0.08; Table S6), transcript abundance changes were calculated as ratio of control (succinate) and test (4-methylbenzoate, benzoate and ternary substrate mixture) C_T values, considering the primer-specific efficiency (formula 1; 28):

$$ratio = E^{\Delta C_t(control-test)} \quad (1)$$

where E is the efficiency of the PCR reaction with the respective primer pair, determined as previously described by Ramakers *et al*. (29).

Nucleotide sequence accession numbers and manual annotation. Nucleotide sequences of genes discussed in this study were submitted to Genbank comprising accession numbers KF941494 to KF941542. A detailed list of accession numbers is provided together with manual annotation records of discussed proteins in Table S2.

RESULTS

Substrate preferences of *Magnetospirillum* sp. pMbN1 were studied during anaerobic growth with mixtures of two or three different substrates using cells adapted to one of six different aliphatic acids or 4-methylbenzoate (Table 1, Fig. 1; Figs. S1–S3).

TABLE 1 Anaerobic growth of *Magnetospirillum* sp. pMbN1 with aliphatic and aromatic acids supplied as single substrate or binary mixture.

Substrate[a]		Results for[b]: Single substrates S_{Ad}		S_{Co}		Mixtures of S_{Ad} and S_{Co}[c] Phase 1		Phase 2	Phase3		
S_{Ad}	S_{Co}	OD_{max}	μ_{max} (h^{-1})	OD_{max}	μ_{max} (h^{-1})	OD_{max}	μ_{max} (h^{-1})	Duration of diauxic lag phase (h)	OD_{max}	μ_{max} (h^{-1})	Further details[d]
Succinate-adapted cells:											
Succinate (5 mM)	**Benzoate (1 mM)**	0.38	0.21	0.18	0.21	0.30	0.19	2.1 ± 0.1	0.42	0.28	Fig. 1
Succinate (5 mM)	4-Methylbenzoate (1 mM)	0.38	0.21	0.15	0.13	0.61	0.24	None	None	None	Fig. 1
Succinate (5 mM)	4-Hydroxybenzoate (1 mM)	0.36	0.20	ND	ND	0.43	0.27	3.3 ± 0.1	0.51	0.50	Fig. S1
Succinate (5 mM)	Phenylacetate (1 mM)	0.36	0.20	0.19	0.16	0.66	0.25	None	None	None	Fig. S1
Succinate (5 mM)	Acetate (8 mM)	0.36	0.20	0.30	0.22	0.53	0.23	None	None	None	Fig. S1
4-Methylbenzoate-adapted cells:											
4-Methylbenzoate (1.5 mM)	**Benzoate (1.5 mM)**	0.27	0.16	0.28	0.15	0.27	0.29	4.0 ± 0.0	0.41	0.29	Fig. 1
4-Methylbenzoate (1.5 mM)	**Phenylacetate (1.5 mM)**	0.27	0.16	0.32	0.16	0.30	0.29	5.0 ± 0.0	0.45	0.28	Fig. S1
Cells adapted to other aliphatic acids:											
Acetate (8 mM)	Benzoate (1 mM)	0.27	0.18	0.17	0.20	0.38	0.18	None	None	None	Fig. S2
Acetate (8 mM)	4-Methylbenzoate (1 mM)	0.27	0.18	0.18	0.19	0.25	0.16	3.5 ± 0.0	0.32	0.24	Fig. S2
Pyruvate (6 mM)	**Benzoate (1 mM)**	0.34	0.18	0.18	0.21	0.30	0.17	1.3 ± 0.3	0.54	0.37	Fig. S2
Pyruvate (6 mM)	4-Methylbenzoate (1 mM)	0.34	0.18	0.19	0.16	0.54	0.22	None	None	None	Fig. S2
Fumarate (5 mM)	**Benzoate (1 mM)**	0.37	0.27	0.19	0.17	0.30	0.17	2.3 ± 0.0	0.43	0.29	Fig. S3
L-Malate (5 mM)	**Benzoate (1 mM)**	0.37	0.33	0.16	0.25	0.31	0.44	2.6 ± 0.1	0.49	0.28	Fig. S3
Oxaloacetate (6 mM)	**Benzoate (1 mM)**	0.27	0.20	0.17	0.23	0.31	0.19	1.0 ± 0.0	0.42	0.34	Fig. S3
Cells adapted to other aromatic acids:											
4-Hydroxybenzoate (1 mM)	**Succinate (5 mM)**	0.13	0.20	0.34	0.10	0.43	0.32	4.3 ± 0.0	0.49	0.50	Fig. S3

[a] Preferentially utilized substrates are highlighted in boldface. Applied substrate concentrations are indicated in parentheses. S_{Ad}, adaptation substrate; S_{Co}, co-substrate.
[b] Values for maximal optical density (OD_{max}) and maximum specific growth rates (μ_{max}) are based on at least two replicate cultures (three for each substrate mixture) yielding standard deviation of <5%. μ_{max} was calculated from the slope of the active growth phase (m): $\mu_{max} = m \times 1/\Delta OD$. If present, the duration of the diauxic lag phase (phase 2) is indicated with standard deviation. ND, not determined.
[c] In the case of diauxic growth, phase 1 corresponds to the first active growth phase. In the case of monophasic growth, phase 1 corresponds to the only observed active growth phase. If present, phase 3 corresponds to a second active growth phase, with phase 2 representing the diauxic lag phase between phases 1 and 3 (see Figs. 1 and 3).
[d] Figs. S1–S3 are in the supplemental material.

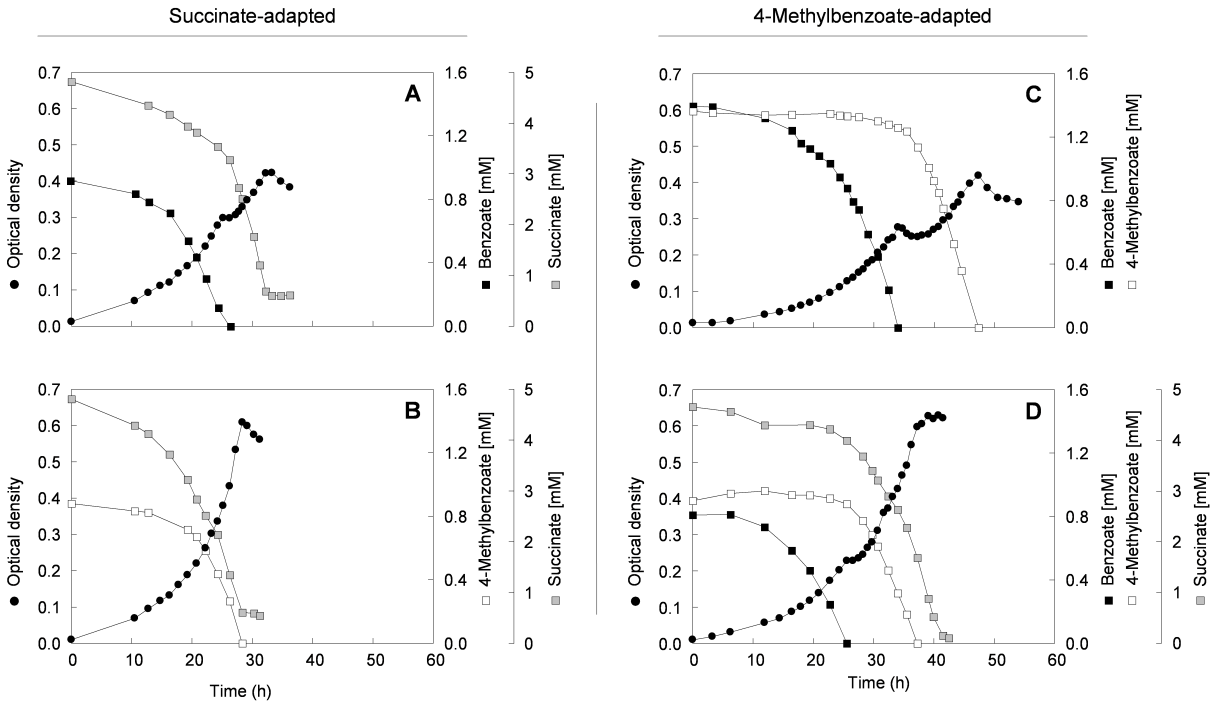

FIG 1 Anaerobic growth of *Magnetospirillum* sp. pMbN1 with binary and ternary substrate mixtures of 4-methylbenzoate, benzoate and succinate. (A, B) Succinate-adapted cells. (C, D) 4-Methylbenzoate-adapted cells.

TABLE 2 Anaerobic growth of 4-methylbenzoate-adapted cells of *Magnetospirillum* sp. pMbN1 with a ternary substrate mixture of 4-methylbenzoate, benzoate and succinate.

Substrate[a]			Results for[b]: Mixture of S_{Ad}, S_{Co1} and S_{Co2}[c]					
			Phase 1		Phase 2	Phase 3		
S_{Ad}	S_{Co1}	S_{Co2}	OD_{max}	μ_{max} (h^{-1})	Duration of diauxic lag phase (h)	OD_{max}	μ_{max} (h^{-1})	Further details
4-Methylbenzoate (1 mM)	**Benzoate (1 mM)**	Succinate (5 mM)	0.23	0.16	1.8 ± 0.1	0.62	0.31	Fig. 1

[a–c] See footnotes to Table 1.

Growth with binary substrate mixtures

(i) Cells adapted to succinate. Succinate-adapted cells of strain pMbN1 growing with a mixture of succinate and benzoate displayed diauxic growth (Fig. 1A). In the first active growth phase (phase 1), the cells preferentially and completely consumed benzoate. Concurrently, 26% of the supplied succinate was depleted from the medium during phase 1. Following a 2.1 h long diauxic lag phase (phase 2), the bulk of succinate was rapidly utilized during the second active growth phase (phase 3).

In contrast to benzoate, other tested co-substrates (4-methylbenzoate, phenylacetate or acetate) were co-utilized with succinate, resulting in monophasic growth (Table 1, Fig. 1B; Fig. S1). Diauxic growth was only observed with 4-hydroxybenzoate as co-substrate; however, succinate was the preferred substrate during phase 1 (Table 1; Fig. S1). The same pattern was also observed with 4-hydroxybenzoate-adapted cells, excluding long induction times of genes for 4-hydroxybenzoate catabolism to account for succinate preference (Table 1; Fig. S3).

(ii) Cells adapted to other aliphatic acids. In addition to succinate, the effect of benzoate as co-substrate was investigated in fumarate-, L-malate-, oxaloacetate-, acetate- or pyruvate-adapted cells (Table 1; Figs. S2 and S3). In most cases, benzoate was preferentially utilized (diauxic growth) from tested substrate mixtures. Only with acetate-adapted cells co-utilization was observed (monophasic growth). In contrast, strain pMbN1 preferentially utilized acetate from a mixture with 4-methylbenzoate (diauxic growth), while pyruvate and 4-methylbenzoate were co-utilized (monophasic growth). The duration of diauxic lag phases (if present) for mixtures with benzoate ranged from 1.0 to 2.6 h, while that for acetate and 4-methylbenzoate lasted 3.5 h.

(iii) Cells adapted to 4-methylbenzoate. The effect of benzoate or phenylacetate as co-substrate was investigated in 4-methylbenzoate-adapted cells (Table 1, Fig. 1C; Fig. S1). Benzoate and phenylacetate were exclusively and completely utilized during phase 1 of diauxic growth, followed by a 4–5 h long diauxic lag phase and utilization of 4-methylbenzoate during phase 3.

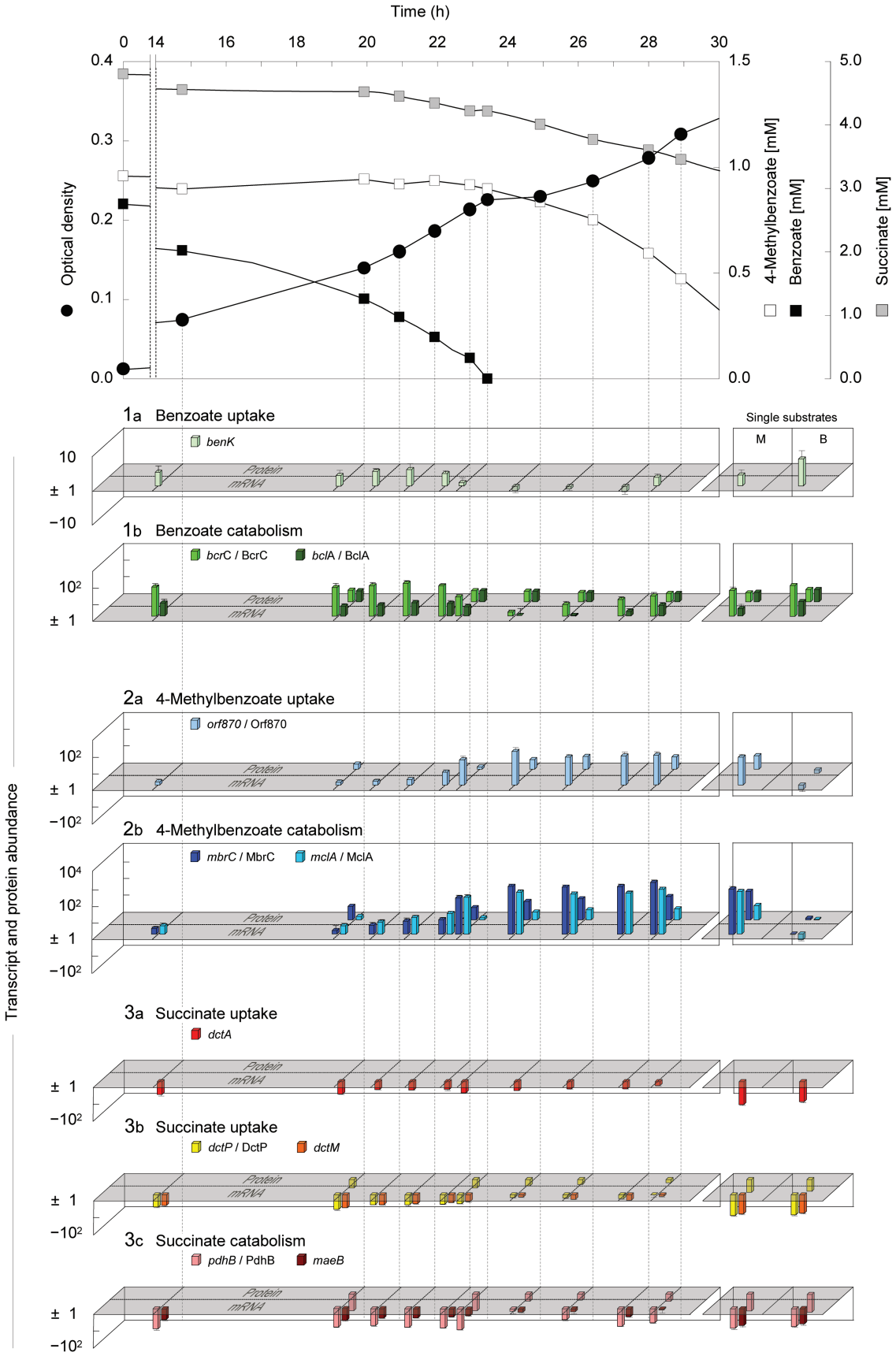

FIG 2 Time-resolved profiles of selected transcripts and corresponding proteins (if identified via 2D DIGE) related to uptake and catabolism of (1) benzoate, (2) 4-methylbenzoate and (3) succinate in cells

of *Magnetospirillum* sp. pMbN1 grown with a ternary substrate mixture of 4-methylbenzoate (M, adaptation substrate), benzoate (B, co-substrate) and succinate (S, co-substrate) (the growth curve shown at the top represents a section of Fig. 1D). Fold changes in protein and ratio of transcript abundances were determined by comparison to succinate-adapted cells as reference. See Fig. 3 for name and predicted functions of selected genes/proteins. For further details see Tables S3–S5 in the supplemental material.

Growth with the ternary substrate mixture

The effect of two different co-substrates was investigated in 4-methylbenzoate-adapted cells grown with a mixture of 4-methylbenzoate, benzoate and succinate (Fig. 1D). Also here, diauxic growth with preferential and complete utilization of benzoate was observed during phase 1. This was accompanied by 15% depletion of the supplied succinate (i.e., half the amount of succinate as compared to the binary mixture with benzoate). Following a short (1.8 h) diauxic lag phase (phase 2), 4-methylbenzoate and the bulk of succinate were co-utilized in phase 3. The same growth behavior and substrate preference was also observed, when succinate-adapted cells were used as inoculum (data not shown). Notably, the substrate utilization preferences observed with binary mixtures of the three different substrates are preserved in the ternary substrate mixture (Fig. 1A–C).

Transcript and protein dynamics during anaerobic diauxic growth with the ternary substrate mixture

Time-resolved abundance profiles of transcripts (11 different across 10 time points) and proteins (soluble and membrane, 5 time points) related to the uptake and catabolism of 4-methylbenzoate, benzoate and succinate were determined during diauxic growth with the ternary substrate mixture (Fig. 1D), and compared to that of succinate-grown cells as reference (Figs. 2 and 3; Tables S3–S5). Respective transcript and/or protein abundances were also determined in cells grown with binary substrate mixtures (Fig. 1A–C) or with single substrates at lower time resolution (1–3 time points, Fig. 3).

(i) Benzoate uptake and catabolism (Fig. 2, part 1). Preferential utilization of benzoate during phase 1 coincided with the largest abundance increases (up to 24.3-fold for the α-subunit of benzoyl-CoA reductase, BcrA) for the protein constituents of the anaerobic benzoyl-CoA pathway (BclA, BcrCBAD, Dch-1, Had-1, and Oah-1), which then constantly decreased during phases 2/3 (Table S3). Upon benzoate depletion from

the medium, transcript levels of benzoate-CoA ligase (*bclA*) and of the C-subunit of benzoyl-CoA reductase (*bcrC*) decreased immediately (within 30 min) from 5- to 3-fold and 60- to 13-fold, respectively. Notably, transcript levels of *bclA* remained low during phases 2/3, whereas that of *bcrC* increased (up to 16-fold) in phase 3 (Table S5).

Benzoate uptake in strain pMbN1 is predicted to proceed via a benzoate/H^+ symporter homologous to BenK (30). The latter was detected in this study in benzoate- or 4-methylbenzoate-, but not in succinate-grown cells. In contrast to the constituents of the anaerobic benzoyl-CoA pathway, the transcript and protein levels of BenK changed only slightly across phases 1–3 (Tables S4 and S5).

(ii) 4-Methylbenzoate uptake and catabolism (Fig. 2, part 2). Despite the apparent non-utilization of 4-methylbenzoate during phase 1 of diauxic growth, four proteins (MbrCBA, Oah-2) of anaerobic 4-methylbenzoate degradation (MclA, MbrCBAD, Dch-2, Had-2, and Oah-2) displayed increased (e.g. 6.5-fold for the γ-subunit of 4-methylbenzoyl-CoA reductase, MbrC) abundances during benzoate utilization. However, protein abundances markedly and steadily increased during the diauxic lag phase, reaching the highest levels during 4-methylbenzoate utilization in phase 3 (e.g. 27.4-fold for MbrC). Transcript levels of predicted 4-methylbenzoate-CoA ligase (*mclA*) and of *mbrC* were initially low (up to 3-fold), but started to increase at benzoate concentrations <~0.3 mM, which was followed by a strong increase upon benzoate depletion (up to 139-fold) and reaching maximal abundances (up to 1046-fold) during 4-methylbenzoate utilization in phase 3 (Table S5). The observed incongruent transcript (low) and protein (medium) levels of MbrC during phase 1 furthermore suggest that the highly abundant proteins of 4-methylbenzoate degradation (14), are only slowly degraded, instead of newly synthesized.

The current proteomic dataset suggests uptake of 4-methylbenzoate by an ABC transporter, all subunits of which (Orf870, 920, 930, 950, 960) were present in cells grown with 4-methylbenzoate, whereas they were absent in those grown with succinate or benzoate (Tables S3 and S4). During diauxic growth with the ternary substrate mixture, the periplasmic solute-binding protein of the ABC transporter (Orf870) started to increase in abundance at the end of the diauxic lag phase, reaching highest levels during phase 3 that were comparable to that in cells grown with 4-methylbenzoate as single substrate. Transcript levels of *orf870* started to increase already at the end of

phase 1 (from 6- to 34-fold within 30 min after benzoate depletion), peaked at the end of the diauxic lag phase (108-fold), and remained at high levels during phase 3 (Table S5). The four membrane-associated components (Orf920–960) of the ABC transporter were identified only in the membrane protein-enriched fraction of cells harvested during phases 2/3 (Table S4).

(iii) Succinate uptake and catabolism (Fig. 2, part 3). Among all identified proteins, the E1 (PdhB) and E3 (LpdA) components of pyruvate dehydrogenase (PdhABC, LpdA) of strain pMbN1 displayed the largest decreases in abundance (up to –10.3-fold) during phase 1 of diauxic growth. During bulk succinate utilization in phase 3, both proteins attained similarly high levels as in the succinate-grown reference (Table S3). The changes in transcript levels of *pdhB* essentially correlated with changes in the protein level. Across phase 1, *pdhB* transcript levels were strongly decreased (–11- to –19-fold) and comparable to that in cells grown only with benzoate or 4-methylbenzoate (Table S5). At the end of the diauxic lag phase, *pdhB* transcript levels were comparable to those in the succinate-grown reference cultures, followed by their decrease in phase 3. In addition to pyruvate dehydrogenase, metabolism of succinate to pyruvate is suggested to also involve $NADP^+$-dependent malic enzyme (MaeB), which was not detected by 2D DIGE. The transcript level of *maeB* was slightly (5-fold) reduced during phase 1 of diauxic growth, but increased with decreasing benzoate concentrations in the growth medium (Table S5). These abundance profiles agree with MaeB and pyruvate dehydrogenase being not required for benzoate or 4-methylbenzoate metabolism. Correspondingly, minimal transcript/protein abundances were observed during utilization of these two aromatic compounds (Fig. 3).

Uptake of succinate in strain pMbN1 is suggested to involve two different transporters. Firstly, a C_4-dicarboxylate TRAP transporter, displaying high amino acid sequence identities (69, 52 and 77%, respectively) to characterized DctPQM from *Rhodobacter capsulatus* (31–33), and secondly, the C_4-dicarboxylate/cation symporter DctA (63% amino acid sequence identity to characterized DctA from *Sinorhizobium meliloti*) (34). 2D DIGE covered only the periplasmic C_4-dicarboxylate-binding protein DctP of the TRAP transporter, which displayed decreased abundance (–3.4-fold) during benzoate utilization in phase 1 and increased continuously upon benzoate depletion during phases 2/3 (Table S3). DctQM were both identified in the membrane protein-enriched fraction of succinate-grown cells, whereas only one of the two subunits was detected in cells grown with benzoate, or across phases 1–3 of diauxic growth.

Symporter DctA was detected in succinate-grown cells and across phases 1–3 of diauxic growth, but not in cells grown with benzoate or 4-methylbenzoate as single substrate (Table S4). Transcript levels of *dctP*, *dctM* and *dctA* displayed similar abundance changes across phases 1–3, the extent and dynamics of which were comparable to that of the *maeB* transcript (see above). Minimal transcript levels of *dctP*, *dctM* and *dctA* were observed in cells grown with benzoate or 4-methylbenzoate as single substrates (Table S5). These transcript profiles are in accord with the known C_4-dicarboxylate-dependent activation of *dctPQM* and *dctA* expression by dedicated C_4-dicarboxylate-responsive two-component sensory/regulatory systems as known from *R. capsulatus* (DctSR) and rhizobia (DctBD) (35, 36).

(iv) Ternary *versus* binary substrate mixtures (Fig. 3). Protein profiles (2D DIGE) of cells grown with binary mixtures (succinate and benzoate or 4-methylbenzoate; 4-methylbenzoate and benzoate) were compared to those from cultures with the ternary substrate mixture (succinate, benzoate and 4-methylbenzoate). Overall, the dynamics in protein abundance changes during growth with binary substrate mixtures corresponded to those observed with the ternary substrate mixture. However, some proteins deviated in their abundances, if only two substrates were present (Fig. 3, Table S3). (i) Absence of 4-methylbenzoate resulted in reduced levels of proteins involved in benzoate catabolism (BclA, BcrCBDA, Dch-1, Had-1, and Oah-1). (ii) In the absence of benzoate, 4-methylbenzoate did not negatively affect DctP and PdhB levels, agreeing with co-utilization of succinate and 4-methylbenzoate (Fig. 1B). Interestingly, if provided together 4-methylbenzoate and benzoate had an additive negative effect on DctP and PdhB levels during phase 1 (i.e. –3.4- and –9.9-fold with the ternary substrate mixture as compared to –2.1- and –3.7-fold with succinate and benzoate, respectively), accompanied also by reduced succinate consumption. It should be noted, however, that the observed differences might be related to using either succinate or 4-methylbenzoate as adaptation substrate in the compared experiments. (iii) In the absence of succinate (benzoate or 4-methylbenzoate applied as single substrate or as mixture), levels of proteins involved in succinate uptake (DctP) and catabolism (PdhB) were minimal. With succinate as the third substrate, however, the levels of these two proteins were affected differently: DctP levels increased in phase 1 (from –5.7 to –3.4-fold), while PdhB levels remained minimal (–9.9-fold) (Fig. 3). This indicates that formation of DctP, but not PdhB, is responsive to externally supplied succinate.

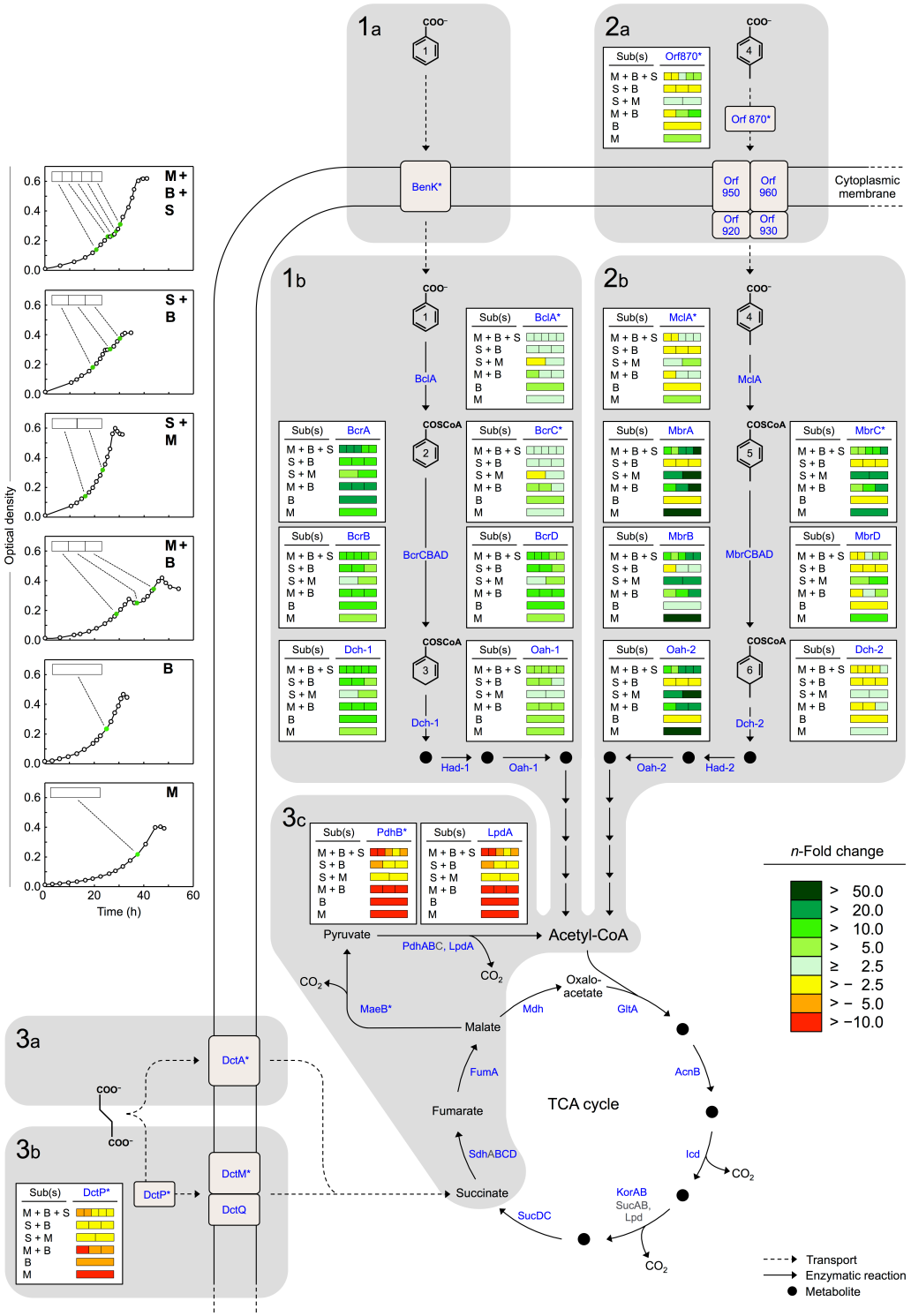

FIG 3 Fold changes in the abundance of proteins (compared to succinate-adapted cells) related to uptake and catabolism of (1) benzoate, (2) 4-methylbenzoate and (3) succinate during anaerobic growth of *Magnetospirillum* sp. pMbN1 with substrate mixtures or single substrates (M, 4-methylbenzoate; B, benzoate; S, succinate; the adaptation substrate is listed first in each case). Sampling points for proteomic analyses are marked with green in the growth curves displayed on the left panel. Identified proteins are marked in blue. For further details (including abundance profiles of Had-1 and Had-2) see Tables S3 and S4 in the supplemental material. *, Transcript profiles of selected genes during growth with the ternary

substrate mixture are shown in Fig. 2. Protein names (in alphabetical order): (AcnB) aconitase 2, (BclA) benzoate-CoA ligase, (BcrCBAD) benzoyl-CoA reductase, (BenK) benzoate/H^+ symporter, (Dch-1) cyclohex-1,5-diene-1-carbonyl-CoA hydratase, (Dch-2) 4-methylcyclohex-1,5-diene-1-carbonyl-CoA hydratase, (DctA) C_4-dicarboxylate/Na^+ symporter, (DctPQM) TRAP-type C_4-dicarboxylate uptake transporter, (FumA) fumarate hydratase, (GltA) citrate synthase, (Had-1) 6-hydroxycyclohex-1-ene-1-carbonyl-CoA dehydrogenase, (Had-2) 6-hydroxy-4-methylcyclohex-1-ene-1-carbonyl-CoA dehydrogenase, (Icd) isocitrate dehydrogenase, (KorAB) 2-oxoglutarate:ferredoxin oxidoreductase, (MaeB) $NADP^+$-dependent malic enzyme, (MbrCBAD) 4-methylbenzoyl-CoA reductase, (MclA) 4-methylbenzoate-CoA ligase, (Mdh) malate dehydrogenase, (Oah-1) 6-oxocyclohex-1-ene-1-carbonyl-CoA hydrolase, (Oah-2) 4-methyl-6-oxocyclohex-1-ene-1-carbonyl-CoA hydrolase, (Orf870–960) predicted ABC-type 4-methylbenzoate uptake transporter, (PdhABC, LpdA) pyruvate dehydrogenase complex, (SdhABCD) succinate dehydrogenase, (SucAB, Lpd) 2-oxoglutarate dehydrogenase complex, (SucDC) succinyl-CoA ligase.

DISCUSSION

(4-Methyl)benzoyl-CoA degradation pathways: one or the other. Anaerobic degradation of benzoate and 4-methylbenzoate in strain pMbN1 proceed via two analogous pathways, each involving a distinctive set of substrate-specifically formed enzymes (14). Despite adaptation to 4-methylbenzoate and "thereby transferred" initial presence of some catabolic proteins (MbrABC, Oah-2), strain pMbN1 clearly preferred utilization of benzoate from binary and ternary substrate mixtures with 4-methylbenzoate, although this necessitated to newly synthesize the respective catabolic enzymes (Fig. 1CD). The observed preference cannot be attributed to markedly different growth rates (i.e. 0.15–0.21 h^{-1} for benzoate, and 0.13–0.19 h^{-1} for 4-methylbenzoate) (Table 1). However, lower growth yields (8.5 g *versus* 9.5 g $(mol\ C)^{-1}$ for benzoate) despite the higher free energies ($\Delta G^{0\prime}$) obtained from complete oxidation of 4-methylbenzoate (–3583 kJ mol^{-1} *versus* –2957 kJ mol^{-1} for benzoate) (13), indicate less efficient metabolism during anaerobic growth with 4-methylbenzoate.

The substrate range of the 4-methylbenzoyl-CoA degradation pathway is limited to 4-methylbenzoate, whereas from the aromatic substrates of strain pMbN1 (13), at least 11 (e.g., phenylacetate) are known from other (facultative) anaerobes to be channeled into the anaerobic benzoyl-CoA pathway (e.g., (20, 37, 38)). Indeed, also phenylacetate repressed 4-methylbenzoate utilization in strain pMbN1 (Table 1). One may speculate that benzoate preference in strain pMbN1 reflects a higher probability to encounter substrates of the benzoyl-CoA pathway in the natural habitat. In *Thauera*

aromatica AR-1, benzoate repressed anaerobic 3,5-dihydroxybenzoate degradation, which is apparently degraded via a catabolic route distinct from the anaerobic benzoyl-CoA pathway (39, 40). It seems thus likely that the anaerobic benzoyl-CoA pathway might generally be preferred over more specialized (i.e. less central) degradation pathways.

Notably, *para*-substituted benzoyl-CoA analogs are not substrates of benzoyl-CoA reductase (Bcr) from *T. aromatica* K172 (41), but as shown for 4-fluorobenzoyl-CoA they can bind to Bcr and inhibit its activity (42). The similarity of benzoate and 4-methylbenzoate with respect to chemical structure and degradation pathways in strain pMbN1 may necessitate strict temporal separation of their degradation to prevent competitive inhibition of involved enzymes.

Benzoate mediates repression of 4-methylbenzoate uptake. The 4-methylbenzoate-specific detection of a putative ABC transporter for 4-methylbenzoate uptake (Orf870–960) and the strong increase in transcript levels of its periplasmic solute-binding protein (*orf870*) upon benzoate depletion (Fig. 2; Tables S3–S5), suggest benzoate to mediate repression of 4-methylbenzoate uptake. This is supported by non-utilization of 4-methylbenzoate during phase 1, despite presence of some proteins (MbrABC, Oah-2) for its degradation (Fig. 3). Since expression of genes for anaerobic catabolism of aromatic acids is typically controlled by regulatory proteins recognizing corresponding aryl-CoA esters (e.g. (43–46)), inhibition of 4-methylbenzoate uptake would prevent its intracellular accumulation and thus generation of 4-methylbenzoyl-CoA as putative inducer for expression of genes for 4-methylbenzoate catabolism (e.g. minimal transcript levels of *mclA*, encoding 4-methylbenzoate-CoA ligase, and *mbrC* during phase 1). It is known that benzoate-CoA ligases of *Azoarcus* sp. CIB (10), *T. aromatica* K172 (47) and *Magnetospirillum* sp. TS- 6 (48) also convert 4-fluorobenzoate at high rates (4-methylbenzoate not tested), and 3-methylbenzoate-CoA ligase (MbdA) of strain CIB exhibits the same activities with 3-methylbenzoate and benzoate (49). The high (50%) amino acid sequence identities of benzoate-CoA ligase (BclA) of strain pMbN1 and MbdA of strain CIB may suggest BclA of strain pMbN1 to possibly also accept 4-methylbenzoate as substrate. In this case, excluding 4-methylbenzoate from entering the cell would most effectively prevent gratuitous induction of the 4-methylbenzoyl-CoA pathway.

At the moment we can only speculate about the mechanism(s) underlying benzoate-mediated repression of 4-methylbenzoate utilization. Chromosomal co-

localization of *orf880,* encoding a TetR-type transcriptional regulator, with *orf870* (upstream in *cis*) and *mclA* (downstream in *trans*) could suggest that this regulator is involved in transcriptional regulation of 4-methylbenzoate uptake and degradation (for detailed gene arrangement see (14)). Notably, expression of transport and catabolic genes for anaerobic degradation of the structurally related 3-methylbenzoate in *Azoarcus* sp. CIB is inducible and was also suggested to be controlled by a TetR-type regulator (49). TetR-type regulators are diverse and often function as repressors of target gene expression in the absence of their inducing ligand (50). DNA binding of characterized TetR-type repressor DesT is relieved upon binding of its inducing ligand (palmitoyl-CoA), whereas binding of the co-repressor (oleoyl-CoA) restores transcriptional repression (51). In strain pMbN1, benzoate and phenylacetate both repressed 4-methylbenzoate utilization (Table 1; Fig. S1). One may speculate that in analogy to the DesT mode of action, benzoyl-CoA as the common intermediate of benzoate and phenylacetate degradation may act as co-repressor of Orf880, re-establishing transcriptional repression of genes for 4-methylbenzoate uptake and catabolism in cells that were originally adapted to 4-methylbenzoate.

Benzoate mediates repression of succinate uptake and catabolism. Benzoate preference of strain pMbN1 from a binary mixture with succinate is in parts reminiscent of a recent study with succinate-adapted "*A. aromaticum*" EbN1. In strain EbN1, benzoate mediates complete repression of succinate uptake by negatively controlling DctSR-dependent activation of *dctPQM* expression (12). Despite the similar diauxic growth phenotypes and presence of homologous DctPQM/DctSR (49–79% amino acid sequence identities), succinate was already partially depleted (i.e. 26% or 15% for binary or ternary substrate mixtures with succinate, respectively) during benzoate utilization in strain pMbN1. This may be due to the presence of an additional transporter for C_4-dicarboxylate uptake (DctA) with a cognate C_4-dicarboxylate-responsive two-component sensory/regulatory system (DctBD). Simultaneous employment of DctA and DctPQM in succinate uptake has been reported for *Pseudomonas aeruginosa* PAO1, where DctBD (*dctSR* is not encoded in strain PAO1) controls the coordinate expression of *dctA* and *dctPQM* (52). In strain pMbN1, succinate-specific detection (transcript/protein) of DctA and DctPQM indeed suggests participation of both transporters in succinate uptake (Figs. 2, 3). Furthermore, the similar changes and dynamics in *dctA* and *dctPM* transcript levels across phases 1–3 (ternary substrate mixture) also suggest their coordinated expression. A notable observation was the

increase in *dctA* and *dctPM* levels in strain pMbN1 when succinate was present in a substrate mixture, whereas in strain EbN1 *dctP* transcripts remained minimal. This indicates that benzoate does not have a similarly strong negative effect on DctSR/DctBD-dependent transcriptional activation of *dctA* and *dctPQM* expression. Together with the partial depletion of succinate already during phase 1, repression of succinate uptake cannot be the only determinant for the observed diauxie in strain pMbN1.

Succinate catabolism involves reactions of the TCA cycle, as well as malic enzyme (MaeB) and pyruvate dehydrogenase (PdhABC, LpdA) to generate acetyl-CoA. Their reduced transcript/protein levels in strain pMbN1 during benzoate utilization in phase 1 (Fig. 2) suggested benzoate to mediate inhibition of succinate conversion to acetyl-CoA. The abundance decrease of the *pdhB* transcript was by far most pronounced and prolonged until complete benzoate depletion (Fig. 2; Table S5). This striking *pdhB* profile indicates that benzoate-mediated repression of gene expression for pyruvate dehydrogenase represents the main target for inhibiting succinate catabolism. Since expression of pyruvate dehydrogenase genes requires intracellular pyruvate as inducer in *S. meliloti* (53) and *E. coli* (54), one may speculate that due to impeded succinate uptake and reduced MaeB-dependent pyruvate generation, intracellular pyruvate levels are insufficient for activation of pyruvate dehydrogenase gene expression. A more direct involvement of benzoate in repressing the expression of pyruvate dehydrogenase genes may be inferred from the experiment with a mixture of pyruvate and benzoate (Fig. S2C). Here, pyruvate was already partially (~1.5 mM) depleted during utilization of the preferred benzoate. Apparently, full expression of the pyruvate dehydrogenase genes viz. maximal pyruvate consumption could only be achieved upon complete depletion of benzoate.

A similar regulatory scenario involving the above mentioned C_4-dicarboxylate transporters as well as pyruvate dehydrogenase may underlie the diauxic growth behavior of strain pMbN1 with binary mixtures of L-malate or fumarate (adaptation substrates) and benzoate (preferred substrate) (Table 1; Fig. S3). The partial and slow depletion of C_4-dicarboxylates during benzoate utilization (phase 1) of diauxic growth of strain pMbN1 (Fig. 1A; Fig. S3) was previously not observed with strain EbN1 (12). In contrast, the latter started utilizing C_4-dicarboxylates only after benzoate was completely depleted. Apparently, the two strains employ different regulatory strategies

to achieve preferential benzoate utilization. Strain EbN1 completely inhibits succinate uptake via a single system (DctPQM), while strain pMbN1 combines partial inhibition of succinate uptake via two systems (DctPQM, DctA) with abundance decrease of its catabolic key enzyme (pyruvate dehydrogenase).

Further aliphatic substrates degraded via pyruvate dehydrogenase and tested in strains pMbN1 and EbN1 are oxaloacetate and pyruvate. They are generally known to be only weak or no substrate of known DctPQM and DctA transporters (31, 55–57) and dedicated alternative uptake systems are presently unknown in both strains. Strain pMbN1 displayed diauxic growth with binary substrate mixtures of oxaloacetate or pyruvate (adaptation substrates; partial and slow utilization in phase 1) and benzoate (preferred substrate) (Table 1; Figs. S2 and S3). This diauxic phenotype may indicate a regulatory combination of uptake and degradation control as described above for C_4-dicarboxylates and benzoate. Apparently, strain pMbN1 utilizes aliphatic acids, the degradation of which involves pyruvate dehydrogenase, in a diauxic manner from substrate mixtures with benzoate. Correspondingly, acetate, which following CoA activation can be directly channeled into the TCA cycle, is co-utilized with benzoate. In contrast, strain EbN1 co-utilizes oxaloacetate or pyruvate with benzoate ((12); unpublished observation). This agrees with DctPQM not being involved in oxaloacetate and pyruvate uptake and with the actual transporter not being affected in the presence of benzoate.

Shared intermediates (i.e. benzoyl-CoA and/or acetyl-CoA) in the degradation of benzoate and other non-repressing co-substrates (4-methylbenzoate, acetate, phenylacetate or 4-hydroxybenzoate), suggest benzoate itself as the inducer, mediating repression of C_4-dicarboxylate uptake and catabolism. The underlying molecular mechanisms for this dual repression are currently unknown. Benzoate may interfere directly or indirectly with DctSR- and/or DctBD-mediated signal transduction, e.g. by modulating kinase activities (58–60). In addition, benzoate may activate transcriptional repressor(s) (61–64), facilitate degradation of target transcripts (65, 66) or prevent translation of transcripts at the sensory/regulatory, uptake and catabolic level (67–69).

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SUPPLEMENTAL MATERIAL

Supplemental Tables

TABLE S1 Primers used for reverse transcription real-time PCR.

TABLE S2 Genes related to anaerobic degradation of 4-methylbenzoate, benzoate and succinate in strain pMbN1. Annotation of genes based on BLASTP hits in the UniprotKB/ Swissprot databases.

TABLE S3 Fold changes in protein abundance during anaerobic growth of strain pMbN1 with binary and ternary substrate mixtures (succinate-grown cells as reference), as determined by 2D DIGE.

TABLE S4 Selected proteins identified (Mascot scores) from membrane protein-enriched fractions of strain pMbN1 grown with benzoate, 4-methylbenzoate and succinate as single substrates, or as ternary substrate mixture.

TABLE S5 Abundance changes (ratios) of selected transcripts in cells of strain pMbN1 anaerobically grown with benzoate, 4-methylbenzoate or succinate (reference) as single substrates, or ternary substrate mixture (see Figs. 1D and 2). Data used for calculation is provided in Table S6.

TABLE S6 Average cycle threshold (C_T) values and efficiencies (E) obtained from reverse transcription real-time PCR reactions.

Supplemental Figures

FIG S1 Anaerobic growth and substrate utilization profiles of strain pMbN1 with binary substrate mixtures (adaptation substrate listed first). (A) Succinate and 4-hydroxybenzoate. (B) Succinate and phenylacetate. (C) Succinate and acetate. (D) 4-Methylbenzoate and phenylacetate.

FIG S2 Anaerobic growth and substrate utilization profiles of strain pMbN1 with binary substrate mixtures (adaptation substrate listed first). (A) Acetate and benzoate. (B) Acetate and 4-methylbenzoate. (C) Pyruvate and benzoate. (D) Pyruvate and 4-methylbenzoate. A correction factor of two was used to calculate final pyruvate concentrations from HPLC-analyzed samples.

FIG S3 Anaerobic growth and substrate utilization profiles of strain pMbN1 with binary substrate mixtures (adaptation substrate listed first). (A) Fumarate and benzoate. (B) L-Malate and benzoate. (C) Oxaloacetate and benzoate. (D) 4-Hydroxybenzoate and succinate.

FIG S4 Proteomic analysis of strain pMbN1 grown anaerobically with substrate mixtures. (A) Anaerobic diauxic growth with a ternary mixture of 4-methylbenzoate, benzoate and succinate (see Fig. 1D). Sampling points for proteomic analyses during phases (Ph) 1–3 are marked in green. (B) Exemplary false-color 2D DIGE gel image comparing the soluble proteins of cells grown with the ternary substrate mixture in phase 1 (benzoate utilization; test state, green color) with succinate-adapted cells (reference state, red color). Selected proteins related to anaerobic 4-methylbenzoate, benzoate and succinate utilization are marked (see Table S3 for protein names and predicted functions) (C) SDS PAGE gel of membrane protein-enriched fractions from cells grown with succinate, benzoate or 4-methylbenzoate as single substrates, or as ternary mixture.

B FURTHER PUBLICATIONS

Physiological and proteomic adaptation of *"Aromatoleum aromaticum"* EbN1 to low growth rates in benzoate-limited, anoxic chemostats.

Trautwein K, Lahme S, Wöhlbrand L, Feenders C, Mangelsdorf K, Harder J, Steinbüchel A, Blasius B, Reinhardt R, Rabus R.

Journal of Bacteriology (2012) **194** (9): 2165-2180

Stereochemical investigations reveal the mechanism of the bacterial activation of *n*-alkanes without oxygen.

Jarling R, Sadeghi M, Drozdowska M, Lahme S, Buckel W, Rabus R, Widdel F, Golding BT, Wilkes H.

Angewandte Chemie International Edition English (2012) **51** (6): 1334-1338

Co-metabolic conversion of toluene in anaerobic *n*-alkane-degrading bacteria.

Rabus R, Jarling R, Lahme S, Kühner S, Heider J, Widdel F, Wilkes H.

Environmental Microbiology (2011) **13** (9): 2576-2786

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Curriculum vitae

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Education

2010-2014	Doctoral thesis "Physiological and molecular investigation of the anaerobic 4-methylbenzoate and benzoate degradation in *Magnetospirillum* sp. strain pMbN1" General and Molecular Microbiology Group of Prof. Dr. Ralf Rabus, ICBM Oldenburg/MPI Bremen (Germany)
Dec. 2009	Diploma thesis "Cultivation of the Denitrifying Bacterium "*Aromatoleum aromaticum"* strain EbN1 in Anoxic Chemostats: Acetate-limited Growth and the Induction of Benzoate Utilization" Supervisor: Prof. Dr. Friedrich Widdel and Prof. Dr. Ralf Rabus
2006-2009	Main studies in biology (University of Bremen) Topics: Microbiology, cell biology and biochemistry
2004-2006	Prediploma in biology (University of Bremen)
2002-2003	Civil service (Arbeiterwohlfahrt Bremerhaven)
1999-2002	Geschwister Scholl Gymnasium Bremerhaven University entrance diploma (Abitur)

Positions and projects

Since Jan. 2014	Research Associate, School of Civil Engineering and Geosciences, Geomicrobiology group, Newcastle University, Newcastle upon Tyne (United Kingdom)
2010-2013	DFG priority project 1319 "Biological transformations of hydrocarbons without oxygen: from the molecular to the global scale"
2007-2009	Research assistant at the Max Planck Institute for Marine Microbiology

Conferences and workshops

2014	Symposium “Novel Anaerobes”, Wageningen, Netherlands Assessing the role of sulfide-oxidizing nitrate-reducing *Epsilonproteobacteria* in oil field corrosion Poster presentation
2013	SYNMIKRO 2nd Summer School for Microbial Cell Biology and Synthetic Signaling Systems LOEWE Center for Synthetic Microbiology, Marburg, Germany
2013	VAAM annual meeting Anaerobic growth of aromatic compound-degrading bacteria with mixtures of succinate and benzoate Oral presentation
2011	Workshop of the DFG priority program 1319 Anaerobic degradation of 4-methylbenzoate by the denitrifying strain pMbN1 involves a novel type of benzoyl-CoA reductase. Oral presentation
2011	VAAM annual meeting Anaerobic degradation of 4-methylbenzoate by the denitrifying strain pMbN1 involves a novel type of benzoyl-CoA reductase. Poster presentation
2010	VAAM annual meeting Growth of “*Aromatoleum aromaticum“* strain EbN1 in acetate-limited chemostats. Poster presentation

Memberships

German Association for General and Applied Microbiology - VAAM

Society for Applied Microbiology - SfAM