Physiology of the anaerobic degradation of naphthalene and benzene by marine sulfate-reducing bacteria

Gao Chen

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DISSERTATION

zur

Erlangung des Grades eines
Doktors der Naturwissenschaften
– Dr. rer. nat. –

dem Fachbereich Biologie/Chemie der
Universität Bremen vorgelegt von

Gao Chen
aus Jiangsu, China

Bremen, 2014

Die vorliegende Doktorarbeit wurde in der Zeit von September 2009 bis Februar 2014 am Max Planck Institut für Marine Mikrobiologie in Bremen durchgeführt

1. Gutachter: Prof. Dr. Friedrich Widdel, Universität Bremen

2. Gutachter: Prof. Dr. Ralf Rabus, Universität Oldenburg

3. Prüfer: Prof. Dr. Ulrich Fischer, Universität Bremen

4. Prüfer: Dr. Florin Musat, Max Planck Institut für Marine Mikrobiologie, Bremen

Tag des Promotionskolloquiums: 11 April, 2014

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Abbreviations

ASW	Artificial sea water
BTEX	Benzene, toluene, ethylbenzene, and xylenes
CoA	Coenzyme A
CODH	Carbon monoxide dehydrogenase
DAPI	4',6'-diamino-2-phenylindole
DCCD	N,N'-dicyclohexylcarbodiimide
FCCP	Carbonyl cyanide 4-(trifluoromethoxy) phenylhydrazone
FDH	Formate dehydrogenase
FISH	Fluorescence *in situ* hybridization
GC-MS	Gas chromatography-mass spectrometry
HMN	2,2,4,4,6,8,8-Heptamethylnonane
HPLC	High performance liquid chromatography
NaphSx	NaphS2, NaphS3, and NaphS6
PAHs	Polycyclic aromatic hydrocarbons
PDA	Photo diode array
PPC	Phenylphosphate carboxylase
rpm	Rounds per minute
UPLC	Ultra performance liquid chromatography
UV-VIS	Ultraviolet-visible

Summary

Hydrocarbons, originated from both geological sources, e.g., crude oil and coal, and biological sources, are widespread in the environment. Anthropogenic activities lead to an increase of distribution and accumulation of hydrocarbons in the environment. Biodegradation of hydrocarbons in the presence of oxygen has been intensively investigated, whereas the study of hydrocarbon biodegradation processes under anoxic conditions is a relatively young research field. A particular interest of the research of anaerobic degradation processes is the initial activation reactions. They functionalize the chemically inert hydrocarbons without the involvement of molecular oxygen. Addition to fumarate, yielding succinate adducts, has been described as a common principle for anaerobic activation of alkanes and alkylated aromatic hydrocarbons, e.g., toluene, xylenes, and 2-methylnaphthalene. However, the mechanism of anaerobic activation of unsubstituted aromatic hydrocarbons, like benzene and naphthalene, are presently not clearly understood. The main focus of the present work was to investigate the physiology and possible activation mechanisms of benzene and naphthalene under sulfate-reducing conditions. Experiments were carried out with marine sulfate-reducing bacteria able to degrade benzene (enrichment culture with benzene [Musat and Widdel, 2008]), or naphthalene (strain NaphS2 [Galushko *et al*., 1999], strain NaphS3 and strain NaphS6 [Musat *et al*., 2009]; further on generically referred as strains NaphSx).

Substrate response experiments were carried out in order to identify conditions for analysis of proteins specifically expressed during growth on naphthalene. The lack of an immediate response of naphthalene-adapted cultures to 2-methylnaphthalene (Musat *et al*., 2009) together with the presence of a long lag phase in incubations of 2-methylnaphthalene-adapted cultures with naphthalene (present work) suggested that activation and degradation of these substrates (at least until the common intermediate 2-naphthoate) was carried out by distinct enzymes. Thus, comparative proteomic analyses of naphthalene- vs. benzoate-grown cells were conducted with a focus on identification of proteins specifically involved in naphthalene degradation. A protein with high similarity as one subunit of phenylphosphate carboxylase was discovered to be specifically up-regulated in naphthalene-grown cells for all three strains, and was proposed as being directly involved in the activation of naphthalene.

Identification of the naphthalene activation product was attempted using ^{14}C-labeled naphthalene as a substrate for concentrated cell suspensions, and following the sequence of metabolites formed during a short incubation time. Metabolites were identified based on their co-elution with authentic standards. 2-Naphthoate was detected as the first metabolite in

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incubations of strains NaphSx with ^{14}C-labeled naphthalene as a substrate. The detection of ^{14}C-2-naphthoate was only possible when unlabeled 2-naphthoate was added for isotope dilution, indicating a fast consumption of 2-naphthoate by further catabolism. In addition to 2-naphthoate, [5,6,7,8]-tetrahydro-2-naphthoate could be identified upon sample extraction and concentration. The detection of 2-naphthoate as the earliest detectable intermediate supported the hypothesis of a carboxylation-like reaction as the first step in anaerobic naphthalene degradation.

Attempts to measure the activities of naphthalene carboxylation or 2-naphthoate decarboxylation in cell-free extracts were unsuccessful. It could be shown that the (de)carboxylation was dependent on the presence of intact cells, and was decreasing during various steps of cell lysis. One possible explanation was that the (de)carboxylation activity was associated with membrane potential, and thus required intact membranes. Therefore, the effects of the uncoupler FCCP, which could dissipate the membrane potential and deprive cellular energy, on the formation and further metabolism of 2-naphthoate were investigated. It was assumed that the addition of FCCP would collapse the carboxylation of naphthalene in intact cells. In contrast to the working hypothesis, the conversion of naphthalene to 2-naphthoate and its reverse reaction were not affected by FCCP addition in whole-cell assays. The application of the ATPase inhibitor DCCD as well as the ionophore monensin in combination with FCCP cannot inhibit, but slightly stimulate the formation and accumulation of 2-naphthoate in whole-cell assays. This finding implied that the carboxylation-like reaction, activating naphthalene to 2-naphthoate, might not require a direct ATP input.

The postulated carboxylation-like activation of naphthalene was further investigated by isotope exchange experiments. Carbon radioisotope exchange between CO_2/HCO_3^- and 2-naphthoate was observed in naphthalene-grown cultures, implying that the reaction of naphthalene functionalization to 2-naphthoate was reversible. The net production of naphthalene was measured as well when cells were fed with 2-naphthoate as an initial substrate, which unambiguously confirmed the reversibility of such a naphthalene carboxylation-like reaction. Both the isotope exchange and the reverse reactions were only observable under sulfate-free conditions, required so as to prevent the rapid consumption of 2-naphthoate by cells of strain NaphSx.

The anaerobic degradation of benzene was studied with the highly enriched sulfate-reducing culture BzS12. In addition to benzene, culture BzS12 could be induced to utilize toluene as a substrate after a long adaptation time. The accumulation of benzoate could be detected in the supernatant of benzene-grown BzS12 culture, suggesting benzoate (or its CoA

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derivative) might be an intermediate in anaerobic degradation of benzene. Together with the exclusion of methylation and hydroxylation hypotheses reported previously (Musat and Widdel, 2008), a carboxylation-like reaction was tentatively postulated as the initial step to activate benzene under sulfate-reducing conditions. Further metabolite analyses from cultures with labeled benzene or labeled bicarbonate showed that the carboxyl group in benzoate, as a detectable metabolite, was not derived from CO_2 or bicarbonate, but might be generated from a downstream intermediate of benzene degradation.

Zusammenfassung

Kohlenwasserstoffe stammen sowohl aus geologischen Quellen, z.B. Erdöl und Kohle, als auch aus biologischen Quellen und sind in der Umwelt weit verbreitet. Anthropogene Aktivität führt zu einer Zunahme der Verbreitung und Anhäufung von Kohlenwasserstoffen in der Umwelt. Während der biologische Abbau von Kohlenwasserstoffen in Gegenwart von Sauerstoff intensiv untersucht wurde, ist deren Abbau unter anoxischen Bedingungen noch ein recht junges Forschungsfeld. Ein besonderes Interesse bei der Erforschung der anaeroben Abbauprozesse gilt der eingänglichen Aktivierungsreaktion, welche den chemisch reaktionsträgen Kohlenwasserstoff ohne Beteiligung von elementarem Sauerstoff funktionalisiert. Eine Addition an Fumarat mit der Bildung substituierter Succinaten wurde als häufiges Prinzip für die anaerobe Aktivierung von Alkanen und Alkylaromaten, z.B. von Toluol, Xylolen und 2-Methylnaphthalin, beschrieben. Hingegen sind die Mechanismen zur anaeroben Aktivierung von unsubstituierten aromatischen Kohlenwasserstoffen wie Benzol oder Naphthalin gegenwärtig noch nicht ausreichend verstanden. Der Hauptfokus der vorliegenden Arbeit lag auf der Untersuchung der Physiologie und möglicher Aktivierungsmechanismen von Benzol und Naphthalin unter Bedingungen der Sulfatreduktion. Experimente wurden mit marinen sulfatreduzierenden Bakterien durchgeführt, die Benzol (Anreicherung mit Benzol [Musat und Widdel, 2008]) oder Naphthalin (Stamm NaphS2 [Galushko *et al.*, 1999], Stämme NaphS3 und NaphS6 [Musat *et al.*, 2009]; im Folgenden zusammenfassen als NaphSx bezeichnet) abbauen können.

Experimente zum Substrat-Anpassungsverhalten wurden durchgeführt, um Bedingungen für die Analyse von Proteinen zu finden, die spezifisch beim Wachstum mit Naphthalin gebildet werden. Das Fehlen einer sofortigen Reaktion Naphthalin-adaptierter Kulturen auf 2-Methylnaphthalin (Musat *et al.*, 2009) sowie eine lange lag-Phase 2-Methylnaphthalin-adaptierter Kulturen gegenüber Naphthalin (vorliegende Arbeit) deuteten an, dass Aktivierung und Abbau dieser Substrate (zumindest bis zum gemeinsamen Intermediate 2-Methylnaphthoat) über verschiedene Enzyme erfolgt. Daher wurden vergleichende proteomische Analysen von Zellen durchgeführt, die auf Naphthalin und Benzoat gewachsen waren, wobei der Fokus auf spezifischen Proteinen für den Naphthalin-Abbau lag. Ein Protein mit großer Ähnlichkeit zu einer Untereinheit von Phenylphosphat-Carboxylase war bei allen drei Stämmen beim Wachstum auf Naphthalin 'hochreguliert'; somit wird eine direkte Beteiligung bei der Naphthalin-Aktivierung vorgeschlagen.

Eine Identifizierung des Produkts der Naphthalin-Aktivierung wurde mit ^{14}C-markiertem Naphthalin in konzentrierten Zellsuspensionen versucht, wobei auf die Abfolge der Metabolitbildung innerhalb kurzer Inkubationszeit geachtet wurde. Metabolite wurden auf Grund ihrer Ko-Elution mit authentischen Standards identifiziert. Als erster Metabolit wurde 2-Naphthoat in Inkubationen von Stämmen NaphSx gefunden. ^{14}C-Naphthoat konnte nur gefunden warden, wenn unmarkiertes 2-Naphthoat zwecks Isotopenverdünnung eingesetzt wurde, was auf eine schnelle weitere Verwertung von 2-Naphthoat im weiteren Katabolismus hindeutete. Zusätzlich zu 2-Naphthoat wurde nach Probenextraktion und Konzentrierung [5,6,7,8]-Tetrahydro-2-naphthoat identifiziert. Das Auffinden von 2-Naphthoat als das früheste erkennbare Intermediat untermauert die Hypothese einer Carboxylierungs-ähnlichen Reaktion als den ersten Schritt im anaeroben Abbau von Naphthalin.

Die vorgeschlagene Carboxylierungs-ähnliche Aktivierungt von Naphthalin wurde über Isotopen-Austauschexperimente weiter untersucht. In 2-Naphthoat-Kulturen wurde ein Kohlenstoff-Radioisotopen-Austausch zwischen CO_2/HCO_3^- und 2-Naphthoat beobachtet, was auf Reversibilität der Funktionalisierung von Naphthalin zu 2-Naphthoat hindeutet. Eine Netto-Bildung von Naphthalin wurde gemessen, wenn Zellen mit 2-Naphthoat versorgt wurden, was eindeutig die Reversibilität der Naphthalin-'Carboxylierung' zeigte. Beides, der Isotopenaustausch und die reverse Reaktion waren nur unter Sulfat-freien Bedingungen zu beobachten, die nötig waren, um eine schnelle Verwertung von 2-Naphthoat durch Zellen von Stamm NaphSx zu verhindern.

Versuche, die Aktivitäten einer Carboxylierung von Naphthalin oder Decarboxylierung von 2-Methylnaphthalin in zellfreien Extrakten zu messen, waren nicht erfolgreich. Es konnte gezeigt werden, dass die (De)Carboxylierung von der Gegenwart noch intakter Zellen abhing und mit zunehmender Zell-Lyse abnahm. Eine mögliche Erklärung dafür war, dass die (De)Carboxylierungs-Aktivität mit dem Membranpotenzial zusammenhängt und daher intakte Membranen benötigt. Daher wurde der Effekt des Entkopplers FCCP, welcher das Membranpotenzial abbaut und die Zellen deenergetisiert, auf die Bildung und den weiteren Metabolismus von 2-Methylnaphthoat untersucht. Es wurde angenommen, dass die Zugabe von FCCP die Carboxylierung von Naphthalin zum Kollabieren bringt. Im Gegenteil zu dieser Arbeitshypothese wurden jedoch die Carboxylierung von Naphthalin zu 2-Naphthoat und deren reverse Reaktion durch FCCP-Zusatz zu Ansätzen mit ganzen Zellen nicht beeinträchtigt. Dieser Befund deutete an, dass die Naphthalin-Carboxylierung' keinen Energieeintrag benötigt.

Der anaerobe Abbau von Benzol wurde mit der hoch angereicherten sulfatreduzierenden Kultur BzS12 untersucht. In Kultur BzS12 konnte die Verwertung von Toluol nach einer langen Inkubationszeit induziert werden. Im Überstand der mit Benzol gewachsenen Kultur BzS12 wurde Benzoat nachgewiesen, was zeigt, dass Benzoat (oder sein CoA-Derivat) ein Intermediat beim anaeroben Abbau von Benzol ist. Zusammen mit dem vorherigen Ausschluss einer Methylierung und Hydroxylierung (Musat und Widdel, 2008) konnte somit eine Carboxylierungs-ähnliche Reaktion für den initialen Schritt bei der Aktivierung von Benzoat unter Bedingungen der Sulfatreduktion angenommen werden. Weitere Metabolitanalysen in Kulturen mit markiertem Benzol oder markiertem Bicarbonat zeigten, dass die Carboxylgruppe in Benzoat als nachweisbarem Metabolit nicht aus CO_2 oder Bicarbonat, sondern von einem Intermediat des Benzolabbaus stammte.

1. Introduction

Hydrocarbons, consisting exclusively of carbon and hydrogen atoms, are mainly naturally formed by long-term geochemical reactions of buried ancient biomass, which is the origin of fossil fuels (crude oil and coal). They are also produced as metabolites of living organisms, e.g., plants, insects, and microorganisms. It is common to classify hydrocarbons into four groups: the alkanes (saturated hydrocarbons), alkenes, aromatic hydrocarbons, and alkynes. Because of similar electronegativity of the carbon and hydrogen atom and the lack of functional groups, hydrocarbons are largely apolar and exhibit low chemical reactivity at room temperature.

The utilization of hydrocarbons by aerobic microorganisms has been well studied. However, oxygen is often unavailable in the environments where hydrocarbons occur, e.g., in deep sediments and in oil reservoirs (Aitken *et al*., 2004). There has been increasing interest during the past two decades in the anaerobic degradation of petroleum hydrocarbons. The main subject of the present study was the degradation of the unsubstituted aromatic hydrocarbon, naphthalene, with sulfate as electron acceptor. Further experiments were also carried out with a highly enriched benzene-degrading sulfate-reducing culture.

1.1 Aromatic hydrocarbons

Aromatic hydrocarbons have unsaturated cyclic ring structures that possess particular stability as a result of the delocalization of π-electrons. According to the number of cyclic rings, aromatic hydrocarbons can be divided into monocyclic compounds, such as benzene and its alkylated derivatives, and polycyclic aromatic hydrocarbons (PAHs), for example naphthalene, anthrancene and their alkylated derivatives. Benzene and naphthalene are the two simplest representatives of unsubstituted monocyclic and polycyclic aromatic hydrocarbons, respectively.

1.1.1 Criteria of aromaticity and the Hückel rule

There is a wide range of compounds, with rings and unsaturated C–H bonds; however, not all of these are aromatic. There are rules which define whether a compound is aromatic. The Hückel rule provides the criterion for defining aromatic compounds. According to this rule, the carbon atoms in the aromatic ring must be sp^2 hybridized and constitute a planar ring so that p_z orbitals, which are perpendicular to the plane of the ring, overlap and form a planar delocalized π-electron system (Fig. 1.1). This significantly contributes to the stability of

aromatic compounds. Furthermore, the ring system must harbor $4n + 2$ ($n = 0, 1, 2, 3…$) π-electrons. For instance, benzene has six π-electrons ($n = 1$) whereas naphthalene has ten π-electrons ($n = 2$).

σ-bonds
sp^2 hybridized orbitals

6 p_z-orbitals

delocalized
π-electron system
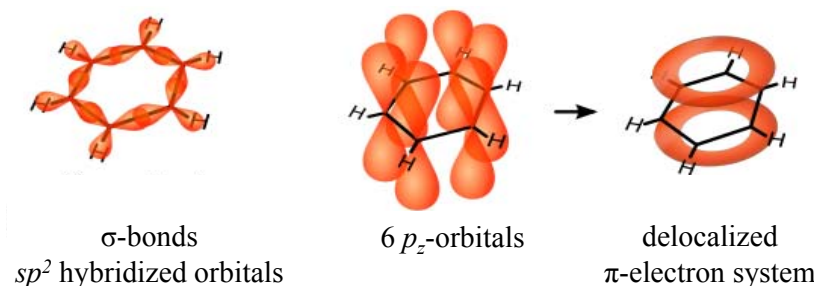
Fig. 1.1. Scheme of molecular orbital structure of benzene (adapted from http://en.wikipedia.org/wiki/Benzene)

The extra gain of stability, the delocalization or resonance energy, is depicted in Fig. 1.2 for benzene (151 kJ mol^{-1}). As a consequence, benzene has much lower enthalpy change of hydrogenation than predicted for a hypothetical compound with three isolated double bonds. In case of naphthalene, the resonance energy is around 255 kJ mol^{-1} (Hepworth *et al*., 2002).
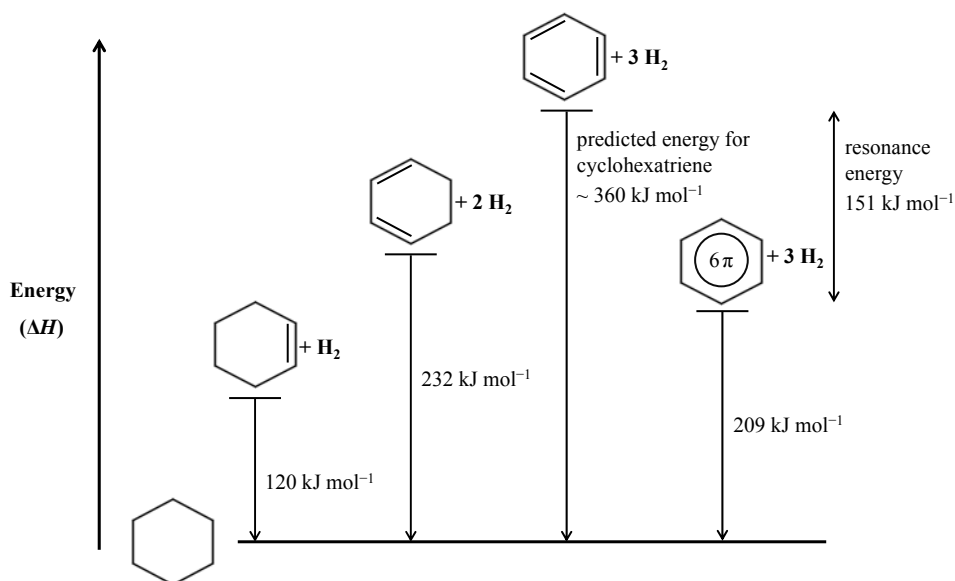
Fig. 1.2. Differences in energy of hydrogenation of cyclohexene, cyclohexadiene, and benzene, illustrating the additional stability resulting from resonance structure in benzene (adapted from Hepworth *et al*., 2002)

1.1.2 Physical and chemical properties of aromatic hydrocarbons

When confronted with many aromatic substances, their olfactory character is the first impression, a property which originally gave rise to the term of "aromaticity". Although the smells are sometimes regarded as "pleasant", many aromatic hydrocarbons are quite toxic to organisms. Their toxicity is attributed to their lipophilic character, which allows them to freely

penetrate into and through biological membranes, including those of the nervous system, and to their partial transformation to reactive aromatic metabolites that may affect DNA and cause mutations. Some aromatic hydrocarbons, e.g., benzene, benz[a]anthracene and benzo[a]pyrene, have been classified as carcinogens. Therefore, aromatic hydrocarbons, e.g., PAHs and BTEX, have been intensively studied with respect to their toxicity, occurrence, and fate in the environment.

In general, due to their apolar character, hydrocarbons are poorly soluble in water at room temperature. Nevertheless, there are variations, and aromatic hydrocarbons may exhibit slightly to significantly higher solubility than saturated ones such as n-alkanes. For example, the solubility of naphthalene in water is only about 240 μM, and benzene even dissolves up to 23 mM (Eastcott *et al*., 1988). The higher solubility of benzene may contribute to bioavailability and adverse health effects.

All six hydrogen atoms are chemically equivalent in benzene, whereas this is not the case in PAHs. For instance, naphthalene contains two sets of four chemically equivalent hydrogen atoms, which are classified as α- and β-positions. Therefore, there are two isomers of monosubstituted naphthalene derivatives, e.g., 1- and 2-methylnaphthalene (Wilkes and Schwarzbaue, 2010).

Despite the lack of functional groups, aromatic hydrocarbons are readily subjected to substitution reactions. The characteristic conjugated π-electron system above and below the aromatic ring plane with high electron density facilitates the attack by electron-deficient electrophiles. Hence, electrophilic aromatic substitution is a common reaction, especially for unsubstituted aromatic hydrocarbons, e.g., benzene and naphthalene. After an attack by electrophiles (E^+) on the π-cloud, a carbocation is formed, first as an σ-complex. Then, a proton is released from the carbocation, regenerating the π-electron system and yielding a substituted aromatic compound (Fig. 1.3A). Although electrophilic aromatic substitution is a favorable reaction for aromatic compounds, nucleophilic aromatic substitution does occur in certain circumstances. This may occur if aromatic compounds have a strong electron-withdrawing group (EWG), causing electron deficiency in their conjugated π-cloud. Then, nucleophiles ($:Nu^-$) can attack and disrupt the π-cloud, resulting in a carboanion intermediate species. Afterwards, aromaticity is restored through elimination of a leaving group (X^-) to yield the substituted aromatic compound (Fig. 1.3B).
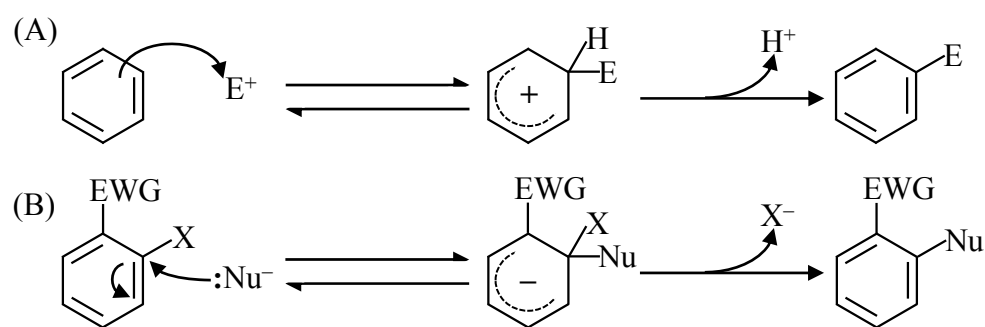
Fig. 1.3. Schematic process of electrophilic aromatic substitution (A) and nucleophilic aromatic substitution (B). E^+, electrophile; EWG, strongly electron-withdrawing group; $:Nu^-$, nucleophile. For detailed explanations, see text (adapted from Carey and Sundberg, 2007).

1.1.3 Aromatic hydrocarbons in the environment

Next to saturated hydrocarbons, aromatic hydrocarbons are the second most important group of constituents in crude oil. The content varies from 20 – 45% in 87% of crude oil types; 10% of crude oil types contain less than 20% aromatic hydrocarbons; and 3% contain more than 45% aromatic hydrocarbons. Among them, benzene, naphthalene, phenanthrene and their alkylated derivatives are usually the most abundant compounds (Tissot and Welte, 1984). Polycyclic aromatic hydrocarbons are also dominant constituents of coal tar; for example, typical coal tar contains about 10% naphthalene by mass (Wise *et al*., 1988).

Besides geochemical sources, aromatic hydrocarbons can also be formed biologically. For example, naphthalene was detected as the main component in extracts of petals, gynoecia, and leaves of *Magnolia* flowers (Azuma *et al*., 1996). Naphthalene was also produced as an insect repellent by the endophytic fungus *Muscodor vitigenus* (Daisy *et al*., 2002). Wilcke *et al*. (2003) showed that termites and woody plants are sources of naphthalene, phenanthrene and perylene in soils in Brazil. The bacterium, *Tolumonas auensis*, has the ability to convert phenylacetate (produced from the fermentative breakdown of phenylalanine) to toluene via a decarboxylation-like reaction (Fischer-Romero *et al*., 1996).

In addition, incomplete combustion of carbon-containing materials such as wood, coal, diesel and tobacco can lead to discharge of large amounts of aromatic hydrocarbons (Oanh *et al*., 1999).

Due to the wide occurrence and extensive use of aromatic hydrocarbons in industry, they can often be detected in the environment, viz. in the atmosphere, soils, water and sediments (Laflamme and Hites, 1978; Harrison *et al*., 1996; Schauer *et al*., 2003). Here, aromatic hydrocarbons can undergo abiotic or biologically-mediated transformations. For instance, photooxidation reactions can effectively decompose aromatic hydrocarbons at both

the laboratory and field scale (Forstner *et al*., 1997; Miller and Olejnik, 2001). Also, chemical reactions in the dark mediated by minerals of aluminum, iron and manganese, are effective for transformation of aromatic hydrocarbons in the environment (Kim, 2002). Oxidation reactions may involve radicals, such as oxygen radicals and hydroxyl radicals (Kochany and Maguire, 1994).

However, abiotic processes alone usually do not lead to complete oxidation and therefore may result in an accumulation of various transformation products. In contrast, biological degradation of aromatic hydrocarbons by microorganisms is usually complete and appears to be the main natural process responsible for the complete mineralization of aromatic hydrocarbons to the terminal inorganic products, CO_2 and H_2O. It has been proven that, besides oxygen (oxic condition), also nitrate, iron(III) or sulfate can be used as alternative terminal electron acceptors under anoxic conditions during the oxidative degradation of hydrocarbons. Biodegradation can even occur under methanogenic conditions, a syntrophic process often involving proton reduction to H_2 as an intermediate (Fig. 1.4).
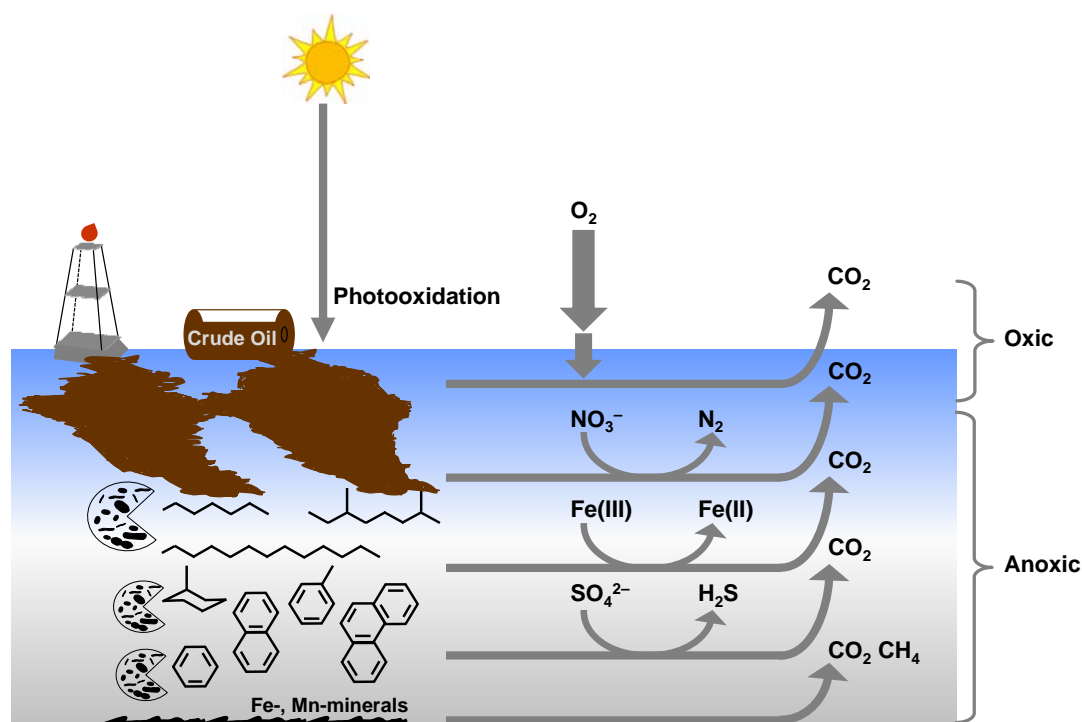
Fig.1.4. Scheme representing a massive crude oil spill from transportation or from a drilling platform in the marine environment, leading to the discharge of large amounts of hydrocarbons. Physico-chemical processes, such as photooxidation and chemical reactions with oxidizing Fe-, or Mn-minerals, can initiate the degradation of crude oil to a certain level. Biodegradation mediated by microorganisms can completely oxidize hydrocarbons to the inorganic products CO_2 and H_2O with oxygen, nitrate, iron(III) or sulfate as terminal electron acceptors. The biodegradation of hydrocarbons can also occur under methanogenic conditions, leading to the formation of CH_4.

1.2 Anaerobic biodegradation of aromatic hydrocarbons — from microcosm studies to pure cultures

Because of environmental concern and biochemical interest, the biodegradation of aromatic hydrocarbons has been intensively investigated. However, most of these studies were conducted under oxic conditions. Due to the chemical stability and assumed need for O_2 as a co-reactant, aromatic hydrocarbons were for a long time thought to be biologically recalcitrant under anoxic conditions. A few decades ago, Ward et $al.$ (1980) showed that ^{14}C-toluene and ^{14}C-benzene could be anaerobically transformed to $^{14}CO_2$ in oil-polluted sediments due to microbial activities. Since then, more and more microcosm studies, several of which led to an isolation of pure cultures, revealed that aromatic hydrocarbons could be completely oxidized with nitrate, iron(III) and sulfate as terminal electron acceptors, or be degraded under conditions of methanogenesis (Fig. 1.4; reviewed in Lovley, 2000; Widdel and Rabus, 2001; Coates et $al.$, 2002; Meckenstock et $al.$, 2004; Vogt et $al.$, 2011; Meckenstock and Mouttaki, 2011).

The first Fe(III)-reducing bacterium shown to utilize several aromatic compounds including toluene and phenol was strain GS-15 (today $Geobacter$ $metallireducens$; Lovely et $al.$, 1989; 1990). Dolfing and coworkers isolated a bacterium, $Pseudomonas$ sp. strain T, from an aquifer column, which was the first pure culture capable of degrading toluene with nitrate or nitrous oxide (N_2O) as terminal electron acceptor (Dolfing et $al.$, 1990). Later, the first pure culture of toluene-degrading sulfate-reducing bacterium strain Tol2 was isolated as well (Rabus et $al.$, 1993). After these studies, more and more pure cultures of toluene-degrading anaerobic bacteria were obtained (Evans et $al.$, 1991; Schocher et $al.$, 1991; Altensehmidt and Fuchs; 1991; Zhou et $al.$, 1995). Pure cultures degrading other substituted aromatic hydrocarbons such as xylenes or ethylbenzene have been isolated and described as well (Rabus and Widdel, 1995, 1996; Ball et $al.$, 1996; Harms et $al.$, 1999; Kniemeyer et $al.$, 2003). Anaerobic microorganisms degrading substituted aromatic hydrocarbons have been recently reviewed (for instance, Widdel et $al.$, 2010).

However, with respect to unsubstituted aromatic hydrocarbons, e.g., benzene and naphthalene, only a few pure cultures have been isolated to date. Because the focus of the present work was on naphthalene and benzene, Table 1.1 presents an overview of pure or highly enriched cultures reported to anaerobically degrade these particular aromatic hydrocarbons.

It was discovered that hyperthermophilic archaeon $Ferroglobus$ $placidus$ could oxidize benzene by coupling to the reduction of Fe(III); this was obviously the first demonstration of a

microorganism in pure culture that can grow on benzene under strictly anoxic condition (Holmes *et al*., 2011). Later on, the capability of anaerobic oxidation of benzene with Fe(III) as electron acceptor was also proven in *Geobacter* species, strain Ben and *Geobacter metallireducens* (Zhang *et al*., 2012; 2013). To date, there is still no pure benzene-degrading sulfate-reducing culture reported; only highly enriched cultures have been described (Phelps *et al*., 1998; Musat and Widdel, 2008; Abu Laban *et al*., 2009). Recently, it was proposed that benzene was degraded by syntrophic consortia via interactions of different types of microorganisms (van der Zaan *et al*., 2012; Taubert *et al*., 2012).

In case of naphthalene, strains NaphS2 and NaphS3 are the only two pure cultures of naphthalene-degrading sulfate-reducing bacteria reported so far, with affiliation to the family *Desulfobacteraceae* of the *Deltaproteobacteria* (Galushko *et al*., 1999; Musat *et al*., 2009). A culture termed strain NaphS6, which may harbor a contaminant with very low cell number, was also described in the same study. The major cell type was highly related to strains NaphS2 and NaphS3. The sulfate-reducing enrichment culture N47, which was enriched from freshwater sediments (Meckenstock *et al*., 2000), has been extensively investigated with respect to anaerobic naphthalene and 2-methylnaphthalene degradation (Safinowski and Meckenstock, 2006; Selesi *et al*., 2010; Bergmann *et al*., 2011a, b; Mouttaki *et al*., 2012).

Recently, an "intra-aerobic" pathway with nitric oxide (NO) dismutation to N_2 and O_2 was proposed in a methane-oxidizing denitrifying enrichment culture *Candidatus Methylomirabilis oxyfera* (Raghoebarsing *et al*., 2006; Ettwig *et al*., 2010; 2012) and also suggested for an alkane-degrading nitrate-reducing bacterium strain HdN1 (Zedelius *et al*., 2011). Then, a few cultures which had been originally obtained as apparent anaerobic degraders of aromatic hydrocarbons may not involve an anaerobic pathway. For example, *Dechloromonas* strains RCB and JJ, *Azoarcus* strains DN11 and AN9 which can degrade benzene and reduce nitrate (Coates *et al*., 2001; Kasai *et al*., 2006), in principle, may also make use of an intra-aerobic pathway. Genome analysis of *Dechloromonas* strains RCB found several mono-/di-oxygenases and other putative enzymes involved in aerobic pathway, supporting such a hypothesis (Salinero *et al*., 2009). Analogously, naphthalene-degrading nitrate-reducing strains NAP-3-1, NAP-3-2, and NAP-4 (Rockne *et al*., 2000) may employ internally formed oxygen as well; however, this assumption awaits further experimental verification. *Alicycliphilus denitrificans* strain BC can grow on benzene with chlorate as an electron acceptor (Weelink *et al*., 2008). It has been proposed that chlorate can provide O_2 via cleavage ('dismutation') of chlorite (ClO_2^-), which might play a role in anaerobic benzene degradation.

Table 1.1. Overview of anaerobic benzene- and naphthalene-degrading pure or highly enriched cultures

Source of enrichment	Substrate	e⁻ acceptor	Dominant phylotypes	Proposed activation pathway	References
Cartwright gasoline station, Toronto, Canada	Benzene	NO_3^-	Betaproteobacteria (Azoarcus, Dechloromonas), Chlorobium sp.	Methylation to toluene (transient production of toluene and benzoate were detected)	Burland and Edwards, 1999; Mancini et al., 2003; Ulrich et al., 2005
Strain RCB was enriched as a hydrocarbon-oxidizing chlorate reducer and strain JJ was isolated as a humic-substances-oxidizing nitrate reducer	Benzene	NO_3^-	Dechloromonas sp. strains RCB* and JJ*	Hydroxylation to phenol, which was subsequently carboxylated and eventually channeled to benzoate	Coates et al., 2001; Chakraborty et al., 2005; Chakraborty and Coates, 2005
Gasoline-contaminated groundwater sample from Kumamoto, Japan	Benzene	NO_3^-	Azoarcus sp. strains DN11* and AN9*	N.D.	Kasai et al., 2006, 2007
Enriched in a chemostat that was running with benzene and nitrate for more than 8 years	Benzene	NO_3^-	Peptococcaceae dominant, Rhodocyclaceae and Burkholderiaceae were in synthrophic interaction	No metabolites detectable	van der Zaan et al., 2012
Strain BC was isolated from a stable benzene-degrading, chlorate-reducing enrichment culture	Benzene	ClO_3^-	Alicycliphilus denitrificans strain BC*	Intra-aerobic pathway	Weelink et al., 2008
Sediment from a shallow refinery-contaminated aquifer near Ponca City, USA	Benzene	Fe(III)	N.D.	Phenol and benzoate as metabolites (carboxyl group in benzoate originated from benzene)	Caldwell et al., 1999; Caldwell and Suflita, 2000
Soil sample from a former coal gasification site in Gliwice, Poland	Benzene	Fe(III)	Peptococcaceae, Desulfobulbaceae, Actinobacteria (synthrophic growth, named enrichment BF)	Carboxylation to benzoate (carboxyl group derived from bicarbonate), while phenol was formed abiotically by autoxidation and genes encoding putative carboxylase were identified	Kunapuli et al., 2007; 2008; Abu Laban et al., 2010
Ferroglobus placidus was originally isolated from a shallow submarine hydrothermal system at Vulcano in Italy, as a Fe²⁺-oxidizing archaeum	Benzene	Fe(III)	Hyperthermophilic archaeon Ferroglobus placidus*	Carboxylation (benzoate was detected as a metabolite and a gene encoding putative carboxylase was up-regulated in benzene-grown cells)	Hafenbradl et al., 1996; Holmes et al., 2011
Sediment from petroleum-contaminated aquifer, Bemidji, USA	Benzene	Fe(III)	Geobacter strain Ben* and Geobacter metallireducens*	Hydroxylation (phenol was detected as a metabolite; genes involved in phenol metabolism were up-regulated during growth on benzene)	Zhang et al., 2012; 2013
Sediment from area of deep-water hydrocarbon seeps in the Guaymas Basin, Gulf of Mexico	Benzene	SO_4^{2-}	Deltaproteobacterium (Desulfobacteriacea)	Carboxylation to benzoate (carboxyl group was not originated from bicarbonate or acetate)	Phelps et al., 1996; 1998; 2001; Oka et al., 2008
Marine sediment from a stagnant part of a Mediterranean lagoon, Étang de Berre, France	Benzene	SO_4^{2-}	Deltaproteobacteria (85% dominance)	Conversion to benzoate, did not utilize phenol and toluene	Musat and Widdel, 2008

Source of enrichment	Substrate	e⁻ acceptor	Dominant phylotypes	Proposed activation pathway	References
Soil sample from a former coal gasification site in Gliwice, Poland	Benzene	SO_4^{2-}	*Pelotomaculum* (95% dominance, named enrichment BPL)	Carboxylation (both phenol and benzoate were detected)	Abu Laban *et al.*, 2009
Ferulic acid-degrading sewage sludge enrichments	Benzene	Methanogenic	N.D.	Hydroxylation to phenol (hydroxyl group was derived from H_2O in culture)	Vogel and Grbic-Galic, 1986; Grbic-Galic and Vogel, 1987
Non-contaminated lotus field soil in Tsuchiura, Ibaraki, Japan	Benzene	Methanogenic	*Deltaproteobacterium* (closed to *Syntrophus gentianae*, named Hasda-A)	No detectable accumulation of extracellular metabolites grown on benzene	Sakai *et al.*, 2009; Masumoto *et al.*, 2012
Coal tar creosote-contaminated sediment obtained from Eagle Harbor in Puget Sound, WA, USA	Naphthalene	NO_3^-	Strain NAP-3-1* (related to *Pseudomonas stutzeri*); strain NAP-4* (related to *Vibrio pelagius*) and strain NAP-3-2*	N.D.	Rockne and Strand, 1998; Rockne *et al.*, 2000
Sediment from a former manufactured gas plant site in Stuttgart, Germany, an aquifer shown to be contaminated with tar oil	Naphthalene	Fe(III)	Dominated by *Peptococcaceae*, named culture N49	Carboxylation (2-naphthoic acid was detected)	Kleemann and Meckenstock, 2011
Sediment from sulfate-rich, coal tar-contaminated aquifer	Naphthalene	SO_4^{2-}	N.D.	Hydroxylation (naphthol was detected as metabolite)	Bedessem *et al.*, 1997
Heavily contaminated sediment collected from the Arthur Kill in New York harbor estuary, USA	Naphthalene	SO_4^{2-}	N.D.	Carboxylation (2-naphthoate and several further reduced products were detected; carboxyl group derived from bicarbonate)	Zhang and Young, 1997; Zhang *et al.*, 2000
Anoxic sediment from a small North Sea harbour (Horumersiel), near Wilhelmshaven, Germany	Naphthalene	SO_4^{2-}	*Deltaproteobacteria* strain NaphS2*	N.D.	Galushko *et al.*, 1999
Sediment sample was from a contaminated aquifer near Stuttgart, Germany	Naphthalene	SO_4^{2-}	*Deltaproteobacteria* enrichment culture N47	Firstly proposed methylation, which was then ruled out and supported carboxylation	Meckenstock *et al.*, 2000; Safinowski and Meckenstock, 2006; Bergmann *et al.*, 2011a,b; Mouttaki *et al.*, 2012
Anoxic sediment was collected from a Mediterranean lagoon, Etang de Berre, France	Naphthalene	SO_4^{2-}	*Deltaproteobacteria* strains NaphS3* and NaphS6 (highly enriched)	Carboxylation (2-naphthoate was detected as a metabolite and methylation was ruled out)	Musat *et al.*, 2009

Note: N.D. = No data; * represents a pure culture.

1.3 Principles in activation of hydrocarbons by microorganisms

Because of their chemical stability, hydrocarbons have to be activated (functionalized) to introduce functional group(s) for further metabolic processing. Hydrocarbon-activating reactions are usually of high interest, in particular those in anaerobes, because they have to overcome the energetic barrier of the inert hydrocarbons. Such reactions may be unprecedented in biochemistry.

1.3.1 Aerobic activation of hydrocarbons

Under oxic conditions, microorganisms always make use of the widespread biological oxidant, dioxygen (O_2). A derived highly active oxygen species acts as a co-substrate to initiate C–H bond cleavage and introduce hydroxyl group(s) via catalysis by mono- or di-oxygenases. In accordance with the diversity of naturally occurring hydrocarbons from petroleum or living organisms, there is a vast number of oxygenases with different substrate specificities. With respect to mechanisms, however, there are some common underlying principles (reviewed for instance in Widdel and Musat, 2010b). Most oxygenases contain a metal atom or metal atoms (mostly Fe, sometimes Cu) in their active centers, undergoing valence changes. They bind to O_2 and reduce it to the peroxide state. A highly reactive oxygen species then attacks the C–H bond to become inserted. Oxygenases are part of multiple component systems, with electron-carrying cofactors, e.g., NADH, NADPH, and protein-bound flavin to accomplish the electron transfer steps in the oxidation processes. The two major groups of oxygenases are heme-iron monooxygenases (cytochrome P450-type) and non-heme iron monooxygenases (Widdel and Musat, 2010b).

The family of aromatic ring hydroxylating dioxygenases that convert aromatic hydrocarbons to *cis*-arene diols has been intensively investigated. Bacterial naphthalene 1,2-dioxygenase, which belongs to Rieske non-heme iron dioxygenases, catalyzes the stereospecific hydroxylation of naphthalene to *cis*-(1*R*,2*S*)-dihydroxy-1,2-dihydronaphthalene. Its crystal structure and catalytic mechanism have been unveiled (Kauppi *et al.*, 1998; Karlsson *et al.*, 2003). Benzene dioxygenase in *Pseudomonas aeruginosa* catalyzes the conversion of benzene to catechol either via phenol or via benzene epoxide as an intermediate. A mechanism has been proposed (Gibson *et al.*, 1968; Bathelt *et al.*, 2008).

1.3.2 Anaerobic activation of hydrocarbons

Anaerobic microorganisms functionalize hydrocarbons by various mechanisms, which are principally different from those employed under oxic conditions.

1.3.2.1 Substituted aromatic hydrocarbons – toluene and ethylbenzene

Toluene as a model compound of substituted aromatic hydrocarbons is the best-studied hydrocarbon with respect to the anaerobic activation mechanism. Initially, based on independent feeding experiments and metabolite analyses with different strains, different reactions were proposed as the initial steps of anaerobic toluene degradation (reviewed in Frazer *et al*., 1995). The first hypothesis was an anaerobic *para*-hydroxylation of toluene to *p*-cresol, which could undergo further oxidation to *p*-hydroxybenzoate, since both of *p*-cresol and *p*-hydroxybenzoate were detected as metabolites in toluene-degrading cultures (Vogel and Grbic-Galic, 1986; Lovley and Lonergan, 1990). The second appealing hypothesis was that toluene was activated by anaerobic hydroxylation of the methyl group to benzyl alcohol, oxidization to benzoate and further ligation with coenzyme-A to yield the central intermediate, benzoyl-CoA. However the crucial toluene methylhydroxylase responsible for the initial reaction could not be detected in cell extracts (Altenschmidt and Fuchs, 1991; Schocher *et al*., 1991).

The identifications of benzylsuccinate and benzylfumarate as metabolites in a toluene-grown denitrifying bacterium strain T1 (Evans *et al*., 1992) as well as in a sulfate-reducing enrichment culture (Beller *et al*., 1992) were important discoveries. However, at that time, they were interpreted as dead-end by-products formed due to an attack by succinyl-CoA (Evans *et al*., 1992). Based on these studies, the formation of benzylsuccinate was then proven as the initial reaction of toluene activation, not a side reaction, in cell-free extracts of a denitrifying bacterium *Thauera aromatica* (Biegert *et al*., 1996), and in permeabilized cells of a denitryfing bacterium strain T (Beller and Spormann, 1997). These pioneering studies led to the recognition of fumarate addition as a common activation mechanism for alkyl-substituted aromatic hydrocarbons. Later on, fumarate addition was also measured as the anaerobic toluene activation reaction in cell extracts of *Aromatoleum aromaticum* strain EbN1 and a sulfate-reducing bacterium, strain Tol2 (Rabus and Heider, 1998).

The mechanism of addition to fumarate was further suggested for anaerobic activation of xylenes (Beller and Spormann, 1997; Morasch *et al*., 2004), ethylbenzene (Kniemeyer *et al*., 2003), and 2-methylnaphthalene (Annweiler *et al*., 2000; Safinowski and Meckenstock, 2004; Musat *et al*., 2009; Selesi, *et al*., 2010).

After addition to fumarate, the resulting benzylsuccinate is activated to benzylsuccinyl-CoA by CoA-transferase and further oxidized to the central intermediate benzoyl-CoA, as demonstrated in cell-free extracts of *Thauera aromatica* (Leutwein and Heider, 1999).
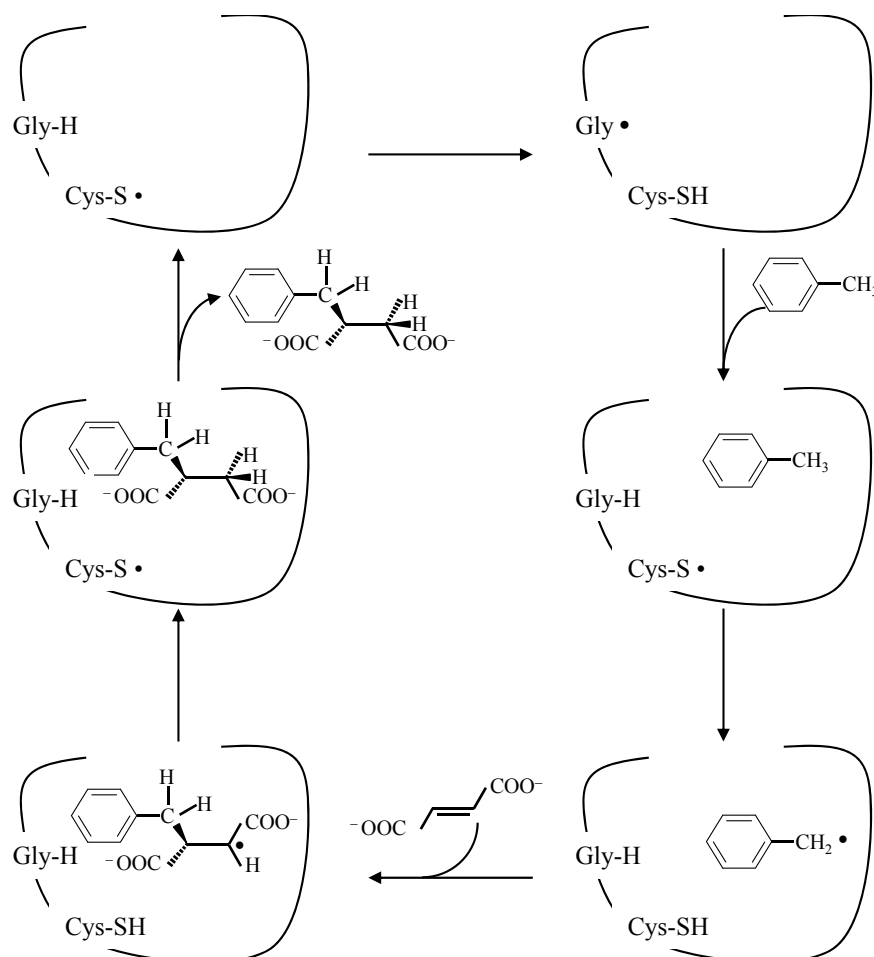
Fig. 1.5. Proposed reaction mechanism of fumarate addition to toluene catalyzed by benzylsuccinate synthase, involving glycyl and thiyl radicals. For detailed explanation, see text (modified from Boll and Heider, 2010).

Benzylsuccinate synthase (BSS), catalyzing the addition to fumarate to activate toluene, has been purified from cell-free extracts of *Thauera aromatica* and characterized as a glycyl radical enzyme, with high similarity to pyruvate formate lyases (Leuthner *et al*., 1998; reviewed in Heider, 2007). BSS from *Thauera aromatica* is composed of three subunits: a large subunit carrying a stable glycyl radical and two smaller subunits carrying FeS clusters. The process of fumarate addition catalyzed by BSS has been proposed to be initiated by abstraction of hydrogen atom from the methyl group of toluene, yielding a benzyl radical. The benzyl radical then adds to fumarate generating a benzylsuccinyl radical, which regains a hydrogen atom from the enzyme yielding benzylsuccinate and the regenerated a cysteyl

radical (Fig. 1.5; Li and Marsh, 2006; Boll and Heider, 2010). The radical mechanism of BSS has been further confirmed by electron paramagnetic resonance spectroscopic (EPR) analysis (Krieger *et al*., 2001; Li *et al*., 2009; for alkanes see Rabus et al., 2001). The encoding genes (*bss*) have been analyzed as well (Leuthner *et al*., 1998).

Ethylbenzene is another well-investigated substituted aromatic hydrocarbon with respect to its anaerobic activation mechanism. It has been shown that ethylbenzene, in the nitrate-reducing bacteria *Azoarcus* sp. strains EB1, EbN1, and PbN1, is functionalized by a stereospecific anaerobic hydroxylation reaction (with oxygen originating from H_2O) to form (*S*)-1-phenylethanol (Rabus and Widdel, 1995; Ball *et al*., 1996; Johnson and Spormann, 1999; Kniemeyer and Heider, 2001). The hydroxylation reaction is catalyzed by ethylbenzene dehydrogenase (EB-DH), which has already been purified and characterized in strain EB1 (Johnson *et al*., 2001) and strain EbN1 (Kniemeyer and Heider, 2001). The encoding genes have also been studied in strain EbN1 (Rabus *et al*., 2002). Enzyme EB-DH is a heterotrimer (αβγ), containing molybdenum (Mo) cofactor and Fe-S clusters, belonging to the dimethylsulfoxide reductase family. The crystal structure of EB-DH from EbN1 has been fully resolved (Kloer *et al*., 2006), based on which a reaction mechanism has been proposed (Szaleniec *et al*., 2007; 2008).

However, there is evidence that sulfate-reducing bacteria employ a different strategy to activate ethylbenzene. In a sulfate-reducing enrichment culture with ethylbenzene as a substrate, the intermediate (1-phenylethyl)succinate was detected (Elshahed *et al*., 2001). Thus, it was proposed ethylbenzene was activated via fumarate addition in SRB. Later, this hypothesis was also suggested in a study with a pure culture of a sulfate-reducing bacterium strain EbS7, where the similar crucial metabolites (1-phenylethyl)succinate and 4-phenylpentanoate were detected. It could be also shown that 1-phenylethanol and acetophenone, the characteristic intermediates in anaerobic ethylbenzene degradation by denitrifying bacteria, neither served as growth substrates nor were detectable as metabolites (Kniemeyer *et al*., 2003). These findings imply that the activating mechanisms employed by different microorganisms might be different even for the same hydrocarbon.

1.3.2.2 Alkanes

Later on, hydrocarbon addition to fumarate was also shown to be involved in anaerobic activation of the less reactive alkanes, a finding further substantiating a common principle. Kropp *et al.* (2000) analyzed the metabolites of an alkane-degrading sulfate-reducing enrichment culture. With isotope labeled *n*-dodecane as a substrate, the postulated first intermediate dodecylsuccinate was identified and the subterminal carbon was suggested to be the site of activation. Rabus *et al.* (2001) conducted sophisticated metabolite analyses to investigate the anaerobic alkane activation mechanism with strain HxN1, a denitrifying bacterium which is capable of oxidizing alkanes anaerobically with nitrate as electron acceptor (Ehrenreich *et al.*, 2000). Using stable isotope labeling, it could be proven that (1-methylpentyl)succinate was the prevalent intermediate in anaerobic *n*-hexane metabolism by strain HxN1. Also, fumarate addition at the subterminal carbon atom (C-2) was demonstrated by chemical analyses including an authentic standard (Rabus *et al.*, 2001). Electron paramagnetic resonance spectroscopic (EPR) analysis showed the presence of an organic radical in *n*-hexane-grown cells, in agreement with the postulated radical mechanism (Rabus *et al.*, 2001). A follow-up study identified 4-methyloctanoic, 4-methyloct-2-enoic, 2-methylhexanoic, 2-methylhex-2-enoic and 3-hydroxy-2-methylhexanoic acids as specific metabolites in *n*-hexane-grown HxN1 cultures. They were suggested to be downstream products of the initial intermediate (1-methylpentyl)succinate, so that a detailed tentative pathway for the anaerobic degradation of *n*-hexane by strain HxN1 could be formulated. This pathway includes carbon skeleton rearrangement and decarboxylation of one carboxyl group (Wilkes *et al.*, 2002).

Other subsequent studies showed that addition to fumarate is a widespread mechanism for anaerobic alkane activation, e.g., in *Desulfatibacillum aliphaticivorans* strain CV2803[T] (Cravo-Laureau *et al.*, 2005), in strain AK-01 (Callaghan *et al.*, 2006), in a short-chain alkane-degrading marine sulphate-reducing bacterium, strain BuS5 (Kniemeyer *et al.*, 2007), and in several enrichment cultures (Davidova *et al.*, 2005; Savage *et al.*, 2010).

The putative enzyme for anaerobic alkane activation, termed (1-methylalkyl)succinate synthase (Mas), and the encoding genes (*mas*) were studied in strain HxN1 (Grundmann *et al.*, 2008). The (1-methylalkyl)succinate synthase was postulated as a heterotrimer (MasDEC), in which the glycyl radical motif may be located in the MasD, according to the protein sequences and comparison with other glycyl radical enzymes. In the genome of sulfate-reducing strain AK-01, two putative alkylsuccinate synthase (ass) gene operons (assA1 and

assA2) were also identified, which encoded glycyl radical type enzymes and were analogous to the well-studied benzylsuccinate synthase (Bss) (Callaghan *et al*., 2008; 2012).

1.3.2.3 Unsubstituted aromatic hydrocarbons – naphthalene and benzene

Naphthalene and benzene are representatives of aromatic hydrocarbons without substituents. The lack of functional group(s) necessitates anaerobic activation mechanisms different from those for the substituted aromatic hydrocarbons. The C–H bond dissociation energies of naphthalene and benzene are around 470 kJ mol^{-1}, which are even higher than that of methane (439 kJ mol^{-1}, compiled for instance in Widdel and Rabus, 2001; Widdel *et al*., 2007). Thus, the direct abstraction of hydrogen from the aromatic ring to yield naphthyl or phenyl radicals requires high activation energy. Hence, addition to fumarate, yielding naphthylsuccinate or phenylsuccinate, respectively, seems unlikely. Also, such intermediates were never detected in naphthalene- or benzene-grown cultures. Even though understanding of the anaerobic activation mechanisms of naphthalene and benzene is presently limited, three rather similar activation mechanisms have been proposed, studied and discussed in the literature. These are hydroxylation, carboxylation and methylation (Fig. 1.6). To date, carboxylation (or more generally a carboxylation-like reaction) is usually the most favored one.

Benzene. The three proposed initial reactions of anaerobic benzene degradation are illustrated in Fig. 1.6. The first hypothesis was anaerobic hydroxylation of benzene to phenol, because phenol could be detected as a metabolite in several benzene-degrading enrichment cultures (Vogel and Grbic-Galic, 1986; Grbic-Galic *et al*., 1987; Chakraborty and Coates, 2005). It could be shown by ^{18}O-labelling that the hydroxyl group in phenol was derived from H$_2$O in the medium (Vogel and Grbic-Galic, 1986; Zhang *et al*., 2013). However, later studies revealed that phenol might be formed abiotically through autoxidation of benzene during sampling and exposure to air (Kunapuli *et al*., 2008). Because of the possibility of O$_2$ generation from strongly oxidizing electron acceptors ("intra-aerobic" pathway; see page 7), benzene-degrading facultative anaerobic bacteria could also employ a similar oxygen species to active benzene yielding phenol as a metabolite. Evidence for such a mechanism was provided in a study of *Alicycliphilus denitrificans* strain BC, which grows with benzene and chlorate as electron acceptor (Weelink *et al*., 2008).

The hypothesis of benzene methylation was based on the identification of toluene in a benzene-degrading enrichment culture (Ulrich *et al*., 2005). An appealing aspect of this hypothesis is that an existing pathway (addition to fumarate) can be employed by including

only one additional enzyme. However, benzylsuccinate or further downstream metabolites were not detected in benzene-degrading cultures.

The hypothesis of benzene carboxylation is based on the frequent detection of benzoate in various benzene-degrading enrichments. However, since benzoyl-CoA is the central intermediate in the metabolism of many aromatic compounds, the detection of its hydrolysis product, benzoate, does not necessarily indicate that carboxylation is the first step in anaerobic benzene degradation. Early labelling studies showed that the carboxyl group in benzoate detectable as a metabolite did not originate from HCO_3^- (Caldwell and Suflita, 2000; Phelps et al., 2001). When ^{13}C-benzene was used as substrate, a labeled ^{13}C-atom could be detected in the carboxyl group, which implied that benzene was not directly carboxylated by external bicarbonate or CO_2.
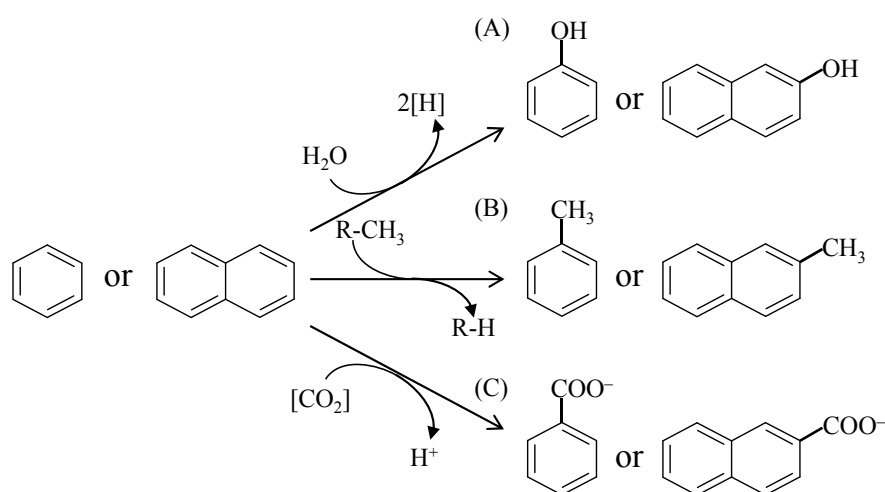
Fig. 1.6. Proposed hydroxylation (A), methylation (B), and carboxylation (C) were proposed as initial steps in anaerobic benzene and naphthalene degradation. The methyl group was assumed to be transferred from a C_1-carrier. For details, see text.

Naphthalene. Activation of naphthalene via anaerobic hydroxylation to naphthol (Bedessem et al., 1997) was subsequently questioned because neither 1-naphthol nor 2-napthol could be utilized as substrates by the enrichment culture N47 (Meckenstock et al., 2000) and the naphthalene-degrading cultures NaphS2, NaphS3, and NaphS6 (Musat et al., 2009).

The methylation hypothesis was appealing, because all the reported naphthalene degraders could utilize 2-methylnapthalene as a substrate and its activation product, (2-naphthylmethyl)succinate, was detected in the naphthalene-degrading freshwater enrichment culture N47 (Safinowski and Meckenstock, 2006; Safinowski et al., 2006). However, a later study with the marine sulfate-reducing bacteria NaphS2, NaphS3, and

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NaphS6 showed that naphthalene-adapted cells were not induced to utilize 2-methylnaphthalene. In addition, the protein band of proposed (2-naphthylmethyl)succinate synthase (Nms) was only specifically present in 2-methylnaphthalene-grown cells. These results excluded methylation as the initial step of naphthalene activation in the marine strains NaphS2, NaphS3, and NaphS6 (Musat *et al*., 2009).

Carboxylation was proposed since 2-naphthoate was detected as an intermediate in naphthalene-degrading cultures. In the presence of labeled $H^{13}CO_3^-$ in the medium ^{13}C could be detected in the carboxyl group of 2-naphthoate (Zhang and Young, 1997). In naphthalene-degrading cultures studied later on, again 2-naphthoate as well as its reduced forms, e.g., 5,6,7,8-tetrahydro-2-naphthoate, and octahydro-2-naphthoate as possible subsequent intermediates were detected (Zhang *et al*., 2000; Meckenstock *et al*., 2000; Annweiler *et al*., 2002; Musat *et al*., 2009). Thus carboxylation became a favored hypothesis for naphthalene activation.

Studies with the highly enriched culture N47 with genomic (Bergmann *et al*., 2011a), proteomic (Bergmann *et al*., 2011b) and cell-extract-based approaches (Mouttaki *et al*., 2012) also ruled out the methylation hypothesis and supported carboxylation as the initial naphthalene activation mechanism.

Carboxylation was also proposed as the initial step of anaerobic activation of the tricyclic aromatic hydrocarbon phenanthrene ($C_{14}H_{10}$), another representative of PAHs, by sulfate-reducing enrichment cultures. Phenanthrene carboxylate with a bicarbonate-derived carboxyl group was a prevalent metabolite (Zhang and Young, 1997; Davidova *et al*., 2007).

1.4 Energetics of microbial utilization of hydrocarbons without oxygen

1.4.1 Energetic aspects of anaerobic degradation of hydrocarbons

Hydrocarbons are highly energy-rich substrates for aerobic microorganisms, which is evident from Gibbs energy changes ($\Delta G°$) of their complete oxidization with O_2. The energetic situation is completely different under anoxic conditions, where the maximum of the available energy is often one order of magnitude lower. Table 1.2 shows the free energy change of the oxidation of benzene and naphthalene with different terminal electron acceptors (oxygen, nitrate, inorganic ferric iron, sulfate, and protons in syntrophic net methanogenesis). The available energy is particularly low under sulfate-reducing and methanogenic conditions. In general, the relative overall energy yield in anaerobic bacteria using hydrocarbons as growth substrates is as follows: nitrate-reduction > iron(III)-reduction > sulfate-reduction > methanogenesis (for instance, Spormann and Widdel, 2000).

Table 1.2. Standard free energy change ($\Delta G°$) of oxidation of benzene and naphthalene with different terminal electron acceptors (O_2, NO_3^-, Fe(III), SO_4^{2-}, and protons in syntrophic net methanogenesis; Widdel and Musat, 2010a)

Reaction equations	$\Delta G°$ (kJ per mol hydrocarbon)
Benzene	
$2\,C_6H_6\,(l) + 15\,O_2\,(g) \rightarrow 12\,CO_2\,(g) + 6\,H_2O$	− 3202
$5\,C_6H_6\,(l) + 30\,NO_3^- + 30\,H^+ \rightarrow 30\,CO_2\,(g) + 15\,N_2\,(g) + 30\,H_2O$	− 3008
$C_6H_6\,(l) + 30\,Fe(OH)_3 + 24\,CO_2\,(g) \rightarrow 30\,FeCO_3 + 48\,H_2O$	− 1423
$4\,C_6H_6\,(l) + 15\,SO_4^{2-} + 30\,H^+ \rightarrow 24\,CO_2\,(g) + 15\,H_2S\,(aq) + 12\,H_2O$	− 214
$4\,C_6H_6\,(l) + 18\,H_2O \rightarrow 15\,CH_4\,(g) + 9\,CO_2\,(g)$	− 135
Naphthalene	
$C_{10}H_8\,(c) + 12\,O_2\,(g) \rightarrow 10\,CO_2\,(g) + 4\,H_2O$	− 5093
$5\,C_{10}H_8\,(c) + 48\,NO_3^- + 48\,H^+ \rightarrow 50\,CO_2\,(g) + 24\,N_2\,(g) + 44\,H_2O$	− 4782
$C_{10}H_8\,(c) + 48\,Fe(OH)_3 + 38\,CO_2\,(g) \rightarrow 48\,FeCO_3 + 76\,H_2O$	− 2247
$C_{10}H_8\,(c) + 6\,SO_4^{2-} + 12\,H^+ \rightarrow 10\,CO_2\,(g) + 6\,H_2S\,(aq) + 4\,H_2O$	− 313
$C_{10}H_8\,(c) + 8\,H_2O \rightarrow 6\,CH_4\,(g) + 4\,CO_2\,(g)$	− 186

The energetic constrains on the one hand determines the lifestyle of anaerobes, which is usually characterized by low growth yields and often also by low growth rates, depending on the type of organic substrate. On the other hand, the low-energy situation with electron acceptors other than nitrate may also demand biochemical pathways for microbial hydrocarbon utilization that may, at least in some steps, differ significantly from aerobic pathways.

An illustrative example of a catabolic strategy under energetic constrains is the reduction of benzoyl-CoA with Bam in comparison to that with Bcr. Although benzoyl-CoA itself is not a hydrocarbon, it is a common intermediate in the anaerobic degradation of many aromatic compounds, including toluene and ethylbenzene. For facultative anaerobic bacteria, such as *Rhodopseudomonas palustris*, *Thauera aromatica*, and *Azoarcus evansii*, the favourable energetic situation obviously allows involvement of the ATP-dependent (2 ATP per ring) class I of benzoyl-CoA reductase (Bcr) is used to reduce the aromatic ring (reviewed by Boll, 2005). For strict anaerobes, like *Geobacter metallireducens*, *Syntrophus aciditrophicus* and sulfate-reducing bacteria (predicted from genome analysis), limited energy is the problem they have to encounter and two ATP equivalents for ring reduction are not or may not be "affordable". Thus, an ATP-independent class II benzoyl-CoA reductase (Bam) with lower energy demand is employed to accomplish dearomatization of benzoyl-CoA (Kung *et al.*, 2009; 2010; Löffler *et al.*, 2011; reviewed by Fuchs *et al.*, 2011).

1.4.2 Reversibility of reactions for anaerobic activation of hydrocarbons

Most catabolic reactions under energy-limited conditions are reversible. Hence, more attention should be paid on the reverse direction especially in natural geochemical habitats, where the energy is often limited for microorganisms (Jin and Bethke, 2007). Considering the energetic situation of hydrocarbon utilization by anaerobes, reverse reactions can be also expected.

For instance, methanogenesis has been formerly shown to include some activity of an apparent simultaneous oxidation of methane (Zehnder and Brock, 1979). More recently, purified Methyl-coenzyme M reductase (MCR), the key enzyme in biological methane formation by methanogenic archaea, has been proven to convert methane to methyl-coenzyme M (Scheller *et al*., 2010). The isotope exchange between $^{12}CH_4$ and $^{13}CH_3$-S-CoM catalyzed by MCR has been detected (Scheller *et al*., 2013). These results suggested the reversibility of methanogenesis. Furthermore, when different anaerobic methane oxidation (AOM) enrichment cultures oxidizing methane with sulfate were incubated with ^{14}C-bicarbonate and ^{35}S-sulfide, radiolabeled methane ($^{14}CH_4$) and sulfate ($^{35}SO_4^{2-}$) could be detected (Holler *et al*., 2011, reviewed in Thauer, 2011).

The reversibility has also been found in the biological Birch-type reduction of benzoyl-CoA catalyzed by benzoyl-CoA reductase, including type I Bcr and type II Bam. It is proposed to be a harsh reaction, due to the extremely low redox potential of the reduction of the aromatic structure. Both Bcr and Bam could catalyze not only the forward dearomatization (reduction) of benzoyl-CoA to dienoyl-CoA, but also the reverse aromatization (oxidation) of dienoly-CoA to benzoyl-CoA (Thiele *et al*., 2008; Kung *et al*., 2010). Reversibility was also shown in anaerobic activation of phenol. Phenol is first phosphorylated to phenylphosphate, which is then carboxylated to generate *p*-hydroxybenzoate (reviewed by Boll and Fuchs, 2005). The observation of phenol formation from *p*-hydroxybenzoate (Tschech and Schink, 1986) as well as isotope exchange between $^{14}CO_2$ and carboxyl group of *p*-hydroxybenzoate, catalyzed by either whole cells or cell-free extracts (Tschech and Fuchs, 1989; Lack *et al*., 1991) again indicated that the reaction of phenol activation to *p*-hydroxybenzoate is reversible.

Hence reversibility can be also expected in low-energy catabolic reactions of aromatic hydrocarbons such as those postulated for steps in the anaerobic degradation of naphthalene and benzene.

2. Objectives of the present work

As introduced in the previous part, the study of anaerobic biodegradation of naphthalene and benzene, the simplest representatives of unsubstituted aromatic hydrocarbons, was rather scarce. Therefore, our understandings of the anaerobic activation of naphthalene and benzene were still matters of discussion. Thanks to previous works, naphthalene-degrading sulfate-reducing bacteria strains NaphS2 (Galushko *et al*., 1999), NaphS3, and NaphS6 (Musat *et al*., 2009) as well as benzene-degrading sulfate-reducing enrichment culture (Musat and Widdel, 2008) had been isolated and are maintained in the laboratory. Thus, this thesis was going to focus on these strains to investigate the metabolism of naphthalene and benzene under sulfate-reducing conditions, with special attentions on the initial reactions of anaerobic naphthalene and benzene degradation.

With the exclusion of anaerobic hydroxylation and methylation as the initial steps for anaerobic naphthalene activation, the discussion focused mostly on the carboxylation hypothesis (Musat *et al*., 2009). However, due to the high energetic barrier posed by resonance structure and the lack of any functional substituents in naphthalene, it was difficult to imagine simple direct carboxylation by CO_2 or HCO_3^-. It was hypothesized that naphthalene is first activated and subsequently carboxylated, similar as the scenario of anaerobic phenol activation, where carboxylation proceeds after phenol phosphorylation. Therefore, it was aimed to investigate if there was an activation step before carboxylation and to look for the potential first intermediate prior to 2-naphthoate in anaerobic naphthalene degradation.

It was speculated that sulfate-reducing bacteria employ largely analogous mechanisms to functionalize benzene and naphthalene, based on the chemical relatedness of the two compounds. So far, there was still no benzene-degrading sulfate-reducing pure culture described. The enrichment culture obtained in our lab was one of the most advanced strictly anaerobic benzene-degrader (Musat and Widdel, 2008). Based on the enrichment culture, a further purified culture, named BzS12, was obtained and used in the studies.

3. Materials and methods

3.1 Microorganisms and anaerobic cultivation

The naphthalene-degrading sulfate-reducing bacterium, strain NaphS2 was isolated from anoxic sediment from a North Sea harbor near Wilhelmshaven, Germany (Galushko *et al*., 1999), whereas strains NaphS3 and NaphS6 were isolated from marine sediment of a Mediterranean lagoon, Etang de Berre, France (Musat *et al*., 2009). The benzene-degrading sulfate-reducing enrichment culture was obtained from the same sediment as that for isolation of strains NaphS3 and NaphS6 (Musat and Widdel, 2008). A largely purified benzene-degrading sub-culture termed as BzS12 was used in this study.

After isolation, all strains were maintained in the laboratory as active cultures by repeated transfer to fresh culture medium with naphthalene or benzene, respectively. Artificial sea water (ASW) medium preparation and anaerobic cultivation techniques were as described elsewhere (Widdel and Bak, 1992; Widdel *et al*., 2006; Widdel, 2010). Cultures for preparation of cell suspensions were routinely grown in 2 l DURAN bottles with a side arm, containing 1.6 l ASW medium, and 200 ml inoculum from a fully grown culture. A volume of 100 ml anoxic 2,2,4,4,6,8,8-heptamethylnonane (HMN) as an inert carrier phase containing naphthalene (20 mg ml^{-1}), 2-methylnaphthalene (10 mg ml^{-1}), or benzene (0.5%, v/v) was added as the substrate source. All cultures were incubated at 28 °C under gentle magnetic stirring (ca. 200 rpm), except for strain NaphS6, which was cultivated in a static state in horizontal position to increase the contact area between medium and the HMN phase.

Cultures were harvested when sulfide concentration had reached around 10 to 15 mM. In order to avoid contact with oxygen, all manipulations were done anoxically under a continuous stream of N_2 gas or inside an anoxic chamber with an atmosphere of $N_2 : CO_2$ (90 : 10, v/v). A volume of 1.6 l from a grown culture was transferred to a separatory funnel, separated from the overlying HMN carrier phase, and collected in plastic beakers (volume = 400 ml). The remaining culture volume was used as inoculum for a subsequent culture. Cells were collected via centrifugation at 16,000 × *g* (Beckman Coulter Avanti J-26XP, USA) for 25 min, washed once with ASW medium, and re-suspended as dense cell suspensions for further experiments.

3.2 Tracing the first intermediate using radiolabeled naphthalene

In order to follow the first intermediate in anaerobic naphthalene degradation, radiolabeled [1,4,5,8-^{14}C]-naphthalene (Hartmann, Germany) was used as a tracer in dense cell suspensions of strains NaphSx. Such metabolite tracing is common in biochemical studies because radioactive signals have high detection sensitivity. For instance, the first intermediates, benzylsuccinate and phenylphosphate, in anaerobic degradation of toluene and phenol, respectively, had been detected by the application of radiolabeled substrates (Lack and Fuchs, 1994; Biegert *et al*., 1996; Rabus and Heider, 1998).

Dense cell suspensions of strains NaphSx were fed with [1,4,5,8-^{14}C]-naphthalene and unlabeled naphthalene. The latter was added to achieve commonly used substrate concentrations; the amount of the purchased radiolabeled naphthalene was too small for regular growth and incubation experiments. For trapping of ^{14}C-2-naphthoate as an intermediate, unlabeled 2-naphthoate (200 µM) was added ('cold' trap). Labeled cell suspensions were incubated on a shaker (ca. 100 rpm) at room temperature. Samples were taken at different time points with N_2-flushed syringes, acidified, centrifuged to remove cells, and analyzed by HPLC equipped with an UV and an online radioflow detector. Details are explained below.

3.3 Isotope exchange reaction between $^{14}CO_2$/$H^{14}CO_3^-$ and 2-naphthoate

Naphthalene-grown cultures (1.6 l) were harvested as described above. Cells were washed once with sulfate- and bicarbonate-free ASW medium buffered with Tris/HCl (30 mM, pH = 7.2) to remove residual sulfate and then re-suspended in 55 ml of the same sulfate-free medium to prepare dense cell suspensions. 2-Naphthoate (400 µM) was supplied as a substrate and radiolabeled bicarbonate (20 µM, 42 kBq ml^{-1}) was additionally injected. The total volume of the reaction was 55 ml. Tris buffer was used instead of bicarbonate to minimize an unlabeled inorganic carbon source and thus increase radiolabel incorporation. Samples were taken at different time points acidified with HCl, and vigorously shaken on a Vortex mixer so as to remove the inorganic carbon label as volatile CO_2. Samples were then extracted with dichloromethane, concentrated, and analyzed by HPLC coupled with an UV-VIS and an online radioflow detector (introduced below).

Samples were also analyzed with a liquid scintillation counter to quantify the actual radioactivity. A volume of 200 µl was diluted in 1 ml Milli-Q H_2O, mixed with 2 ml scintillation cocktail solution (PerkinElmer), and analyzed in a scintillation counter (TR-2900, Canberra-Packard).

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3.4 Preparation of cell-free extracts and assay conditions

After harvesting, cells were washed once with anoxic Tris/HCl buffer (100 mM, pH = 7.5, reduced with 5 mM DTT) and re-suspended in small volume (1 or 2 ml) of the same buffer depending on the amounts of biomass. Cells were then lyzed physically by vigorous vortexing with fine glass beads (0.1 mm diameter, Roth, Germany). Then, unbroken cells and cell debris were removed via centrifugation at $20,238 \times g$ (Eppendorf centrifuge 5424, Germany) for 15 min. All the manipulations were carried out in an anoxic chamber with an N_2 atmosphere, and the ambient temperature in the chamber is 4 °C.

Assays with cell-free extracts were performed to test the activity of 2-naphthoate formation with naphthalene as substrate. The basic conditions for cell-free extract assays were as following: 100 mM Tris/HCl buffer (pH = 7.5), 5 mM DTT, 5 mM Mg^{2+}, 5 mM ATP, 25 mM HCO_3^-, and around 200 μM naphthalene substrate; naphthalene was added from a stock solution in methanol (20 mM). Reactions were run in a volume of 1 ml in small serum vials closed with Teflon-lined septa fixed by aluminum crimps and incubated in an anoxic chamber under $N_2 : CO_2$ (90 : 10) gas mixture. Defined amounts of cell-free extract were added to start the reaction. At defined time points, samples (50 μl) were taken with a Hamilton (Australia) microliter syringe and then mixed with 50 μl of 70% acetonitrile and 1 μl diluted H_2SO_4 (1 M). Sample mixtures were then centrifuged and filtered through 0.2 μm nylon filters, which were analyzed with an UPLC system (Acquity H-Class, Waters) to determine the concentrations of naphthalene and 2-naphthoate.

3.5 Whole-cell assays with the application of FCCP

The harvesting procedure was the same as mentioned above. Cells (from 1.6 l cultures) were washed once with 330 ml ASW medium without sulfate, and then re-suspended in 5 ml of the same medium. This was used as a stock of cell suspensions for assays. The assays were performed in an anoxic chamber and carried out in small serum vials closed with Teflon-lined septa fixed by aluminum crimps. A defined volume (50 μl, unless otherwise indicated) of the cell suspension stock was diluted with sulfate-free ASW medium to a final volume of 1 ml. FCCP was injected from a 1 mM stock solution in methanol to a final concentration of 20 μM (unless otherwise indicated). Reactions were started by addition of defined concentrations of naphthalene (ca. 200 μM) or 2-naphthoate (ca. 1 mM) as described above. Aliquots of 50 μl samples were withdrawn at different time points with a Hamilton microliter syringe, mixed with 50 μl of 70% acetonitrile, acidified by 1 μl of H_2SO_4 (1 M), centrifuged, and filtered through 0.2 μm nylon filters (Wicom, Germany). Sample analyses were performed on an

UPLC system (Acquity H-Class, Waters) coupled with a PDA detector to quantify the concentrations of naphthalene, 2-naphthoate, and [5,6,7,8]-tetrahydro-2-naphthoate.

Further assays to test the effects of the ATPase inhibitor DCCD and the Na^+/H^+ antiporter monensin were conducted in the same way.

3.6 Cultivation, extraction, and sample preparation for metabolite analysis

Benzene-grown cultures designed for metabolite analysis were cultivated in round bottles with a side arm; volumes were around 100 ml. After inoculation, $^{12}C_6$- or $^{13}C_6$-benzene dissolved in HMN (0.5%, v/v) was injected as a substrate. Because benzoate had been a presumed metabolite, in order to examine if its carboxyl group was derived from bicarbonate, ^{13}C-bicarbonate was added into cultures with $^{12}C_6$-benzene at the concentration of 5 mM from a 1 M stock solution. To favor accumulation of metabolites at detectable concentrations, the metabolism was limited by the concentration of the electron acceptor; only 5 mM instead of 28 mM sulfate were added. After the expected sulfide concentration (5 mM) had been formed, cultures were inactivated at 80 °C for 10 min and acidified to ca. pH = 1 with concentrated HCl. Metabolites in cultures were repeatedly extracted with dichloromethane. The total volume of dichloromethane used for extraction was around 200 ml per culture.

Extracts were evaporated to about 1 ml in a TurboVap (R) 500 concentration evaporator (Biotage, Sweden). Samples were then methylated using 500 µl a solution of diazomethane in diethylether; diazomethane was freshly prepared from Diazald (Sigma-Aldrich). After 1 min of reaction, 10 µl of formic acid (Merck) were added to avoid side reactions of the diazomethane. Samples were stored at 4 °C until their analysis by GC-MS.

3.7 Analytical methods

3.7.1 Sulfide measurement

Development of cultures was monitored by sulfide measurement via a photometric method described by Cord-Ruwisch (1985). An aliquot was withdrawn with an N_2-flushed syringe, and 0.1 ml was directly injected (to avoid loss of the volatile H_2S) into 4 ml of reagent containing $CuSO_4$ (5 mM) and HCl (50 mM). CuS precipitation occurred according to the reaction: $Cu^{2+} + H_2S \rightarrow CuS + 2H^+$. The absorbance of the colloidal brown CuS was determined spectrophotometrically at 480 nm. The H_2S concentration in the cultures was calculated based on a calibration curve acquired in the same procedure with a defined Na_2S solution. The latter was prepared from fresh crystals of $Na_2S \cdot 9H_2O$ under the flush of N_2.

3.7.2 HPLC analysis including radiolabel detection

Metabolites in samples were separated by an HPLC system (Sykam, Germany) equipped with a reverse phase Nucleodur C_{18} Isis column (250 mm × 5.0 μm; Macherey-Nagel, Germany) and analyzed by an UV-VIS detector (Linear 206 PHD, USA) as well as by a consecutively connected online radioflow detector (Berthold LB509, Germanny) for radioactivity measurement. Identification of radiolabeled intermediates was based on the co-elution of authentic standards under the same conditions. The column temperature was set to 25 °C, and a gradient of 10% to 70% acetonitrile with 1 mM H_3PO_4 was delivered at a flow rate of 1 ml min^{-1} for 45 min. Naphthalene, 2-methylnaphthalene, 2-naphthoate, and tetrahydro-2-naphthoate were detected at 254 nm. Under these conditions, 2-naphthoate (23.1 min), [5,6,7,8]-tetrahydro-2-naphthoate (25.7 min), 2-naphthylaldehyde (27.1 min), naphthalene (32.3 min), and 2-methylnaphthalene (37.0 min) can be well separated and quantified.

3.7.3 UPLC analysis

Naphthalene, 2-naphthoate, and tetrahydro-2-naphthoate in samples were also analyzed by an UPLC system (Acquity H-Class, Waters) equipped with an Acquity UPLC BEH shield reverse phase C_{18} column (1.7 μm; 2.1 × 50 mm) and a Photo diode array (PDA) detector. The column temperature was set to 30 °C, and a gradient of 10% to 70% acetonitrile with 1 mM H_3PO_4 was delivered at a flow rate of 0.6 ml min^{-1} for 5 min. Naphthalene was detected at 220 nm, while 2-naphthoate and tetrahydro-2-naphthoate were detected at 235 nm. Identification and quantification of compounds were based on comparison with peaks of authentic standards and calibration curves.

3.7.4 GC analysis of benzene and toluene

Concentrations of benzene and toluene were measured by a gas chromatograph (Shimadzu GC-14B, Japan) equipped with a Supel-Q PLOT fused silica capillary column (30 m × 0.53 mm; Sigma Aldrich) and a flame ionization detector (FID). Nitrogen (N_2) was used as a carrier gas. The oven was operated isothermally at 140 °C; the injector was set to 150 °C and the detector to 280 °C. Gas samples (0.1 ml) from the head space of cultures were withdrawn at room temperature by N_2-flushed gas-tight syringes (Hamilton, Australia) and then injected into a gas chromatograph. Concentrations were calculated based on a calibration curve.

3.7.5 GC-MS analysis to detect metabolites in benzene cultures

GC-MS analysis was performed on a Trace GC-MS system (Thermo Scientific, Bremen, Germany). The gas chromatograph was equipped with a fused silica capillary column (BPX-5, SGE; 50 m × 0.22 mm i.d., 0.25 μm film thickness). The column temperature was initially held at 50 °C for 1 min, and then programmed to 310 °C at a rate of 3 °C min^{-1} with a final hold time of 30 min. Helium was used as the carrier gas. The PTV injector temperature was programmed from 50 °C to 300 °C (10 min isothermal) at a rate of 10 °C s^{-1}. The injection volume of 1 μl was processed in the splitless mode. The mass spectrometer was operated in EI mode (70 eV) at a source temperature of 260 °C. Full scan mass spectra were recorded from m/z 50 to 600. GC-MS analyses were carried out by Rene Jarling in Deutsches GeoForschungsZentrum (GFZ), Potsdam.

4. Results and discussion

4.1 Naphthalene

As introduced previously, methylation of naphthalene to 2-methylnaphthalene was proposed as the initial step of anaerobic naphthalene activation in the freshwater enrichment culture N47 (Safinowski and Meckenstock, 2006). This hypothesis had been appealing because it could resemble a Friedel-Crafts electrophilic substitution by employing a methyl group (CH_3^+ equivalent) donor such as methyl-coenzyme B_{12} or *S*-adenosylmethionine (Widdel *et al*., 2007). Furthermore, all known naphthalene-degrading cultures showed the capacity to utilize 2-methylnaphthalene which can be activated by the widely-acknowledged addition to fumarate, yielding naphthyl-2-methylsuccinate. However, later studies with the marine sulfate-reducing strains NaphS2, NaphS3, and NaphS6 showed that naphthalene-grown cells were not immediately activated towards 2-methylnaphthalene. Moreover, protein analyses showed that a subunit of naphthyl-2-methylsuccinate synthase (Nms) was specifically present in 2-methylnaphthalene-grown cells, but was missing in naphthalene-grown cells. These results indicated that methylation was unlikely as a naphthalene activation mechanism in the three marine strains (Musat *et al*., 2009).

4.1.1 Response of 2-methylnaphthalene-grown NaphSx cells to naphthalene and comparative proteomic analyses

Since naphthalene-grown cells were not induced to utilize 2-methylnaphthalene according to a previous study (Musat *et al*., 2009), further experiments were designed to examine whether *vice versa* 2-methylnaphthalene as a growth substrate could induce the capacity for naphthalene utilization of strains NaphSx. Knowledge of this was decisive for completing the differential proteomic analyses. In order to investigate the response of 2-methylnaphthalene-grown cells to the addition of naphthalene or 2-methylnaphthalene for control in a highly resolved time course, each compound was dissolved directly in artificial sea water (ASW) medium (viz. without carrier phase). The bottles were then inoculated with cells of 2-methylnaphthalene-grown cultures of all three strains. Samples were taken periodically to determine the consumption of naphthalene and 2-methylnaphthalene via HPLC analysis.

Naphthalene degradation by cells of all three strains started only after about 10 days of adaptation, and complete degradation took around 30 to 40 days (Fig. 4.1). In contrast, the control substrate 2-methylnaphthalene was consumed within three to four days, indicating the cells had remained active during the preparation procedure. As another positive control, cells

of strain NaphS2 pre-grown on naphthalene were shown to completely degrade naphthalene within five days of incubation (Fig. 4.1). The results showed that the capacity to degrade naphthalene was not expressed in 2-methylnaphthalene-grown cultures; rather, it had to be induced. According to these results together with those from a previous study (Musat *et al.*, 2009), it can be concluded that there is no 'cross induction' for the two substrates in all three strains. With other words, the three marine strains express the capacities for the degradation of naphthalene and 2-methylnaphthalene with high substrate specificity. This property is useful for further comparative proteomic analyses (naphthalene- vs. benzoate-grown cells) to identify proteins specifically involved in naphthalene degradation.
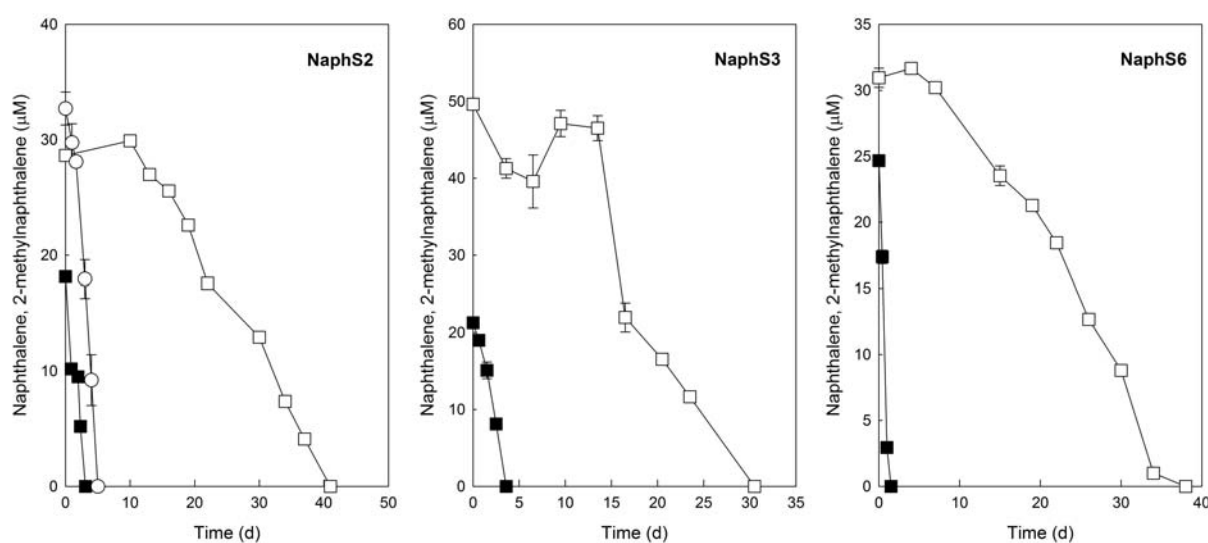

Fig. 4.1. Response of 2-methylnaphthalene-grown cells of strains NaphSx to the addition of naphthalene (□) or 2-methylnaphthalene (■). The degradation of naphthalene by naphthalene-grown cells of strain NaphS2 was shown as a positive control (○). All three strains consumed the original growth substrate 2-methylnaphthalene rapidly; naphthalene degradation started only after an adaptation time of about 10 days. Results showed that the capacity to degrade naphthalene was not expressed in 2-methylnaphthalene-grown cultures. The experiments were carried out in 150 ml serum bottles with Teflon-coated septa, provided with 100 ml ASW medium containing naphthalene (around 30 to 50 μM) or 2-methylnaphthalene (around 20 μM) and inoculated with 1 ml of concentrated cell suspensions of strains NaphSx. Aqueous samples (1 ml) were taken at defined time points and analyzed by a HPLC system to monitor the consumption of naphthalene or 2-methylnaphthalene. The error bars represent standard deviation calculated based on two independent incubations.

Therefore, cells adapted to naphthalene (at least five passages) were subjected to proteomic analyses via two-dimensional gel electrophoresis (2DE). The reference substrate was benzoate. One protein (spot 2) was discovered to be specifically up-regulated in naphthalene-adapted NaphS2 cells in comparison with benzoate-grown cells (Fig. 4.2A). Sequence analysis showed that it had high similarity to one subunit of phenylphosphate

carboxylase (PPC), involved in anaerobic phenol metabolism by catalyzing the carboxylation of phenylphosphate to *p*-hydroxybenzoate (Schmeling *et al*., 2004; Schuhle and Fuchs, 2004). A similar protein spot was also detected in naphthalene-grown cells of strains NaphS3 and NaphS6 (Fig. 4.2B, C). It gave a hint that PPC-like protein might involve in anaerobic metabolism of naphthalene. However, the initial reaction of naphthalene still cannot be fully analogous due to the absence of a hydroxyl group and thus a naphtholphosphate as the first intermediate. Hence, the PPC-like protein might be involved in a modified carboxylation reaction of naphthalene in strains NaphSx. Putative genes encoding PPC-like proteins had been identified in the draft genome of strain NaphS2 and shown to be up-regulated in naphthalene-grown cells (DiDonato, *et al*., 2010). In agreement with this finding, PPC-like proteins were also detected in naphthalene-degrading sulfate-reducing freshwater enrichment culture N47 based on comparative proteomic analyses (Bergmann *et al*., 2011b).
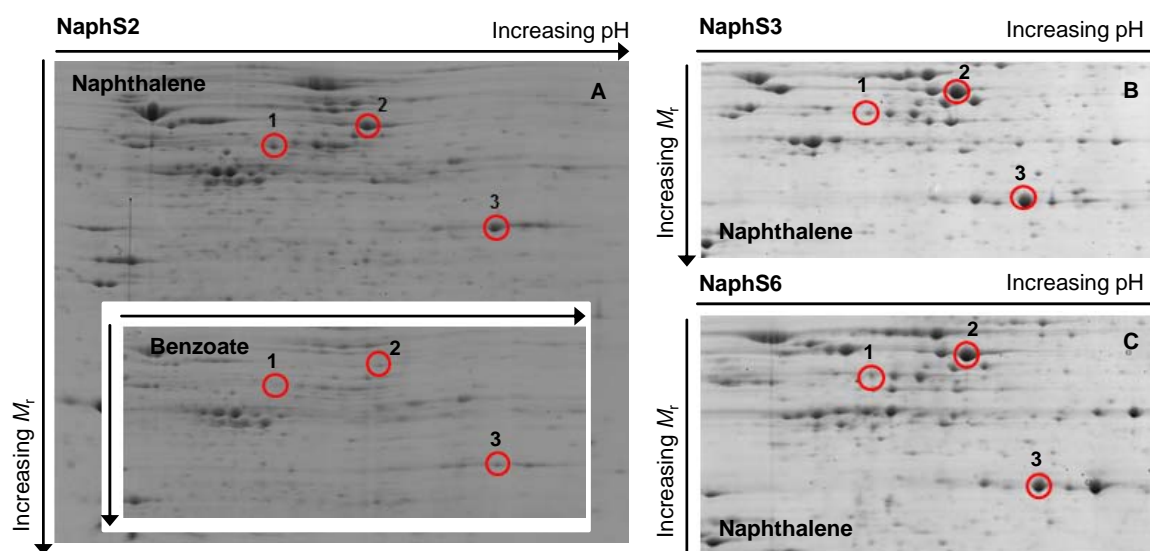

Fig. 4.2. Detection of a phenylphosphate carboxylase (PPC)-like protein in naphthalene-grown cells. 2DE protein profile of strain NaphS2 grown on naphthalene and benzoate (A). A particular protein (spot 2) was specifically up-regulated in naphthalene-grown cells; it showed high similarity to a subunit of PPC through the sequence analysis. Such a specifically expressed protein was also detected in naphthalene-grown cells of strains NaphS3 (B) and NaphS6 (C). Courtesy of Ralf Rabus, unpublished results.

4.1.2 Tracing the first intermediate of naphthalene utilization by radiolabeling

With the identification of PPC-like proteins formed specifically in naphthalene-grown cells, further attempts were carried out to detect 2-naphthoate and other metabolites formed by cultures from radiolabeled naphthalene. Previous studies (Zhang and Young, 1997; Meckenstock *et al*., 2000; Musat *et al*., 2009) attempting to unveil the activation step were mostly based on radiolabel-independent metabolite analyses of naphthalene-grown cultures. However, because the metabolites were extracted from naphthalene-grown cultures at a certain growth period, this method can not discriminate the first intermediate from downstream metabolites.

Incubations were done with concentrated cell suspensions in order to yield detectable amounts of metabolites within a short incubation time. Some known metabolites from the upper naphthalene degradation pathway were included as standards, e.g., 2-naphthoate and [5,6,7,8]-tetrahydro-2-naphthoate. Since 2-methylnaphthalene was previously proposed as the activation product of naphthalene (Safinowski and Meckenstock, 2006), it was also included in the mixture of standards.

When cell suspensions were incubated with [1,4,5,8-^{14}C]-naphthalene as a substrate, radiolabeled 2-naphthoate was formed as the first intermediate at a relatively short incubation time (10 min; Fig. 4.3A). However, it was detectable only upon the addition of unlabeled 2-naphthoate as a 'cold trap' (200 μM). In the absence of 2-naphthoate trap, complete oxidation of ^{14}C-naphthalene to ^{14}C-bicarbonate without formation of a detectable intermediate was observed (data not shown). The requirement for an unlabeled trap (isotope dilution) indicated that the amount of radiolabeled naphthalene alone was growth-limiting so that intermediates were scavenged by subsequent reactions below their detection limit.
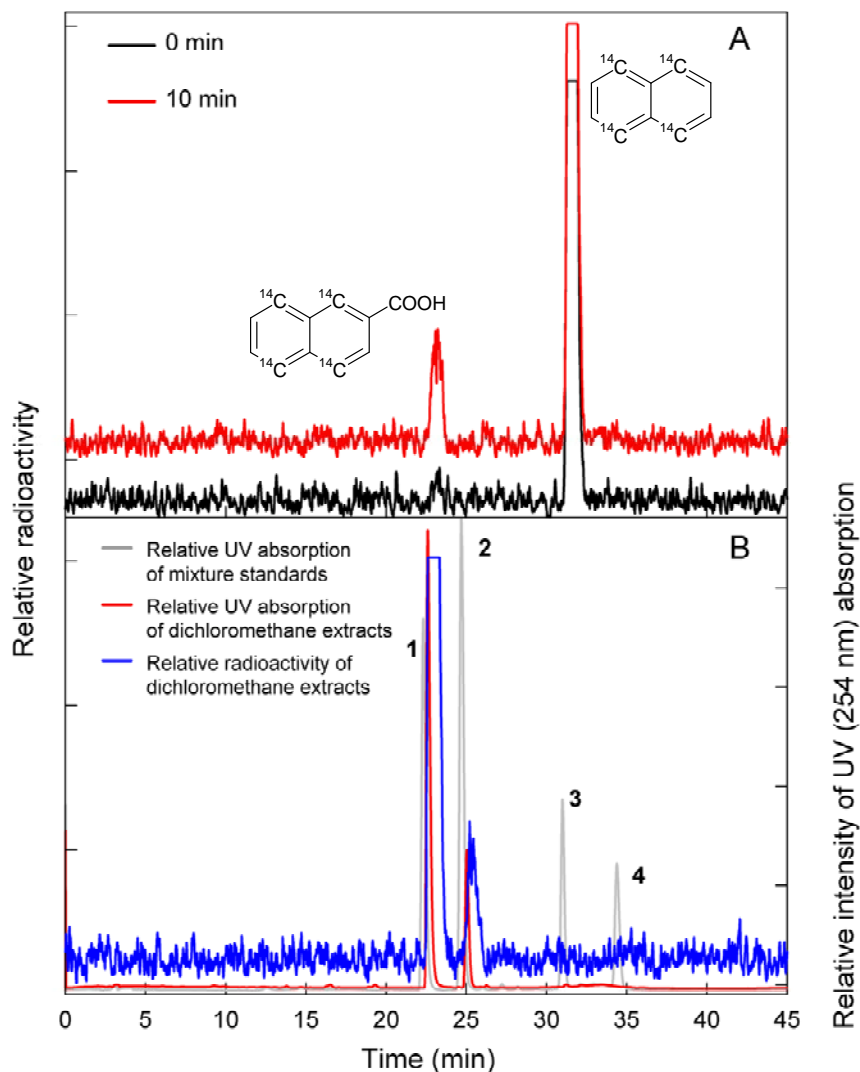
Fig. 4.3. Detection of radiolabeled intermediates formed in dense cell suspensions of strains NaphSx incubated with [1,4,5,8-^{14}C]-naphthalene (26.7 μM, 14.8 kBq ml^{-1}) and 200 μM unlabeled 2-naphthoate as a cold trap. Radiolabeled 2-naphthoate was apparently formed as the first intermediate, becoming detectable after 10 min in cell suspensions of strain NaphS6 (A). Both intermediates ^{14}C-2-naphthoate and ^{14}C-tetrahydro-2-naphthoate were detected in the dichloromethane extracts of NaphS3 cell suspensions incubated with ^{14}C-naphthalene for 9 h (B). The intermediates were identified based on co-elution with authentic standards. Sample elution profile with overlay of UV spectrum of mixed authentic standards is shown in gray line, and peaks represent: (1) 2-naphthoate; (2) [5,6,7,8]-tetrahydro-2-naphthoate; (3) naphthalene; and (4) 2-methylnaphthalene.

In the same sample, the proposed intermediate, [5,6,7,8]-tetrahydro-2-naphthoate was initially not detectable. However, small amounts became detectable after stronger concentration of dichloromethane extract (Fig. 4.3B). The result suggests that also the marine strains first reduce 2-naphthoate (CoA-thioester) at the unsubstituted ring (by 2-naphthoyl-CoA reductase), like the highly enriched freshwater culture N47 (Eberlein *et al*., 2013a; b;

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recently reviewed by Boll *et al.*, 2014) and other enriched naphthalene-degrading anaerobes (Zhang *et al.*, 2000; Phelps *et al.*, 2002).

2-Naphthoate formation at a significant concentration was also observed in whole-cell assays with naphthalene as a substrate in sulfate-free medium, viz. if oxidation was prevented due to the absence of an external electron acceptor. In such an experiment, the dependence of 2-naphthoate production on bicarbonate could be shown (Fig. 4.4). Stable isotope-labeling with ^{13}C-NaHCO$_3$ confirmed that the carboxyl group in 2-naphthoate originated from bicarbonate, as shown in a previous study (Zhang and Young, 1997). Label incorporation into the carboxyl group was further confirmed by using ^{14}C-bicarbonate in present study (data not shown).
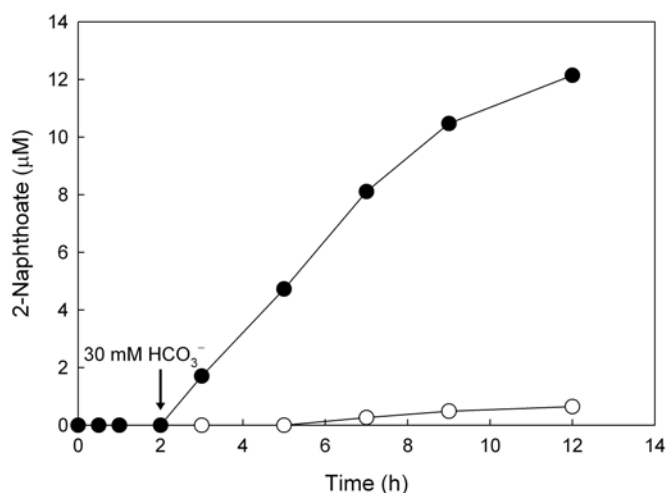

Fig. 4.4. Dependence of 2-naphthoate production on the presence of bicarbonate in whole-cell assays in the absence of sulfate. 2-Naphthoate production started immediately upon the addition of HCO$_3^-$ (30 mM) after 2 hours, while in the control assay without HCO$_3^-$ only marginal formation of 2-naphthoate was observed. The experiment was conducted with dense cell suspensions of strain NaphS6 in sulfate-free ASW medium with Tris/HCl buffer (30 mM) instead of bicarbonate buffer. Assays were performed in a volume of 1 ml in closed serum vials with the addition of naphthalene (around 100 μM). Samples were analyzed with an UPLC system.

In conclusion, the present radiolabeling experiment with the marine strains indicates that introduction of a CO$_2$/HCO$_3^-$-derived carboxyl function for subsequent reduction of the CoA-thioester is a general mechanism of anaerobic naphthalene degradation by sulfate-reducing bacteria from various environments.

4.1.3 Attempts to further characterize the naphthalene activation reaction

After naphthalene-derived 2-naphthoate had been detected in cell suspensions of the marine strains, in accordance with the findings in freshwater culture N47 (Mouttaki *et al.*, 2012), the reaction was investigated in some more details. It was of interest whether the postulated naphthalene carboxylation-like reaction could be established in cell-free extracts, and to which extent the reaction depended on an energy source.

4.1.3.1 Experiments with cell extracts vs. intact cells, and with different co-substrates

The formation of 2-naphthoate from naphthalene in the presence of CO_2/HCO_3^- was examined in cell suspensions, crude cell lysate, and cell-free extracts (subjected to high-speed centrifugation) of strain NaphS3, respectively. Formation of 2-naphthoate was absent in cell-free extracts, whereas there was a reduced activity in crude cell lysate (Fig. 4.5). Because microscopy of the latter revealed a fraction of intact cells that obviously had escaped lysis, it can be concluded that the postulated naphthalene carboxylation-like reaction under the presently established conditions apparently depends on intact cells. Control measurements of CO dehydrogenase (CODH) and formate dehydrogenase (FDH) with cell-free extract indicated high activity (87 nkat mg^{-1} protein and 59 nkat mg^{-1} protein, respectively) for strain NaphS3 (Appendix, Fig. 9.1) and strain NaphS6 (data not shown), which implied that the basic conditions, e.g. means to prepare cell-free extracts and buffer solutions, for assaying oxygen-sensitive enzymes were reliable.
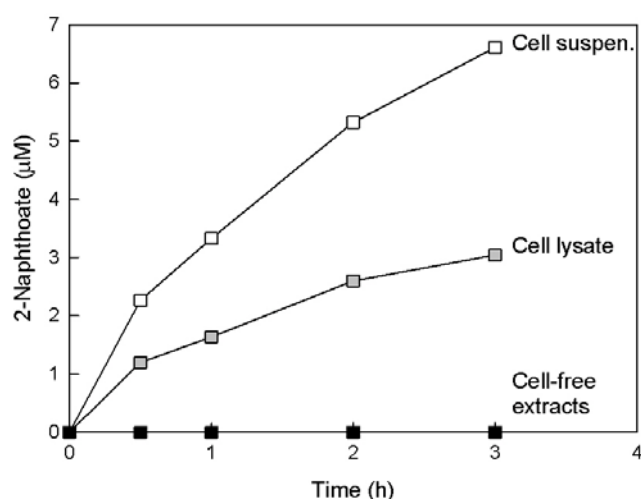

Fig. 4.5. Formation of 2-naphthoate in the presence of naphthalene as a substrate in cell suspensions (□), crude cell lysate (▣), and cell-free extracts (■) of strain NaphS3, respectively. The activity was obviously lost during cell lysis. The activity still detectable in crude cell lysate was apparently due to incomplete cell disruption and the presence of intact cells. Details of the experimental conditions are described in page 23 of the "Materials and methods" section.

Further tests were carried out to examine whether the loss of the 2-naphthoate-formation activity upon cell lysis may be due to the lack or dilution of the actual *in vivo* carboxyl donor which may not be CO_2 or bicarbonate but rather a compound derived from these. CO, formate, and acetyl-CoA were tested as such possible carboxyl group donors in cell-free extracts. However, formation of 2-naphthoate from naphthalene was not observed under the given experimental conditions.

The lack of 2-naphthoate-forming activity observed so far in cell-free extracts may be explained by (a) the lack or strong dilution of the *in vivo* carboxyl donor (other than CO_2, HCO_3^-, CO, formate, and acetyl-CoA), (b) disintegration of an enzyme complex, or (c) lack of an energy source such as the membrane potential.

4.1.3.2 Effect of FCCP on 2-naphthoate formation and on its further catabolism

Since one of the effects of cell disintegration is the collapse of the membrane potential which may be needed for the activity of certain energy-dependent reactions, the effect of energy-deprivation on 2-naphthoate formation and its further degradation in whole-cell assays was examined by the addition of the protonophore carbonyl cyanide 4-(trifluoromethoxy) phenylhydrazone (FCCP). FCCP is a weakly proton-binding membrane-permeable compound which dissipates the proton gradient across the cytoplasmic membrane and thus also causes ATP deprivation (Fig. 4.6; Terada, 1990; Nicholls and Ferguson, 1992).
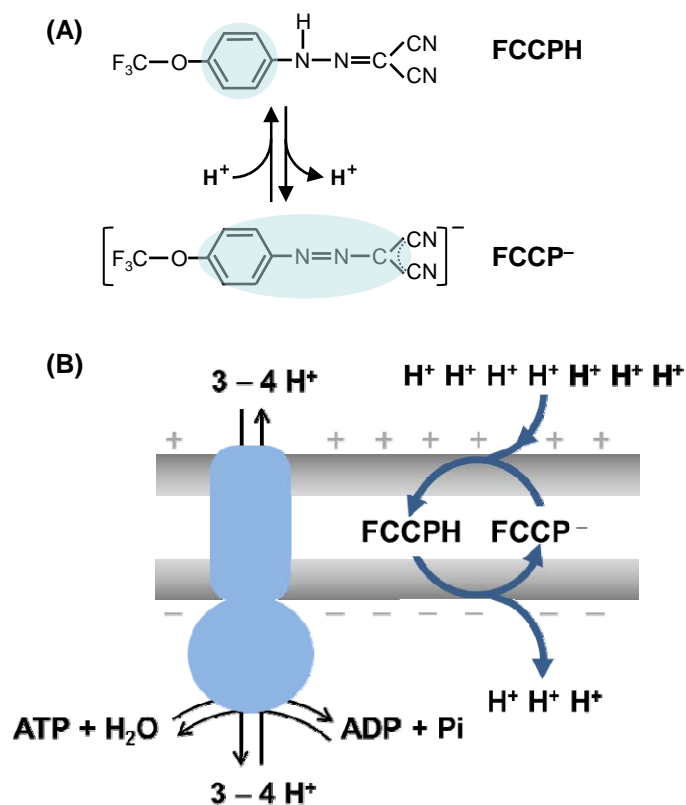
Fig. 4.6. Scheme of transmembrane proton equilibration by uncoupler FCCP. FCCP, one of the most commonly employed protonophore, is a lipophilic weak acid able to permeate across lipid bilayers in either the protonated or deprotonated forms; the shaded area represents the π-electron system, extension of which facilitates N-deprotonation (A). Scheme of uncoupling including FCCP and F_OF_1-ATPase (B) (modified from Nicholls and Ferguson, 1992).

(A) Effect of FCCP on 2-naphthoate formation in whole-cell assays

If whole cells of strains NaphS3 and NaphS6 were incubated with naphthalene and sulfate in bicarbonate-buffered ASW medium, 2-naphthoate did not accumulate (Fig. 4.7B, D). However, if sulfate was omitted, there was always a clear accumulation of 2-naphthoate in whole-cell assays of both strains, NaphS3 and NaphS6 (Fig. 4.7A, C). Obviously, scavenge of 2-naphthoate (or an *in vivo* intermediate yielding 2-naphthoate in the analytical assay) by subsequent reactions under conditions of sulfate reduction was faster than 2-naphthoate formation, preventing detectable steady-state concentrations. This finding is in agreement with the previous observations in assays with radiolabeled [1,4,5,8-^{14}C]-naphthalene as a tracer, where the formation of [1,4,5,8-^{14}C]-2-naphthoate as an intermediate was detectable only upon isotope dilution with unlabeled 2-naphthoate as a cold trap.

Rather than inhibiting 2-naphthoate formation, the addition of the uncoupler FCCP to sulfate-free cell suspensions of strains NaphS3 and NaphS6 promoted the accumulation of 2-naphthoate in comparison to controls without FCCP (Fig. 4.7A, C). When sulfate was

present in the assays, 2-naphthoate did not accumulation; however, with an addition of FCCP, 2-naphthoate accumulated even in the presence of sulfate (Fig. 4.7B, D). The 2-naphthoate production in whole-cell assays was strictly dependent on naphthalene, and in controls with the addition of FCCP without naphthalene, there was no detectable 2-naphthoate production (data not shown), which confirmed the production and accumulation of 2-naphthoate after FCCP addition was not due to the liberation of a storage form of 2-naphthoate.
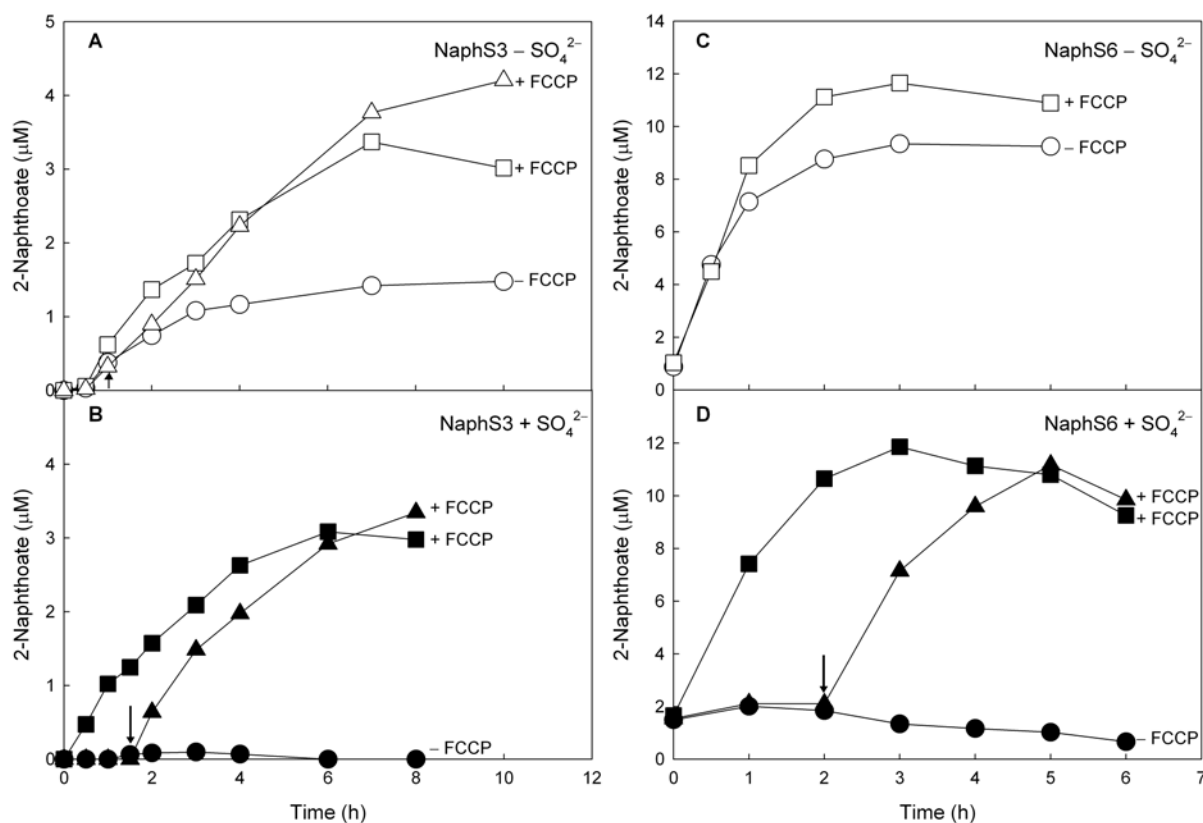

Fig. 4.7. Influence of sulfate depletion and FCCP addition on the formation of 2-naphthoate from naphthalene in whole-cell assays. Time course of 2-naphthoate formation in whole-cell assays of strains NaphS3 (A and B) and NaphS6 (C and D) incubated with naphthalene as a substrate with (B and D) or without (A and C) sulfate as a terminal electron acceptor. According to the time course, for both strains, 2-naphthoate could not accumulate when sulfate present, due to the fast downstream degradation; when sulfate was omitted from the medium, significant 2-naphthoate accumulation could be observed. Application of FCCP in cell suspensions of both strains NaphS3 and NaphS6 could slightly stimulate 2-naphthoate formation rather than inhibit it.

2-Naphthoate formation despite the presence of FCCP suggested the functionalization of naphthalene to 2-naphthoate itself might be an ATP-independent reaction. To further investigate the apparent energy-independent conversion of naphthalene to 2-naphthoate at the observed concentrations, further whole-cell incubation experiments were carried out with the ATPase inhibitor, *N,N'*-dicyclohexylcarbodiimide (DCCD). DCCD can covalently bind to the carboxyl groups of glutamyl residues and effectively inhibits the function of the ATPase

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complex without destroying the membrane potential, viz. the use of the proton gradient for ATP synthesis (Sebald *et al.*, 1980). Like FCCP, application of DCCD at the concentrations of 50 μM or 1 mM did not inhibit the formation of 2-naphthoate, but rather caused the accumulation of 2-naphthoate in whole-cell assays of strain NaphS6, independently of the presence of sulfate (Fig. 4.8A).
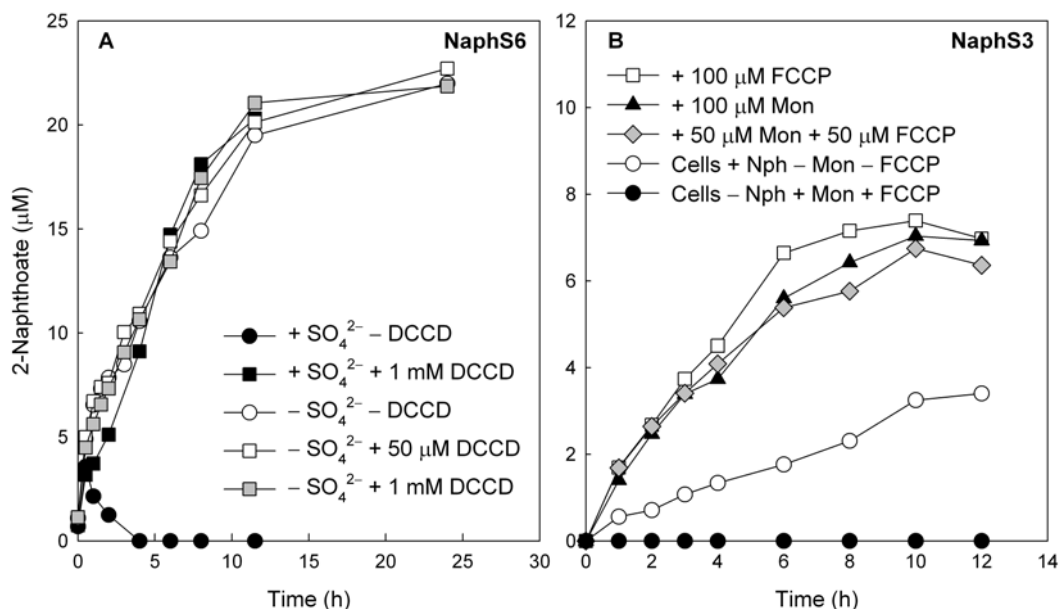

Fig. 4.8. Effect of the ATPase inhibitor DCCD and ionophore monensin (Mon) on the formation of 2-naphthoate in whole-cell assays of strains NaphS6 (A) and NaphS3 (B), respectively. Similar to FCCP, addition of DCCD could not inhibit 2-naphthoate formation. When sulfate was present, 2-naphthoate could not accumulate without DCCD (A, ●), whereas application of DCCD led to a remarkable accumulation of 2-naphthoate (A, ■). The addition of monensin alone as well as with the combination of FCCP could not impede 2-naphthoate formation and the influence was similar as the addition of FCCP alone (B). 2-Naphthoate formation in whole-cell assays of strain NaphS3 was naphthalene-dependent (B, ●).

It has been known that some decarboxylation processes, e.g., the decarboxylation of oxaloacetate, methylmalonyl-CoA, and glutaconyl-CoA, are coupled with the translocation of Na^+, and thereby, lead to build up the Na^+ gradient across cell membranes, which can be used for energy generation by Na^+-dependent ATP synthase (Dimroth, 1987; Buckel, 2001). Because naphthalene-degrading strains NaphSx were isolated from the marine environment, Na^+ is certainly playing an important role in their physiology. Therefore, it had been speculated whether *vice versa* the naphthalene carboxylation was driven by a Na^+ gradient. Uncoupler FCCP can only abolish proton gradient without influencing sodium gradient. Therefore, in order to examine this hypothesis, monensin was employed in whole-cell assays. Monensin is a Na^+/H^+ antiporter, which shuttles Na^+ versus H^+ cations across lipid membranes of cells and collapses a possible transmembrane Na^+ gradient if a protonophore is

present that dissipates the H^+ (Mollenhauer *et al*., 1990). However, similar to FCCP, addition of monensin alone or with the combination of FCCP in sulfate-free cell suspensions of strain NaphS3 could not inhibit 2-naphthoate formation, and even showed slight stimulatory effect (Fig. 4.8B).

The conclusions from the above experiments with impediment of the catabolism are as follows: Under sulfate-free conditions, regular electron transport driving the anaerobic respiratory mechanisms and thus ATP generation cannot proceed. This explains the accumulation of 2-naphthoate to a certain level before the entire metabolism ceases. Additionally, application of certain amounts of FCCP (up to 200 µM tested) in the whole-cell assays apparently collapsed the proton gradient across the cellular membranes, shutting down the ATP recharging system and subjecting the cells to energy deprivation. The application of monensin in combination with FCCP can completely abolish both H^+ and Na^+ gradient across cell membranes. Addition of DCCD inhibits the synthesis of ATP at ATPase, while the membrane potential can further build up. If naphthalene carboxylation would have been dependent on ATP or membrane-potential (either H^+ or Na^+) under these conditions, the application of FCCP, monensin, or DCCD should have caused an inhibition effect on 2-naphthoate formation. However, experiments revealed the opposite results. The unexpected stimulating effect of FCCP, monensin, and DCCD on 2-naphthoate formation led to the speculation that the functionalization reaction of naphthalene to 2-naphthoate itself might be energy- or membrane-potential-independent. It may thus involve a pre-formed carboxyl donor from a preceding energy-consuming formation of 'activated CO_2'. Further catabolism of 2-naphthoate again is expected to involve energy-requiring steps. Energy deprivation could therefore affect further processing of 2-naphthoate. This may explain why FCCP, monensin, and DCCD addition or sulfate omission caused the higher accumulation of 2-naphthoate.

As a positive control, sulfide production in cultures of strains NaphSx were completely inhibited after the addition of FCCP and DCCD at the comparable concentrations applied in the whole-cell assays, confirming both FCCP and DCCD can effectively de-energize cells of sulfate-reducing bacteria and impede the energy-requiring sulfate-reduction process (data not shown).

(B) Effect of FCCP on further catabolism of 2-naphthoate in whole-cell assays

To further verify the hypothesis that the subsequent catabolism of 2-naphthoate involves energy-requiring steps, the effect of FCCP addition on 2-naphthoate degradation was investigated in whole-cell assays of strains NaphS3 and NaphS6, in which 2-naphthoate was supplied as the initial substrate. Without sulfate, 2-naphthoate degradation was not significant and FCCP addition did not reveal any influence within a relatively short incubation time (3 hours; Fig. 4.9A, C). As expected from other growth experiments, 2-naphthoate consumption in the presence of sulfate was very fast. If FCCP was added under such conditions, a clear inhibitory effect was observed (Fig. 4.9B, D). These results further supported the working hypothesis, that energy deprivation could impede subsequent catabolism of 2-naphthoate by cells of strains NaphSx.
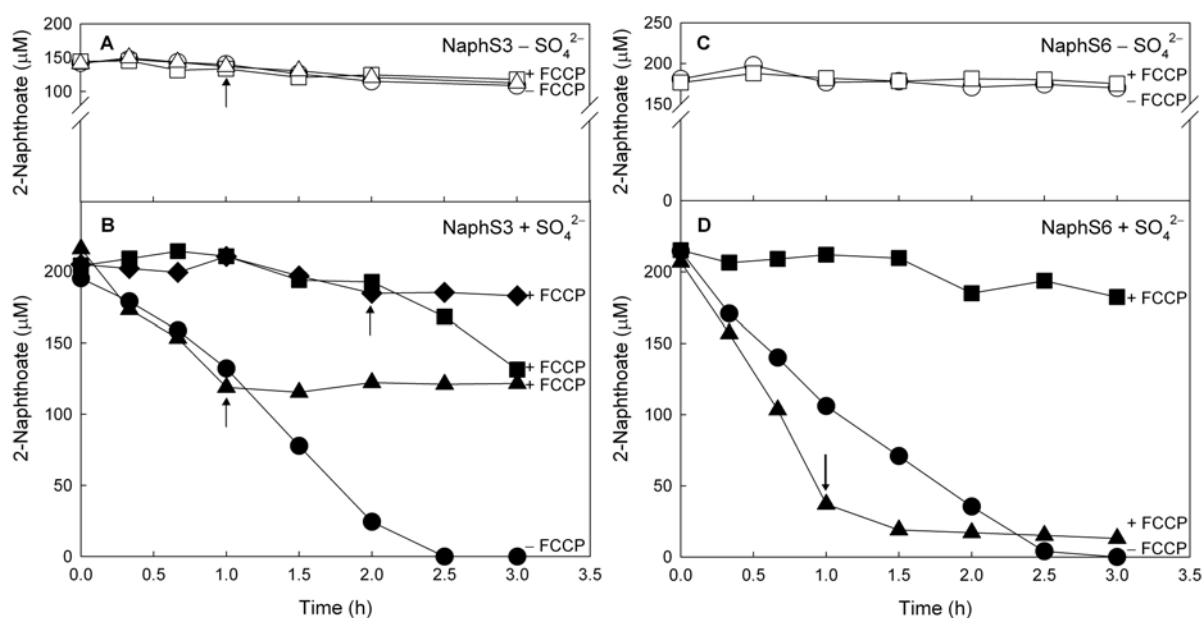

Fig. 4.9. Effect of FCCP addition on 2-naphthoate degradation in whole-cell assays of strains NaphS3 (A and B) and NaphS6 (C and D) incubated with 2-naphthoate as a substrate with (B and D) or without (A and C) sulfate as terminal electron acceptor. When sulfate was present (●), 2-naphthoate could be degraded immediately and addition of FCCP at time zero (■,◆) or 1 hour (▲) clearly showed inhibition on 2-naphthoate further degradation. The inhibition effect of FCCP only lasted for 2 hours and degradation started again afterwards in assays of NaphS3 (B, ■), which might be due to the instability of FCCP in cell suspensions. Reinjection of FCCP at 2 hour (◆) showed a continuous inhibition effect. Without sulfate (○, □, Δ), 2-napthoate could not be degraded within 3 hours and addition of FCCP (□, Δ) could not reveal any difference.

Based on current study, a pathway of initial reactions of anaerobic naphthalene degradation is proposed (Fig. 4.10). Naphthalene is likely activated by a carboxylation-like reaction to form 2-naphthoate, which appears to be an ATP-independent reaction. Then 2-naphthoate most likely undergoes an ATP-dependent CoA-ligation reaction generating

2-naphthoyl-CoA. Thus, sulfate omission or addition of FCCP, DCCD as well as monensin could certainly affect the energy-demanding 2-naphthoyl-CoA formation, thereby inhibiting further processing of 2-naphthoate in both strains.
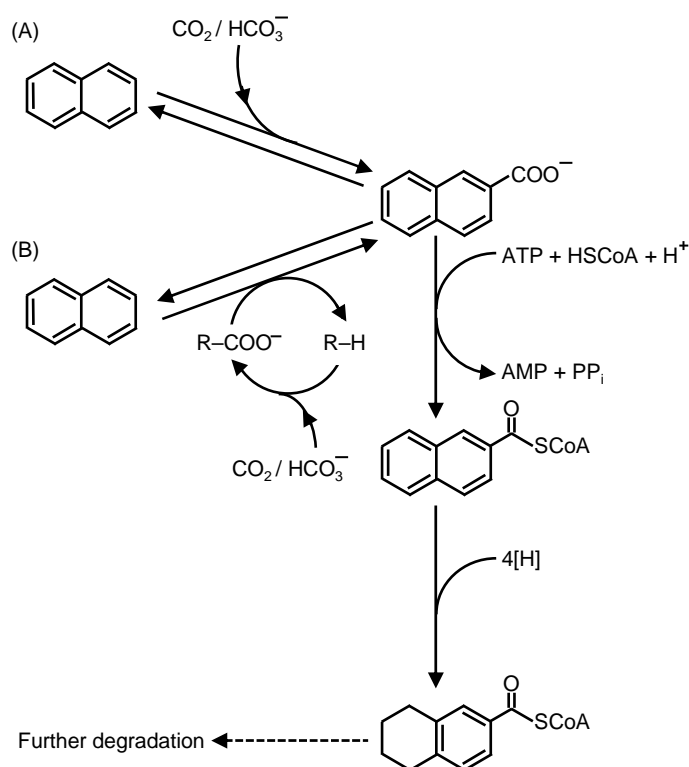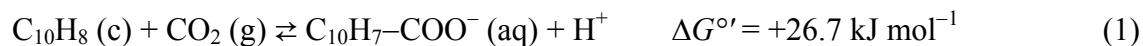

Fig. 4.10. Present hypotheses of anaerobic naphthalene activation and subsequent two reactions. Because of the quite high energetic barrier of aromatic structure of naphthalene, it is difficult to imagine simply direct carboxylation by CO_2 or HCO_3^- (A). It is possible that CO_2/HCO_3^- is first activated to generate a carboxyl group donor R–COO$^-$ (B); then carboxylation leads to 2-naphthoate, which is subsequently subjected to an ATP-dependent CoA-ligation reaction, forming 2-naphthoyl-CoA. 2-Naphthoyl-CoA is further reduced to tetrahydro-2-naphthoyl-CoA, which may be an energy-independent reaction according to recent enzymatic studies on freshwater enrichment culture N47 (Eberlein *et al.*, 2013a, b). Afterwards, it will be further completely degraded to CO_2.
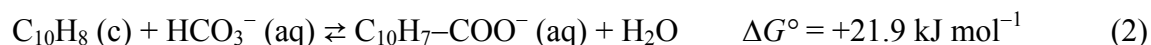
(C) Discussion of naphthalene carboxylation from energetic perspectives

According to the results obtained, the direct *in vivo* conversion of naphthalene to 2-naphthoate itself apparently does not require an energy input, at least up to the observed concentrations. On the other hand, from a thermodynamic point of view, the standard free energy change of direct naphthalene carboxylation is expected to be endergonic, like many other direct carboxylation reactions of non-activated carboxyl acceptors. This urged upon refined calculation of the free energy and equilibrium constant of the reaction. As ΔG_f° data for the 2-naphthoate anion are not available in reference tables, it was calculated based on the group

contribution method described by Mavrovouniotis (1991; for all calculations, see Appendix) According to this, the free energy of formation of 2-naphthoate is -126.8 kJ mol^{-1}. If CO_2 is the reactant in naphthalene carboxylation, then the equation and $\Delta G°'$ are as follows:

$$C_{10}H_8 \text{ (c)} + CO_2 \text{ (g)} \rightleftharpoons C_{10}H_7\text{--}COO^- \text{ (aq)} + H^+ \qquad \Delta G°' = +26.7 \text{ kJ mol}^{-1} \qquad (1)$$

Or if HCO_3^- is the carboxylation reactant, then the equation and $\Delta G°$ are:

$$C_{10}H_8 \text{ (c)} + HCO_3^- \text{ (aq)} \rightleftharpoons C_{10}H_7\text{--}COO^- \text{ (aq)} + H_2O \qquad \Delta G° = +21.9 \text{ kJ mol}^{-1} \qquad (2)$$

Our present investigations shed light on the energetics of net carboxylation of aryl species. Carboxylation reactions with free inorganic carbon species are usually thermodynamically unfavorable processes and require energy input to drive the formation of new C–C bond (Glueck *et al.*, 2010; Bar-Even *et al.*, 2012). The most noticeable examples are acetone carboxylase in the aerobic bacterium *Xanthobacter* strain Py2 (Sluis and Ensign, 1997) and the recently revealed acetophenone carboxylase in *Aromatoleum aromaticum* strain EbN1 (Jobst *et al.*, 2010), in which carboxylation of acetone and acetophenone are directly coupled with ATP hydrolysis. In case of anaerobic phenol carboxylation, phenol is first activated by an ATP-dependent phosphorylation to phenylphosphate, which is subsequently carboxylated with CO_2 to yield *p*-hydroxybenzoate (Boll and Fuchs, 2005). Current study showed 2-naphthoate formation in both strains NaphS3 and NaphS6 was independent on the presence of the terminal electron acceptor sulfate and application of FCCP, DCCD as well as monensin could not inhibit 2-naphthoate formation in cells, indicating naphthalene conversion to 2-naphthoate by both strains might not directly require neither an ATP input nor the membrane potential.

In view of the changes of Gibbs free energy of equations (1) and (2), naphthalene carboxylation is still a slightly endergonic reaction, which leaves open possibilities that naphthalene carboxylation probably couples with a downstream exergonic reaction. It is also possible that a high-affinity consumption of 2-naphthoate by subsequent reactions will keep it at very low *in vivo* concentration facilitating the activation reaction.

4.1.4 Reversibility of the carboxylation-like reaction of naphthalene

4.1.4.1 Carbon radioisotope exchange between CO_2/HCO_3^- and 2-naphthoate

As introduced previously, reversibility is expected in energy-limited catabolic reactions. In anaerobic phenylphosphate carboxylation catalyzed by phenylphosphate carboxylase (PPC), carbon isotope exchange reaction between $^{14}CO_2$ and *p*-hydroxybenzoate was observed, suggesting the reversibility of phenol carboxylation (Lack *et al*., 1991). Because of the detection of PPC-like protein specifically formed in naphthalene-grown cells of strains NaphSx as discussed previously, experiments were designed to investigate possible isotope exchange reaction between CO_2/HCO_3^- and 2-naphthoate in strains NaphSx, with the use of radiolabeled bicarbonate. To prevent the rapid consumption of 2-naphthoate, cells were incubated without the electron acceptor sulfate. Bicarbonate-buffer was replaced with Tris/HCl-buffer to avoid label dilution. Details of the experimental set up are described in page 22 in the section of materials and methods.

HPLC coupled with UV and radioflow detection indeed revealed the incorporation of ^{14}C into the added 2-naphthoate. Peak identity with 2-naphthoate was confirmed by co-elution of an authentic standard (Fig. 4.11A). During incubation, the radioactivity of 2-naphthoate increased continuously, as determined precisely by additional liquid scintillation analysis of dichloromethane extracted samples (Fig. 4.11B). Assuming that the label was incorporated into the carboxyl group of 2-naphthoate, the experiment showed the expected carbon isotope exchange between CO_2/HCO_3^- and the carboxyl group of 2-naphthoate.
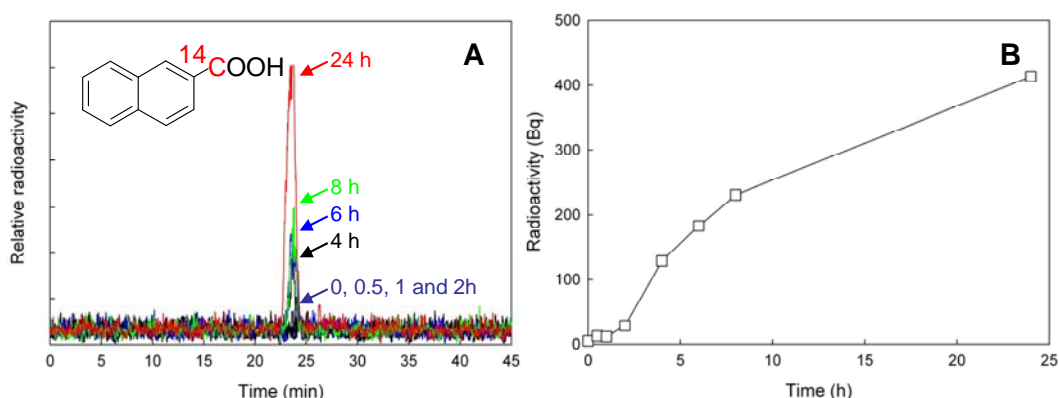

Fig. 4.11. Formation of radiolabeled 2-naphthoate in cell suspensions of strain NaphS6 incubated with unlabeled 2-naphthoate (0.4 mM) and radiolabeled bicarbonate (20 μM, 42 kBq ml^{-1}) in sulfate-free Tris/HCl-buffered ASW medium. Detection via HPLC coupled with an UV and an online radioflow detector (A); Time course of radiolabel incorporation determined through liquid scintillation analysis (B).
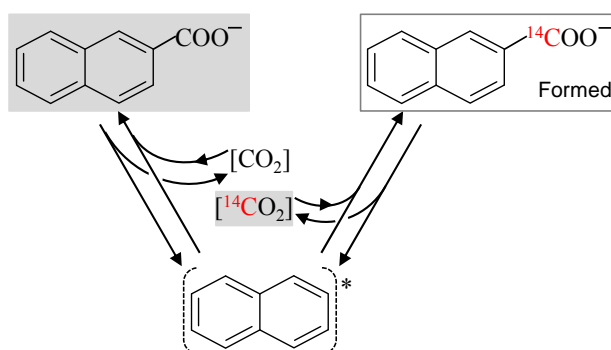
Fig. 4.12. Scheme of carbon radioisotope exchange between of CO_2/HCO_3^- and the carboxyl group of 2-naphthoate as catalyzed by cells of strains NaphSx. The substrate of the exchange reaction could be free naphthalene or an enzyme-bound activated form of naphthalene (asterisk).

A similar isotope exchange reaction was also observed in cell extracts of the highly enriched freshwater culture N47 (Mouttaki *et al.*, 2012).

The carbon radioisotope exchange indicated reversibility of the carboxylation-like reaction of naphthalene. The substrate for carboxylation is not necessarily naphthalene itself. It may be also an enzyme-bound activated form of naphthalene (marked with asterisk in Fig. 4.12).

Based on the currently employed method using whole cells, we cannot distinguish between CO_2 and HCO_3^- as the primary reactant. Both species can be used in carboxylation reactions (reviewed by Erb, 2011). In anaerobic phenylphosphate carboxylation, CO_2 was proven as the actual reagent (Lack *et al.*, 1991).

If the aqueous samples were directly analyzed by HPLC without extraction with dichloromethane, several other unknown radioactive compounds could be detected besides ^{14}C-2-naphthoate, which might be side products from ^{14}C-bicarbonate (data not shown).

4.1.4.2 Naphthalene production from 2-naphthoate

The observation of carbon radioisotope exchange reaction indicated that the carboxylation-like reaction converting naphthalene to 2-naphthoate catalyzed by NaphSx cells is reversible. To directly examine this hypothesis, possible net production of naphthalene was monitored with 2-naphthoate as a substrate in dense cell suspensions.

Naphthalene-grown cultures of all three strains were carefully separated from the carrier phase with the growth substrate and washed once with sulfate-free ASW medium to remove residual aqueous naphthalene and sulfate. The cells were subsequently re-suspended in sulfate-free ASW medium to prepare concentrated cell suspensions. 2-Naphthoate was added to start the reaction, and samples were taken periodically to determine naphthalene

concentration via an UPLC analysis. Sulfate was omitted to prevent the quick consumption of 2-naphthoate and thus maintain its concentration high.

Naphthalene production from 2-naphthoate was indeed observed in cell suspensions of strain NaphS3 (Fig. 4.13A). There was no naphthalene production in control incubations without 2-naphthoate or without NaphS3 cells. The capability for net decarboxylation was confirmed with 6-fluoro-2-naphthoate as an analogous substrate. In incubations of cell suspensions with 6-fluoro-2-naphthoate, its corresponding decarboxylation product 2-fluoronaphthalene could be measured as well (Fig. 4.13B).
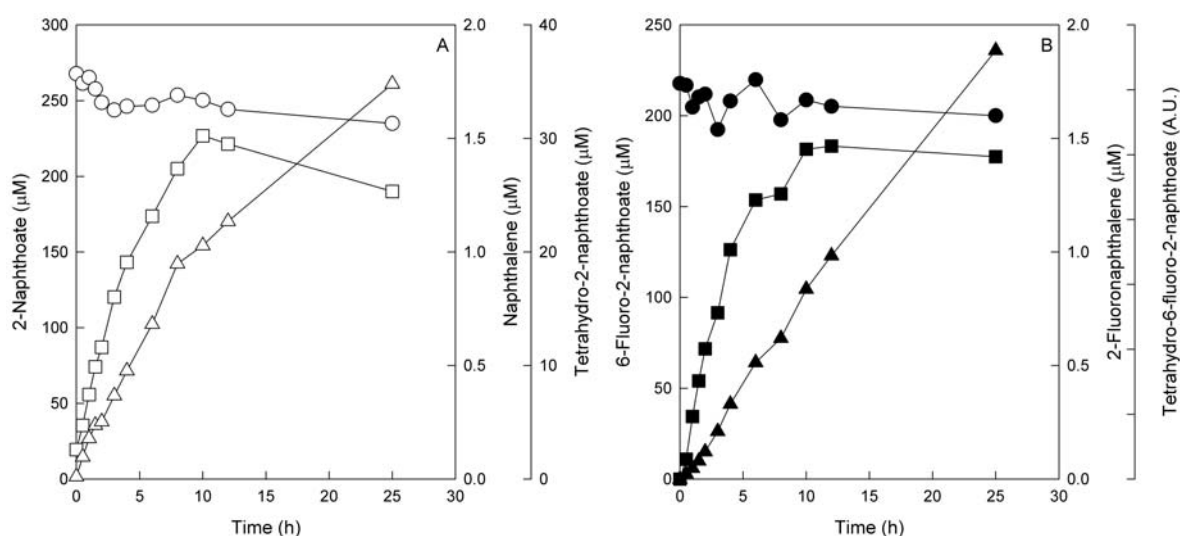

Fig. 4.13. Demonstration of the net decarboxylation of 2-naphthoate (○) and 6-fluoro-2-naphthoate (●) to naphthalene (□) and 2-fluoronaphthalene (■), respectively, by cell suspensions of strain NaphS3 in sulfate-free ASW medium. Formation of [5,6,7,8]-tetrahydro-2-naphthoate (△) and putative [5,6,7,8]-tetrahydro-6-fluoro-2-naphthoate (▲) as products of subsequent ring reduction was also observed. Because of the lack of a standard, the increase of tetrahydro-6-fluoro-2-naphthoate is indicated on a relative concentration scale.

Besides naphthalene, also the downstream product of 2-naphthoate, [5,6,7,8]-tetrahydro-2-naphthoate was detected (Fig. 4.13A). Its concentration was significantly higher than in incubation experiments in the presence of sulfate. In accordance with 2-naphthoate reduction, 6-fluoro-2-naphthoate yielded an increasing peak that was tentatively identified as [5,6,7,8]-tetrahydro-6-fluoro-2-naphthoate based on analogous comparisons of retention times (Fig. 4.13B). However, due to the lack of an authentic standard, further proof of identity and quantification of [5,6,7,8]-tetrahydro-6-fluoro-2-naphthoate was not possible.

4.1.5 Investigation of the hypothesis of carbonylation as naphthalene activation step

Aldehyde decarbonylation was reported as the last step in alkane production in the plant *Pisum sativum* (Cheesbrough and Kolattukudy, 1984) and in cyanobacteria (Schirmer *et al*., 2010; Li *et al*., 2011), catalyzed by an aldehyde decarbonylase. This principle has already been used in bioengineering to develop genetically modified *Escherichia coli* strains that are capable of producing alkanes (Choi and Lee, 2013). Vice versa, carbonylation of aliphatic and possibly also of aromatic hydrocarbons may be envisaged as a working hypothesis. Moreover, carbon monoxide (CO) as a reagent may exhibit higher chemical reactivity than CO_2. Thus, an initial attack of naphthalene by CO or another C_1-compound of the same oxidation state (e.g., a carrier-bound formyl group) may be envisaged. This would lead to 2-naphthaldehyde, which can be subsequently oxidized to 2-naphthoate.

Very recently, carbonylation was proposed as the initial reaction in anaerobic acetone activation by a sulfate-reducing bacterium, *Desulfococcus biacutus*, and CO instead of CO_2 was discovered as the co-substrate (Acosta *et al*., 2013; 2014). However, in aerobic and nitrate-reducing bacteria, it is widely accepted that acetone is initially activated via an ATP-dependent carboxylation reaction (Sluis and Ensign, 1997).

To investigate whether carbonylation of naphthalene to 2-naphthaldehyde was the step prior to 2-naphthoate formation, radiolabeled [1,4,5,8-^{14}C]-naphthalene was used again to examine whether [1,4,5,8-^{14}C]-2-naphthaldehyde could be identified as the first intermediate. Similar as the method introduced before (chapter 4.1.2), experiments were conducted with radiolabeled naphthalene in NaphS3 cell suspensions in the presence of unlabeled 2-naphthaldehyde (300 μM) as a cold trap, i.e. by making use of isotope dilution. Samples were taken at different time points and analyzed with HPLC coupled with an UV and an online radioflow detector.

Under sulfate-reducing conditions, the added 2-naphthaldehyde was immediately oxidized to 2-naphthoate, which was further catabolized (Fig. 4.14A). At the same time, naphthalene was slowly metabolized by NaphS3 cells at a constant rate (Fig. 4.14A). However, radiolabeled ^{14}C-2-naphthaldehyde could not be trapped as an intermediate, whereas ^{14}C-2-naphthoate was detected again as the first metabolite (Fig. 4.14B). The reason for the detection of ^{14}C-2-naphthoate might be due to the rapid accumulation of 2-naphthoate from the fast oxidation of 2-naphthaldehyde, serving as a trap for the 2-naphthoate derived from radiolabeled naphthalene. Current results do not support the hypothesis of naphthalene carbonylation as the anaerobic activation step.
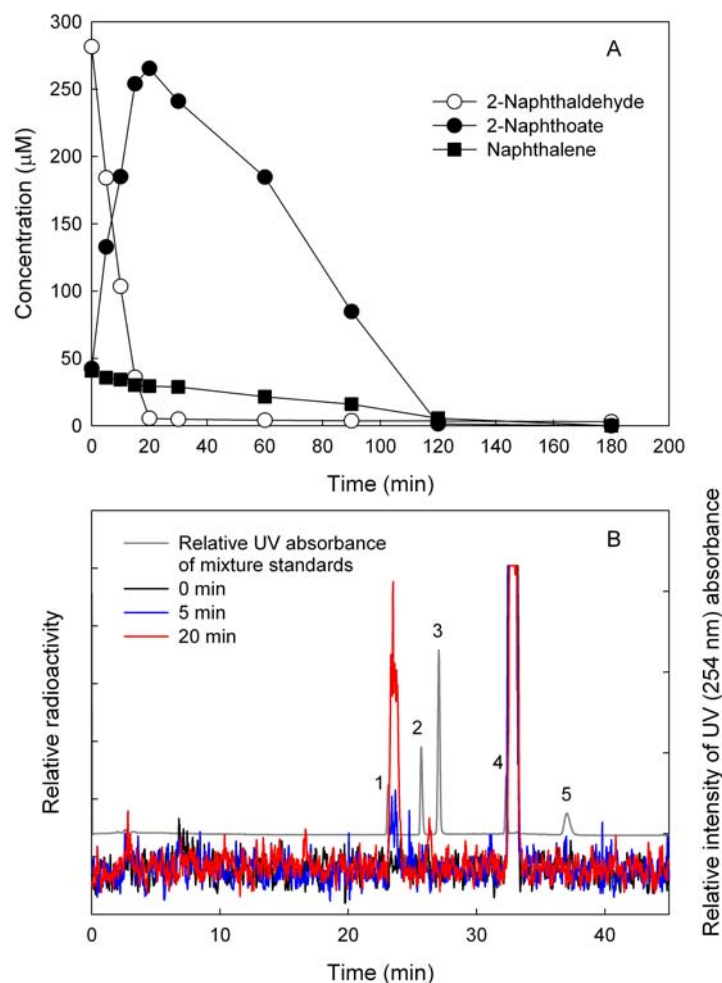
Fig. 4.14. Time course of incubations of NaphS3 cell suspensions supplied with radiolabeled and unlabeled naphthalene in the presence of 2-naphthaldehyde (300 μM) as a trap. 2-Naphthaldehyde (○) was quickly degraded via 2-naphthoate (●) as an intermediate, which was transiently formed during the incubation; naphthalene (■) was continuously degraded by NaphS3 cells at a relatively slow rate (A). Formation of ^{14}C-2-naphthoate as the first detectable intermediate from ^{14}C-naphthalene (B). The speculated intermediate, ^{14}C-2-naphthaldehyde, was not formed during the incubation with ^{14}C-naphthalene, suggesting that naphthalene was not carbonylated. Grey line shows the mixture of authentic standards detected by their UV absorption; the peaks represent: (1) 2-naphthoate, (2) [5,6,7,8]-tetrahydro-2-naphthoate, (3) 2-naphthaldehyde, (4) naphthalene, and (5) 2-methylnaphthalene.

Because 2-naphthaldehyde was rapidly oxidized, the corresponding alcohol, (2-naphthyl)methanol was also tested as a substrate. (2-Naphthyl)methanol was indeed utilized by suspensions of naphthalene-grown cells of strain NaphS3 in the presence of sulfate (Fig. 4.15). Obviously, the specific alcohol dehydrogenase and aldehyde dehydrogenase are constitutive in strain NaphS3. However, neither 2-naphthaldehyde nor 2-naphthoate accumulated during the incubation, most probably due to the effective oxidation of 2-naphthaldehyde and 2-naphthoate by downward reactions. Thus, a pathway of oxidation of

(2-naphthyl)methanol to 2-naphthaldehyde, which is further oxidized to 2-naphthoate, is proposed (Fig. 4.16).

If an alcohol dehydrogenase and aldehyde dehydrogenase are present, added aldehyde may in principle be also disproportionated. However, the 2-naphthoate/2-naphthaldehyde stoichiometry (nearly 1:1) in Fig. 4.14A indicates that the rapid consumption of the aldehyde was due to net oxidation rather than to disproportionation.
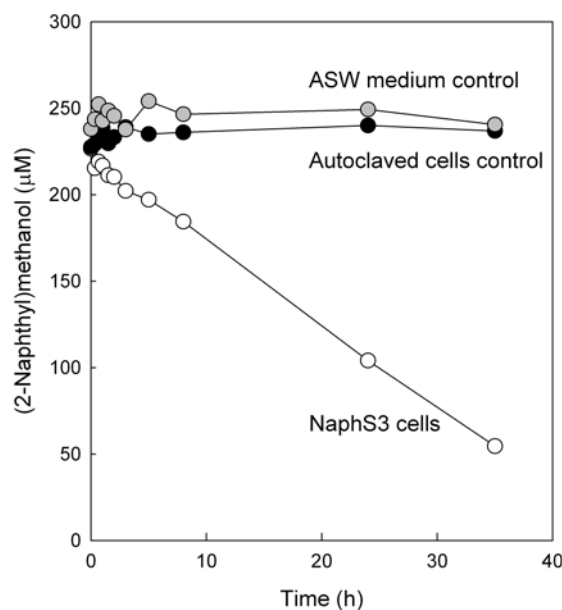

Fig. 4.15. Degradation of (2-naphthyl)methanol by cells of strain NaphS3 (○) under sulfate-reducing conditions; (2-naphthyl)methanol was not degraded in un-inoculated ASW medium (◐) or in control incubations with autoclaved cells (●).
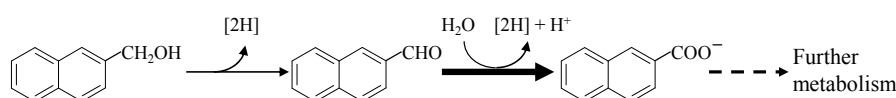

Fig. 4.16. Proposed pathway of oxidation of (2-naphthyl)methanol to 2-naphthoate via 2-naphthaldehyde as an intermediate. Thickness of arrows relatively represents the rate of metabolism. Oxidation of 2-naphthaldehyde to 2-naphthoate was much faster in comparison with oxidation of (2-naphthyl)methanol to 2-naphthaldehyde, suggested by the lack of detection of 2-naphthaldehyde during incubations with (2-naphthyl)methanol.

These findings expand our knowledge on the substrate spectrum of the strain NaphS3. 2-Naphthoate might be an important central intermediate in the metabolism of bicyclic aromatic compounds, similar with the role of benzoate in the metabolism of mono-aromatic compounds. Even though experiments were conducted only with strain NaphS3, similar metabolic behavior was expected for the other two marine strains NaphS2 and NaphS6, based on their close phylogenetic and physiological relationship with strain NaphS3.

4.2 Benzene

A benzene-degrading sulfate-reducing enrichment culture was obtained from marine sediment of a Mediterranean lagoon, Etang de Berre, France. Molecular sequence analyses and probing showed that the culture was dominated (over 85%) by a particular type of sulfate-reducing *Deltaproteobacteria*, closely related to other aromatic hydrocarbon-degrading members (Musat and Widdel, 2008). Lack of immediate responses to phenol and toluene could principally exclude hydroxylation and methylation as possible mechanisms for anaerobic benzene activation by this enrichment culture (Musat and Widdel, 2008). After enrichment, attempts to purify the benzene-degrader via series agar dilution method (Widdel and Bak, 1992) had been carried out and a more purified enrichment culture, termed culture BzS12, was obtained. In the present work, culture BzS12 was used to carry out further investigations on anaerobic degradation of benzene.

4.2.1 Characterization of further purified benzene-degrading sulfate-reducing culture BzS12

The highly enriched culture BzS12 was characterized through hybridization with Cy3-labeled 16S rRNA-targeted oligonucleotide probe BZN649 reported in a previous study (Musat and Widdel, 2008) to quantify the abundance of the dominant phylotype. Phase contrast microscopy revealed that oval to elongated cells were dominant. In addition, morphologically distinct thin spirochaete-like cells could be observed as well at lower numbers (Fig. 4.17A). The dominant, oval cell type could be targeted by the specific probe BZN649, indicating that the dominant phylotype was the same as that in the previous enrichment culture (Fig. 4.17B, C). The percentage of the dominant phylotype was increased from 85% to around 98%, calculated via FISH-based cell counting.
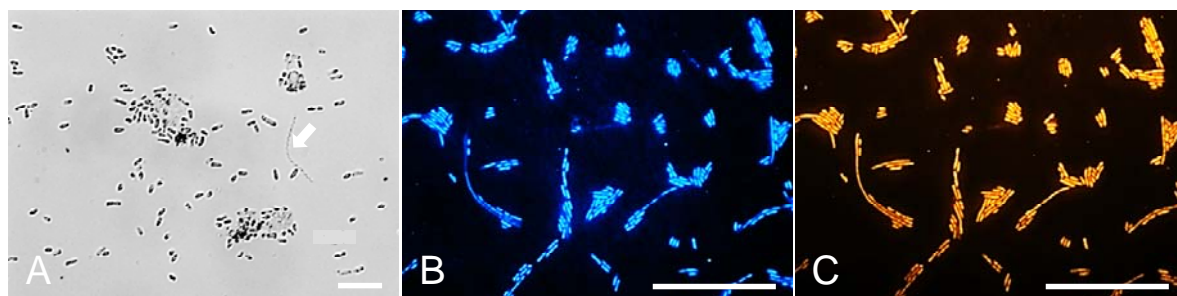

Fig. 4.17. Phase contrast microscopic image of the highly enriched benzene-degrading culture BzS12. In addition to the dominant cell type, only a single, spirochaete-contaminant can be seen (arrow; A); DAPI staining signal (blue; B) and specific probe BZN649 hybridization signal (orange; C) obtained by FISH, showing very low number of contaminant cells. Scale bars = 10 μm.

4.2.2 Differential growth of culture BzS12 with toluene and benzene and further substrate tests

The further-purified culture BzS12 still possesses the original metabolic capability to completely degrade benzene with sulfate as terminal electron acceptor (Fig. 4.18). In addition, after around three weeks of incubation, the benzene-grown cultures began to utilize also toluene as a substrate (Fig. 4.18). Still, growth of culture BzS12 with toluene was much slower in comparison with growth on benzene, or with growth of sulfate-reducing strain Tol2 that had been directly isolated with toluene (Rabus *et al*., 1993).
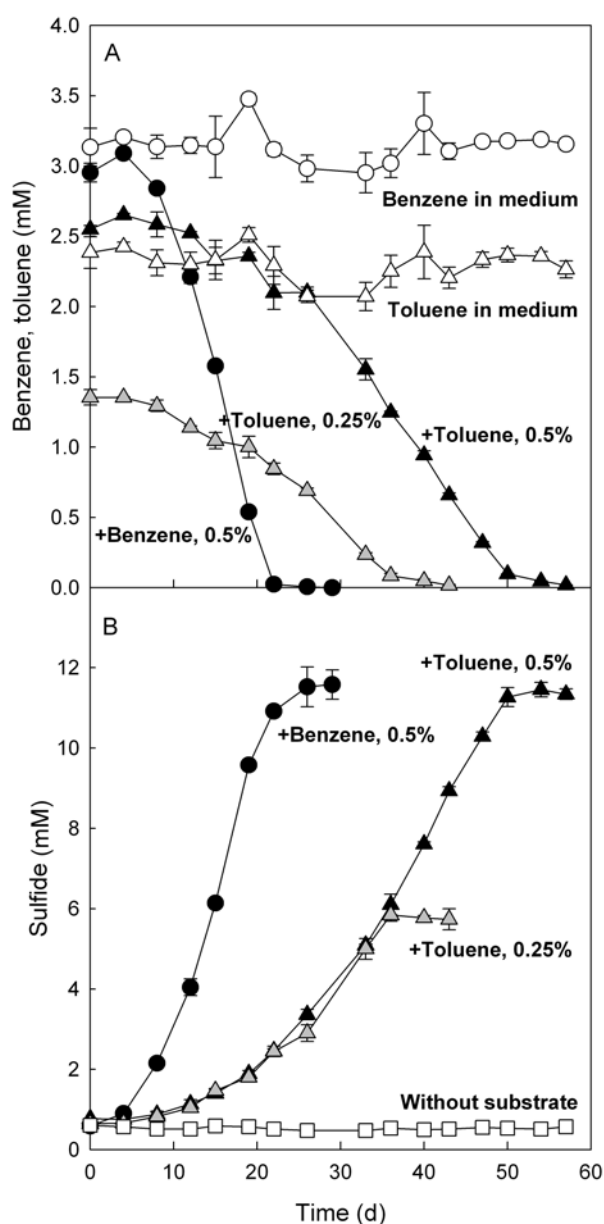

Fig. 4.18. Time course of the concentrations (A) and corresponding sulfide production (B) during incubation of benzene-adapted enrichment culture BzS12 with benzene (●) or toluene (▲, △) as substrates under sulfate-reducing conditions. The cultural volume was 110 ml. Benzene and toluene were added from HMN stock with the concentration of 0.5% or 0.25% (v/v); for each incubation, the volume of added HMN stock was 6 ml. Toluene degradation started after a long adaptation time (around 20 days) and was much slower compared with benzene degradation, which was reflected also by the profile of sulfide production. In abiotic controls, with benzene (○) or toluene (△) in ASW medium without inoculums, there was no significant decrease during the incubation time. Inoculated cultures without substrate (□) did not produce sulfide. Error bars represent standard deviation which was calculated based on duplicate incubations.

The stoichiometric calculation revealed that almost all the electrons generated from the complete oxidation of benzene or toluene were used for sulfate reduction (Table 4.1). The relatively lower electron recovery in incubations with toluene might be due to the adsorption of hydrocarbons to the butyl rubber stopper during the relatively longer incubation time than with benzene. The presence of long induction time indicated that the capacity of toluene utilization by BzS12 was not originally preserved in benzene-adapted culture, while it could be induced after a long adaptation. According to the previous study, the original benzene-degrading enrichment culture could not immediately response to the addition of toluene (Musat and Widdel, 2008).

Table 4.1 Stoichiometric calculation of electron balance during anaerobic degradation of benzene and toluene by the culture BzS12 under sulfate-reducing conditions

Equation: $C_6H_6 + 3.75\ SO_4^{2-} + 1.5\ H^+ + 3\ H_2O \rightarrow 6\ HCO_3^- + 3.75\ H_2S$	
Benzene added (mmol):	0.34
Benzene degraded (mmol):	0.34
Electrons from benzene oxidation (mmol):	10.28
Sulfide produced (mmol):	1.28
Electrons used for sulfate reduction (mmol):	10.20
Electron recovered ratio (%):	99.3

Equation: $C_6H_5(CH_3) + 4.5\ SO_4^{2-} + 2\ H^+ + 3\ H_2O \rightarrow 7\ HCO_3^- + 4.5\ H_2S$		
	Experiment	
	1	2
Toluene added (mmol):	0.30	0.16
Toluene degraded (mmol):	0.29	0.16
Electrons from toluene oxidation (mmol):	10.57	5.65
Sulfide produced (mmol):	1.23	0.59
Electrons used for sulfate reduction (mmol):	9.81	4.73
Electron recovered ratio (%):	92.8	83.6

Note: benzene or toluene was added from HMN stock with the concentration of 0.5% or 0.25% (v/v); the volume of added HMN stock was 6 ml for each incubation.

The toluene-adapted culture BzS12 was re-examined via FISH. The results showed that the same dominant phylotype could be targeted with the specific probe BZN649, confirming that was the original benzene-degrader rather than an enriched contaminant with a similar cell shape (image not shown). The finding of toluene as a growth substrate opens up the possibility of using toluene as an ideal reference substrate to follow differential proteomic analyses to study proteins specifically involved in anaerobic benzene degradation. Anaerobic toluene degradation has been already studied in much detail on the metabolic, genomic, and proteomic level (reviewed by Heider, 2007).

Consistent with previous studies, neither benzoate nor phenol could be utilized by the culture BzS12. However, as discussed previously (Musat and Widdel, 2008), the lack of the capacity to use added benzoate can not exclude the carboxylation hypothesis, since the cells might lack a transport systems for effective uptake of the polar (charged) benzoate anion (benzoic acid, pK_a = 4.22) or lack enzymes, like CoA ligase or CoA transferase, for further processing benzoate to benzoyl-CoA. In a former study, it was reported that sulfide production started after four days upon benzoate addition, which was interpreted as the selection of benzoate-degrading bacteria in the original enrichment culture (Musat and Widdel, 2008). However, after further purification, the culture BzS12 could not grow with benzoate, which might be due to the removal of the benzoate-degrading community. The formation and accumulation of benzoate was detected in the supernatant of benzene-grown BzS12 culture (Fig. 4.19). It gave a hint that benzoate or benzoyl-CoA (hydrolysis leads to benzoate) might be a possible intermediate in anaerobic benzene degradation.
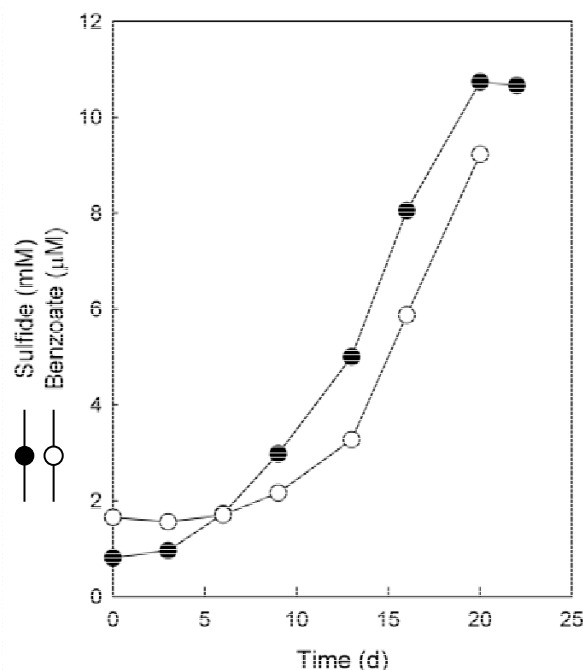
Fig. 4.19. Formation and accumulation of benzoate and sulfide production in culture BzS12 with benzene (5 ml, 0.5% in HMN) as a growth substrate. In controls without benzene there was no benzoate and sulfide production (not shown).

With respect to phenol, the absence of a response can essentially exclude hydroxylation as the benzene activation mechanism. Since phenol has hydrophobic properties under physiological conditions (phenol, pK_a = 10) and can diffuse through cell membranes, added phenol would be accessible to the cells. Additionally, substrate tests showed that apart from benzene and toluene, culture BzS12 can also utilize acetate and pyruvate as growth substrates (data not shown).

4.2.3 Isotope labelling study of metabolites of benzene-grown BzS12

The mechanism of anaerobic activation of benzene is still controversially discussed. Whereas hydroxylation and methylation can be essentially excluded in the presently investigated sulfate-reducing culture (Musat and Widdel, 2008), experiments carried out so far still leave open ring carboxylation as a possible mechanism for anaerobic benzene activation. Carboxylation to benzoate was an appealing hypothesis explaining anaerobic benzene activation (Caldwell and Suflita, 2000; Abu Laban *et al.*, 2010).

To elucidate the activation reaction, culture BzS12 was supplied with $^{13}C_6$- or $^{12}C_6$-benzene, and metabolites of the cultures were extracted and analyzed via GC-MS. Furthermore, to examine the hypothesis of benzene activation via a carboxylation-like reaction, ^{13}C-bicarbonate was added to incubations. In order to limit the metabolic process

and to accumulate detectable metabolites, cultures were grown with lower sulfate concentration (5 mM instead of 28 mM). After the expected sulfide concentration (5 mM) had been reached, metabolites from cultures were extracted with dichloromethane, which were then further concentrated and analyzed by GC-MS.
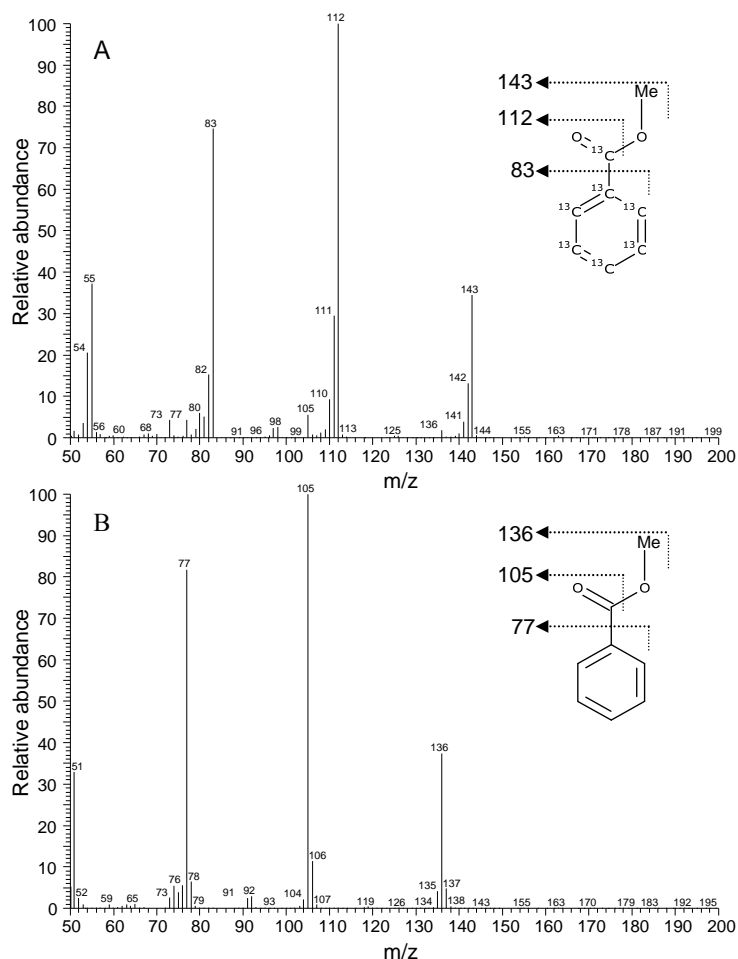

Fig. 4.20. Mass spectra of $^{13}C_7$-benzoic acid methyl ester (A) and unlabeled benzoic acid methyl ester (B) detected as metabolites in anaerobic benzene-degrading culture BzS12. $^{13}C_7$-benzoic acid (A) was detected from culture BzS12 incubated with $^{13}C_6$-benzene as substrate in unlabeled bicarbonate-buffered ASW medium, while unlabeled benzoic acid (B) was detected from culture BzS12 with unlabeled benzene in ^{13}C-labeled bicarbonate-buffered ASW medium.

Benzoate was the prevalent metabolite in all benzene-grown cultures (Fig. 4.20). Detailed examination of the labelling patterns of metabolites showed the carboxyl group in benzoate was derived from the benzene ring itself rather than from bicarbonate. In the incubation of culture BzS12 with $^{13}C_6$-benzene and unlabeled bicarbonate, most of the benzoate was labeled with seven ^{13}C-atoms, viz. labeling included the carboxyl group (m/z 143; Fig. 4.20A). We could also identify benzoate with six ^{13}C-atoms and one ^{12}C-atom (m/z 142), but the location of ^{12}C-atom within the molecule was uncertain. If benzoate would have

an exclusive ^{12}C-atom at its carboxyl group, there would be no or much less signal at *m/z* 82, which represented the aromatic ring with only five ^{13}C-atoms and one ^{12}C-atom. The mass spectra results indicated that the ^{12}C-atom was spread randomly over the molecule rather than restricted in the carboxyl group. The presence of peak at *m/z* 142 was probably due to a contamination of the $^{13}C_6$-benzene with $^{13}C_5$-benzene.

On the other hand, there was almost no incorporation of ^{13}C into the carboxyl group in benzoate when the culture was grown with unlabeled benzene in the presence of ^{13}C-bicarbonate. Most of the benzoate contained seven ^{12}C-atoms (*m/z* 136; Fig. 4.20B). The isotope labelling studies thus indicated that CO_2 or HCO_3^- did not serve as carboxyl group donors to activate benzene, but that rather the carboxyl group was generated from an unknown organic, benzene-derived intermediate. This finding with culture BzS12 is in agreement with other studies of anaerobic benzene degradation by sulfate-reducing enrichment cultures (Caldwell and Suflita, 2000; Phelps *et al.*, 2001), which tentatively excluded anaerobic activation of benzene via a direct carboxylation reaction.

Considering the above observations, the pathway of benzene was reconsidered. One hypothesis was considered within the scope of the present work: Activated acetate or its downstream products such as bound CO or tetrahydrofolate-bound C_1-units may serve as the source of the carboxyl group.

As a first test of this hypothesis, isotope labeled compounds, [1-^{13}C]-acetate or [2-^{13}C]-acetate were added to BzS12 cultures with benzene as a substrate. As mentioned above, culture BzS12 could utilize acetate. However, analysis of labeling patterns in the extracted benzoate did not reveal incorporation of ^{13}C from labeled acetate as a co-substrate (data not shown). As mentioned above, culture BzS12 could utilize acetate. Hence, the labeling experiment carried out here apparently excludes the hypothesis of acetate as the presumptive carboxyl group source.

5. Summary and conclusions

Activation by addition to fumarate, yielding succinate adducts, catalyzed by glycyl radical enzymes, has been described as a common principle to activate alkanes and alkyl-substituted aromatic hydrocarbons by microorganisms under anoxic conditions (reviewed in Widdel and Rabus, 2001; Boll and Heider, 2010). With respect to unsubstituted aromatic hydrocarbons, e.g., naphthalene and benzene, the activation mechanisms are not fully understood. Considering the lack of any functional groups and the high C–H bond dissociation energy (470 kJ mol^{-1}), it is unlikely that naphthalene and benzene are activated via addition to fumarate. Therefore, other, possibly unprecedented mechanisms are expected for the activation of these compounds. In the present thesis the anaerobic degradation of naphthalene and benzene by sulfate-reducing bacteria was studied, focusing on the physiology and their activation mechanisms.

5.1 Naphthalene

Naphthalene-grown cells of strains NaphSx did not immediately respond to the addition of 2-methylnaphthalene, thereby excluding methylation as the initial step to activate naphthalene (Musat *et al*., 2009). Further substrate-response experiments revealed that 2-methylnaphthalene-grown cells required a long adaptation time to start to utilize naphthalene, whereas they responded rapidly to 2-methylnaphthalene. Hence, there was no apparent cross-induction of activating enzymes between both substrates, naphthalene and 2-methylnaphthalene, suggesting separated regulation for the degradation of each substrate. Hence, a proteomic comparison of naphthalene-grown with 2-methylnaphthelene-grown cells may provide insights into naphthalene-specific enzymatic reactions. Comparison with 2-naphthoate-grown cells might be even more straight-forward because proteins from the 'upper pathway' of 2-methylnaphthalene may be absent. Within the scope of this thesis, cells for comparison so far had been grown only with benzoate. Still, separation of proteins by 2D gel electrophoresis and sequence analyses did reveal PPC-like protein synthesized specifically in naphthalene-grown cells.

Incubations with ^{14}C-naphthalene and analyses of the formed products identified 2-naphthoate as the first intermediate in short time incubations. Radiolabeled 2-naphthoate was detectable only upon the addition of unlabeled 2-naphthoate as an intermediate cold trap, implying the faster consumption of 2-naphthoate by subsequent reactions. With the detection of a PPC-like protein as well as the identifications of 2-naphthoate as intermediates, a

carboxylation-like reaction was proposed as the initial step for anaerobic naphthalene activation. In addition to 2-naphthoate, [5,6,7,8]-tetrahydro-2-naphthoate was also identified when samples were extracted with dichloromethane and concentrated. The detection of [5,6,7,8]-tetrahydro-2-naphthoate confirms that the reduction of 2-naphthoate (CoA thioester) by marine strains NaphSx also takes place at the unsubstituted aromatic ring as suggested by studies of freshwater enrichment culture N47 (Eberlein *et al.*, 2013a, b).

Attempts to measure the activities of naphthalene functionalization to 2-naphthoate and its reverse reaction in cell-free extracts were unsuccessful, whereas reference enzymes, e.g., CODH and FDH, were active under the presently established assay conditions. To test whether the failure was due to energy deprivation caused by cell disruption, the uncoupler FCCP was applied in whole-cell assays to study its effect on 2-naphthoate formation. However, instead of inhibiting metabolite formation, the addition of FCCP even slightly stimulated 2-naphthoate formation in whole-cell assays, independent of the presence of sulfate. The stimulatory effect of FCCP was probably due to the inhibition of the energy-requiring further catabolism of 2-naphthoate coupled to sulfate reduction. Similar results were obtained with the application of the ATPase inhibitor DCCD and the ionophore monensin in combination with FCCP. These results suggested an energy-independent formation of 2-naphthoate from naphthalene via a carboxylation-like reaction with the involvement of an unknown precursor that was present in the cells.

The assumed reaction of naphthalene carboxylation catalyzed by cells of strain NaphSx was discovered to be reversible. The first hint of the reversibility of naphthalene carboxylation-like reaction was the carbon isotope exchange reaction between CO_2/HCO_3^- and carboxyl group in 2-naphthoate. In incubations with 2-naphthoate as a substrate in the presence of ^{14}C-bicarbonate in the cultural medium, radiolabeled 2-[carboxyl-^{14}C]-naphthoate could be detected. Moreover, the net production of naphthalene was observed when 2-naphthoate was supplied as the initial substrate, which directly proved the reversibility of the conversion of naphthalene to 2-naphthoate. Carbon isotope exchange and net naphthalene production from 2-naphthoate were only observed under sulfate-free conditions, which prevented the rapid further catabolism of 2-naphthoate.

Carbonylation of naphthalene to 2-naphthaldehyde appeared unlikely as a possible activation mechanism, based on the study employing radiolabeled naphthalene as a tracer. Cells of strains NaphSx could quickly oxidize 2-naphthaldehyde to 2-naphthoate and (2-naphthyl)methanol could be degraded as well by strains NaphSx.

5.2 Benzene

The anaerobic degradation of benzene was studied with a 'pre-purified', highly-enriched benzene-degrading sulfate-reducing culture termed BzS12. In comparison to the preceding state of enrichment (Musat and Widdel, 2008), the abundance of the dominant phylotype had increased from 85% to around 98%, as determined via FISH-based cell counting. After a pronounced adaptation period, culture BzS12 could also grow with toluene as a substrate. During growth on benzene, free benzoate accumulated in the supernatant of the culture. Isotope labeling and metabolite analyses confirmed that the carboxyl group in benzoate was not derived from bicarbonate in the culture medium, but might originate from an unknown benzene-derived intermediate. Acetate was unlikely to serve as the source of the carboxyl group in benzoate.

6. Outlooks

Based on the present research, a carboxylation-like reaction has been suggested as the initial step to activate naphthalene by strains NaphS2, NaphS3, and NaphS6 under sulfate-reducing conditions. However, the detailed mechanism underlying such a carboxylation-like reaction is still unclear, since only partial enzymatic studies were carried out within the scope of this thesis. In principle, there might be particular activating steps or states prior to naphthalene carboxylation to 2-naphthoate.

In order to fully illuminate the initial reaction of anaerobic naphthalene activation, further researches should focus on enzymatic studies. A convenient enzyme assay should be established to measure either the forward or the reverse reaction of naphthalene carboxylation. Considering that naphthalene carboxylation to 2-naphthoate might be a thermodynamically unfavorable reaction, it might be advisable to pay more attention to the reverse reaction, viz. the net production of naphthalene from 2-naphthoate. Possibly functional cofactors like biotin or metal ions should also be given consideration in enzyme assays.

Genome analysis has been proven as a powerful tool in microbiological metabolic studies. The draft genome of the first naphthalene-degrading sulfate-reducing bacterium, strain NaphS2, was reported by DiDonato *et al*. (2010). The study of naphthalene metabolism by strains NaphSx under sulfate-reducing conditions would certainly benefit from further investigations into the genomes and comparison with those of other aromatic hydrocarbon-degrading bacteria as well as transcriptom studies to analyze differential gene expression during anaerobic growth on naphthalene.

Aside from the focus of the initial activation reaction, more detailed metabolite analyses and physiological studies are desirable to fully resolve the whole anaerobic naphthalene degradation pathway. An additional, interesting aspect is the energy metabolism of strains NaphSx. Sulfate-reducing bacteria, which utilize a 'low-potential' electron acceptor, are often confronted with energetic constrains in the environment. Gaining further insights into the energy metabolism of strains NaphSx will help to clarify questions concerning the energetics of the hypothetical functionalization of naphthalene to 2-naphthoate.

With respect to the research of anaerobic degradation of benzene, there is a definite need for obtaining a pure culture based on the current enrichment BzS12 which contains low numbers of spirochaete-like bacteria.

Because toluene could be used as a substrate by culture BzS12 after long-time adaptation, further comparative proteomic analysis with toluene as a reference substrate could be carried out to study proteins specifically involved in benzene degradation.

Similar as in the study of anaerobic naphthalene degradation, enzymatic studies as a direct approach would be most appropriate to unveil the activation reaction of benzene.

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8. Manuscript

Anaerobic interconversion of naphthalene and 2-naphthoate in energy-deprived cells of sulfate-reducing bacteria

Gao Chen, Friedrich Widdel, Florin Musat*

Department of Microbiology, Max Planck Institute for Marine Microbiology, Celsiusstraße 1, D-28359 Bremen, Germany

Manuscript in preparation

*For correspondence. E-mail fmusat@mpi-bremen.de

Summary

Naphthalene, the simplest representative of unsubstituted polycyclic aromatic hydrocarbons, can be degraded by aerobic and anaerobic bacteria. The marine sulfate-reducing bacteria NaphS2, NaphS3, and NaphS6 could anaerobically oxidize naphthalene to CO_2, a pathway expected to involve 2-naphthoyl-CoA as an intermediate for further processing through dearomatization and complete oxidation to CO_2. Whereas cell suspensions of all strains incubated with naphthalene and sulfate did not reveal apparently detectable 2-naphthoate, omission of sulfate caused accumulation of 2-naphthoate and its production concentration depends on cell density rather than naphthalene concentration in the assays. If the protonophore carbonyl cyanide 4-(trifluoromethoxy)phenylhydrazone (FCCP) was added, which dissipates the membrane potential and depletes ATP, the formation of 2-naphthoate continued to higher concentrations, independent of the presence of sulfate in the assay. This may indicate a limited, carboxyl donor pre-formed in an energy-dependent process. Furthermore, some net naphthalene formation from 2-naphthoate was measured in whole-cell assays as well when sulfate was absent, confirming the reversibility of naphthalene carboxylation. Results obtained support that naphthalene is activated via a carboxylation-like reaction to form 2-naphthoate, which might not directly require ATP input, and such a carboxylation reaction is reversible.

Introduction

Naphthalene ($C_{10}H_8$), the simplest representative of unsubstituted polycyclic aromatic hydrocarbons, is a major component of coal tar, typically amounting to 10% by mass (Wise *et al*., 1988), and it is also an abundant constituent of petroleum (Tissot and Welte, 1984). Besides geochemical sources, living organism such as fungi (Daisy *et al*., 2002), plants (Azuma *et al*., 1996) and some insects (Chen *et al*., 1998) can biologically form naphthalene. Large amounts of naphthalene are additionally generated by the incomplete combustion of industrial, domestic and natural carbon-containing materials (Oanh *et al*., 1999). Naphthalene is used as raw material in the production of phthalic anhydride in industry and also once commonly used (now obsolete) in mothballs in household (Jia and Batterman, 2010).

Biodegradation of naphthalene under oxic conditions has been documented in great detail and the degradation pathway as well as involved genes and enzymes have been well elucidated (Habe and Omori, 2003). Aerobic microorganisms activate naphthalene, using reactive oxygen species generated from molecular oxygen, by a naphthalene dioxygenase,

yielding *cis*-(1*R*,2*S*)-dihydroxy-1,2-dihydronaphthalene (Kauppi *et al.*, 1998; Kalsson *et al.*, 2003). In the absence of oxygen, in the more recently established research of anaerobic biodegradation of aromatic compounds, employment of naphthalene has also been of heuristic value. It was discovered in microcosm studies that naphthalene could be biologically degraded under nitrate-reducing (Mihelcic and Luthy, 1988), sulfate-reducing (Coates *et al.*, 1997; Bedessem *et al.*, 1997), and methanogenic conditions (Chang *et al.*, 2006). Pure or highly enriched cultures have been isolated which could degrade naphthalene anaerobically coupled with nitrate, iron, or sulfate reduction (Galushko *et al.*, 1999; Rocken *et al.*, 2000; Meckenstock *et al.*, 2000; Musat *et al.*, 2009; Kleemann and Meckenstock, 2011).

Knowledge of the anaerobic activation and further metabolism of naphthalene still awaits better understanding. Several mechanisms of activation under anoxic conditions have been proposed, including anaerobic hydroxylation to naphthol (Bedessem *et al.*, 1997), methylation to 2-methylnaphthalene (Safinowski and Meckenstock, 2006), and carboxylation to 2-naphthoate (Zhang and Young, 1997). The hypothesis of methylation of naphthalene was once appealing, since all the naphthalene-degrading cultures showed the capacity to utilize 2-methylnaphthalene and the further activation of 2-methylnaphthalene was widely-acknowledged as addition to fumarate, yielding naphthyl-2-methylsuccinate (Safinowski and Meckenstock, 2004). It has become more and more evident that the anaerobic metabolism of naphthalene involves 2-naphthoate or 2-naphthoyl-CoA as an early intermediate, with a CO_2-derived carboxyl group, further metabolized by reductive dearomatization at the unsubstituted ring (Zhang and Young, 1997; Mouttaki *et al.*, 2012; Eberlein *et al.*, 2013a, b). Studies with marine strains NaphS2, NaphS3 and NaphS6, in principle ruled out the hypotheses of hydroxylation and methylation as possible naphthalene activation mechanisms (Musat *et al.*, 2009). More recently, the formation of 2-naphthoate from naphthalene could be measured *in vitro* with a highly enriched culture N47 obtained from freshwater sediments, showing that naphthalene carboxylation did not require addition of ATP in the assay (Mouttaki *et al.*, 2012).

In this study, we employed marine isolates, sulfate-reducing bacteria NaphS2 (Galushko *et al.*, 1999), NaphS3 and NaphS6 (Musat *et al.*, 2009), to study the anaerobic metabolism of naphthalene, focusing on the initial steps. Because 2-naphthoate had been detected as a metabolite in these cultures in previous study (Musat *et al.*, 2009), the formation of 2-naphthoate was investigated in whole-cell assays in a short incubation time and radiolabeled naphthalene was firstly employed as a tracer to study the early intermediates in anaerobic degradation of naphthalene. The dependence of 2-naphthoate formation on cellular

energy was examined by the application of the uncoupler FCCP in whole-cell assays. The reversibility of the hypothetical naphthalene carboxylation reaction was also investigated.

Results

2-Naphthoate formation from naphthalene in whole-cell assays

2-Naphthoate has been consistently detected as a metabolite in naphthalene-grown cultures (Zhang and Young, 1997; Meckenstock *et al*., 2000; Musat *et al*., 2009). However, in assays where whole cells were incubated with naphthalene and sulfate in bicarbonate (30 mM) buffered artificial seawater medium, 2-naphthoate did not accumulate to significant extent. If sulfate was omitted, apparent 2-naphthoate accumulation could be observed in both NaphS3 and NaphS6 whole-cell assays (Fig. 1). Obviously, scavenge of 2-naphthoate (or an intermediate yielding 2-naphthoate in the analytical assay) by subsequent reactions under conditions of sulfate reduction was as fast as 2-naphthoate formation, preventing detectable steady-state concentrations. The fast consumption of 2-naphthoate as an intermediate in anaerobic naphthalene degradation was observed also in assays with radiolabeled [1,4,5,8-^{14}C]-naphthalene, where the formation of [1,4,5,8-^{14}C]-2-naphthoate was detectable only upon addition of unlabeled 2-naphthoate as a cold intermediate trap (Fig. S1). In addition, in experiments with radiolabeled naphthalene, radiolabeled 2-naphthoate was the first detectable intermediate (Fig. S1). The employment of radiolabeled naphthalene as a tracer to investigate the early intermediates of anaerobic naphthalene degradation in dense cell suspensions within short incubation times was used for the first time in the present study. The finding of 2-naphthoate apparently as the earliest intermediate detected is in agreement with the postulate of a carboxylation-like reaction (Zhang and Young, 1997; Mouttaki *et al*., 2012).
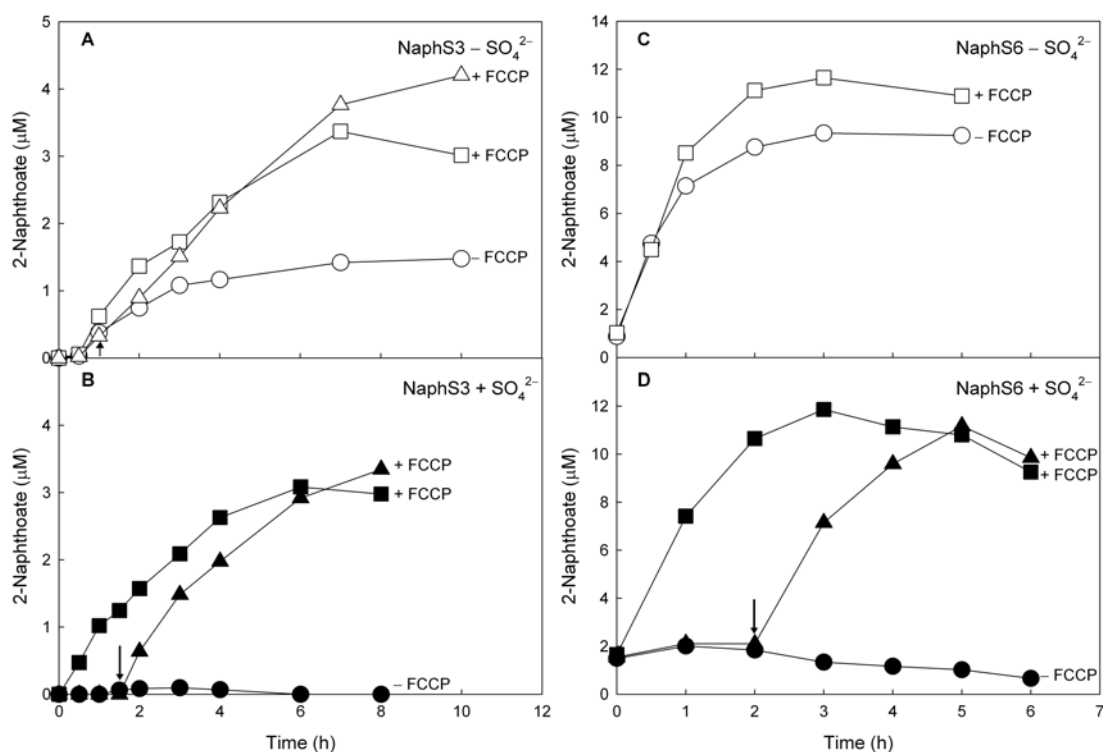
Fig. 1. Time course of 2-naphthoate formation in cell suspensions of strains NaphS3 (A and B; $OD_{600} \approx 2.7$) and NaphS6 (C and D; $OD_{600} \approx 6.4$) incubated with naphthalene as a substrate with (B and D) or without (A and C) sulfate as a terminal electron acceptor. According to the time course, for both strains, 2-napthoate could not accumulate when sulfate present, due to the fast downstream degradation; when sulfate was omitted from medium, significant 2-naphthoate accumulation could be observed. Application of the protonophore FCCP in cell suspensions of both strains NaphS3 and NaphS6 could slightly stimulate 2-naphthoate formation rather than inhibit it.

Effect of the protonophore FCCP on 2-naphthoate formation in whole-cell assays

Attempts to measure the formation of 2-naphthoate from naphthalene in cell-free extracts (*in vitro*) were unsuccessful, whereas the reference enzymes CO dehydrogenase (CODH) and formate dehydrogenase (FDH), could be measured in parallel with relatively high activity (86.7 nkat mg^{-1} protein and 59.4 nkat mg^{-1} protein) for both strains NaphS3 and NaphS6 (only results of NaphS3 were shown in Fig. S2). Such a loss of activity upon cell lysis despite strictly anoxic conditions could be due to, for instance, excessive dilution of essential cofactors, disintegration of enzyme complexes, or deprivation of membrane potential which may have been necessary for 2-naphthoate formation. Therefore, the effect of energy-deprivation on 2-naphthoate formation by addition of FCCP, a weakly proton-binding membrane-permeable compound, dissipating the proton gradient was examined in whole-cell assays.

Rather than inhibiting 2-naphthoate formation, FCCP allowed 2-naphthoate concentration to increase slightly higher than in controls without the protonophore, independently of addition of sulfate (Fig. 1). When sulfate was present, 2-naphthoate did not accumulate; addition of FCCP led to an obvious accumulation of 2-naphthoate. If sulfate was omitted in the assay from the start, slight accumulation of 2-naphthoate could be observed; addition of FCCP led to an even slightly higher accumulation (Fig. 1).

Energy-requiring reactions in the metabolism of naphthalene can be also impaired by omission of the electron acceptor. If sulfate is omitted, i.e. if bacterial anaerobic respiratory chain with APS and sulfite as electron acceptors can not function, a lack of ATP is expected. Additionally, application of certain amount of FCCP (up to 200 μM tested) in whole-cell assays, would collapse the proton gradient across cellular membranes, shutting down the ATP recharging system and subjecting the cells to energy deprivation (Nicholls and Ferguson, 1992). If naphthalene carboxylation would have been energy-dependent under these conditions, FCCP application should show an inhibition effect on 2-naphthoate formation. However, experiments revealed the opposite results. The unexpected stimulating effect of FCCP on 2-naphthoate formation in naphthalene-degrading sulfate-reducing bacteria led to the speculation that naphthalene functionalization to 2-naphthoate itself might be energy-independent, but involves a pre-formed carboxyl donor from an energy-consuming formation of 'activated CO_2'. Further catabolism of 2-naphthoate also requires an energy input. Instead of affecting naphthalene carboxylation, energy deprivation could thus affect further processing of 2-naphthoate.

Effect of the protonophore FCCP on further catabolism of 2-naphthoate

In order to further test the hypothesis that forward catabolism of 2-naphthoate required an energy input, the effect of FCCP addition on 2-naphthoate degradation by cells of NaphS3 and NaphS6 was investigated in whole-cell assays, in which 2-naphthoate was supplied as the initial substrate. Without sulfate, 2-naphthoate degradation was not significant, and FCCP addition did not reveal any difference within a relatively short incubation time (3 h; Fig. 2). However, when sulfate was present, 2-naphthoate consumption by NaphS3 and NaphS6 cells were very fast and FCCP showed a clear inhibition effect (Fig. 2). These results further supported the working hypothesis, that energy deprivation could impede subsequent catabolism of 2-naphthoate.

In the proposed scenario of initial steps involving naphthalene degradation in sulfate-reducing condition (Fig. 5), naphthalene is likely activated by a carboxylation-like reaction to

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form 2-naphthoate, which then undergoes a coenzyme A ligation reaction generating 2-naphthoyl-CoA, a step possibly requires ATP input. Thus, sulfate omission or FCCP addition could certainly affect the energy-demanding 2-naphthoyl-CoA formation, thereby inhibiting further processing of 2-naphthoate in both strains.
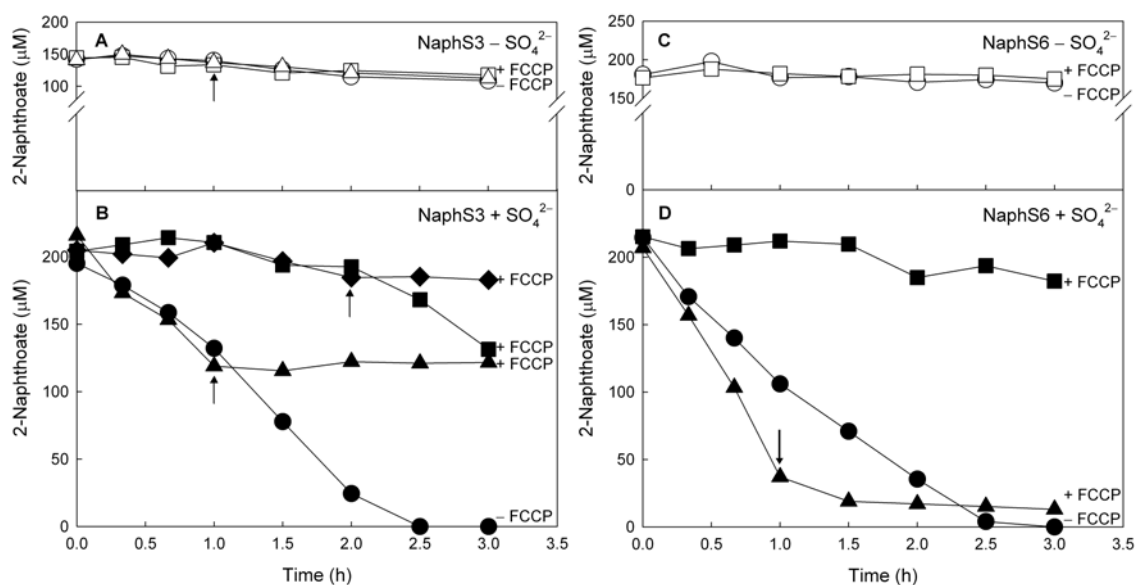

Fig. 2. Effect of FCCP addition on 2-naphthoate degradation in cell suspensions of NaphS3 (A and B; $OD_{600} \approx$ 14.4) and NaphS6 (C and D; $OD_{600} \approx 20$) incubated with 2-naphthoate as a substrate with (B and D) or without (A and C) sulfate as a terminal electron acceptor. When sulfate was present (●), 2-naphthoate could be degraded immediately and addition of FCCP at time zero (■, ◆) or 1 hour (▲) clearly showed inhibition on 2-naphthoate further degradation. The inhibition effect of FCCP lasted for 2 hours and degradation started again afterwards in NaphS3 (B, ■), which might be due to the instability of FCCP in cell suspensions. Reinjection of FCCP at 2 hour (◆) showed a continuous inhibition effect. Without sulfate (○, □, Δ), 2-napthoate could not be degraded within 3 hours and addition of FCCP (□, Δ) could not reveal any difference.

Thermodynamic calculation of naphthalene carboxylation and dependence of 2-naphthoate concentration on cell density in whole-cell assays

According to the results obtained, the direct *in vivo* conversion of naphthalene to 2-naphthoate does not require an energy input, at least up to the observed concentrations. On the other hand, the standard free energy change of direct naphthalene carboxylation is expected to be endergonic, like many other direct carboxylation reactions of non-activated carboxyl acceptors. This urged upon refined calculation of the free energy and equilibrium constant of the reaction. As ΔG_f° data for the 2-naphthoate anion is, to our knowledge, not available in reference tables, it was calculated to be –126.8 kJ mol^{-1} based on the group contribution

method described by Mavrovouniotis (1991). If CO_2 is the reactant in naphthalene carboxylation, then the equation and $\Delta G^{\circ\prime}$ are as follows (for all calculations, see Appendix):

$$C_{10}H_8 \text{ (c)} + CO_2 \text{ (g)} \rightleftarrows C_{10}H_7\text{--}COO^- \text{ (aq)} + H^+ \qquad \Delta G^{\circ\prime} = +26.7 \text{ kJ mol}^{-1} \qquad (1)$$

Or if HCO_3^- is the carboxyl donor, then the equation and ΔG° are:

$$C_{10}H_8 \text{ (c)} + HCO_3^- \text{ (aq)} \rightleftarrows C_{10}H_7\text{--}COO^- \text{ (aq)} + H_2O \qquad \Delta G^{\circ} = +21.9 \text{ kJ mol}^{-1} \qquad (2)$$
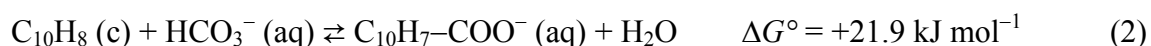
Since the changes of Gibbs energy ($\Delta G^{\circ\prime}$ or ΔG°) of equations (1) and (2) are very close, equation (1) is chosen for simplicity in the further discussion. Accordingly, the equilibrium constant (for pH = 7) is (via $K_{eq} = e^{-\Delta G^{\circ}/(RT)}$)

$$K_{eq} = \frac{\{C_{10}H_7COO^-\}\{H^+\}}{\{C_{10}H_8\}\{CO_2\}} = 2.16 \times 10^{-12} \qquad (3)$$

Hence, in the assay conditions, with pure naphthalene and H_2O, pH = 7, and an equilibrium CO_2 pressure of 0.1 atm, the equilibrium concentration of 2-naphthoate would be around 2.16 µmol l^{-1} (activity coefficients not considered). A similar value of equilibrium concentration of 2-naphthoate can be obtained based on the calculation of equation (2), with bicarbonate as the carboxyl group donor.

However, further experiments showed that the concentration of 2-naphthoate formed was dependent on the cell density in whole-cell assays (Fig. 3) rather than on the equilibrium constant. The final 2-naphthoate concentration in the current whole-cell assays was much higher than the equilibrium concentration, implying the existence of a possible driving force of such an activation reaction, e.g., unknown activated precursors in the cells.
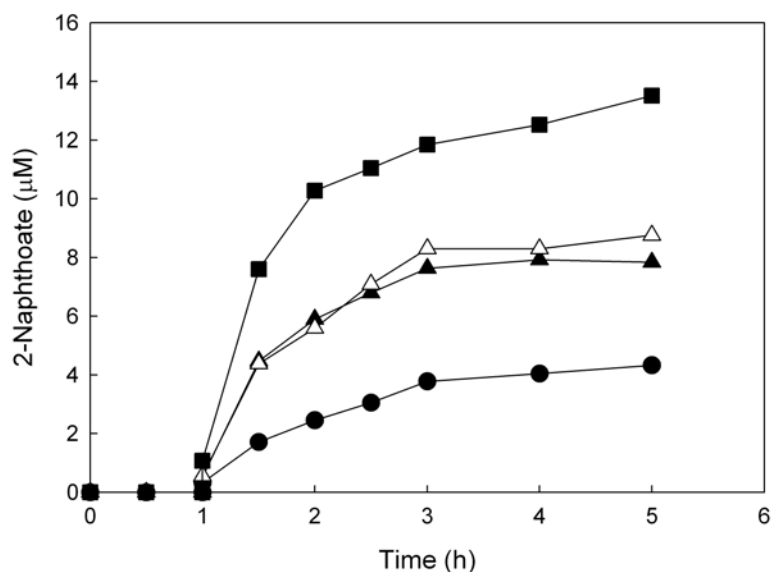
Fig. 3. Time course of 2-naphthoate production in NaphS6 whole-cell assays without sulfate. Initial naphthalene concentrations were around 100 µM, and 100 µl (■), 50 µl (▲) and 20 µl (●) NaphS6 cell suspension stock ($OD_{600} \approx 128$) were added in the assays (volume = 1 ml). Open triangle (Δ) showed assay where naphthalene initial concentration was about 200 µM and 50 µl NaphS6 cell suspensions was added. In all assays, naphthalene was injected after 1 hour incubation, and 2-naphthoate production started immediately after naphthalene addition. According to the time course, the formed 2-naphthoate concentration showed a dependency on the cell density rather than on the naphthalene initial concentration.

Reversibility of naphthalene carboxylation

Most metabolic reactions are reversible, which is more noticeable in energy-limited conditions (Jin and Bethke, 2007). The reversibility has been observed, for instance, in anaerobic methane oxidation (Holler *et al*., 2011), in anaerobic phenol activation (Lack *et al*., 1991), and also in biological Birch-type reduction of benzoyl-CoA (Thiele *et al*., 2008; Kung *et al*., 2010). In view of the indicated equilibrium, changes of Gibbs energy ($\Delta G°'$) of naphthalene carboxylation is in the reversibility range (Bar-Even *et al*., 2012), indicating it should be also possible to convert 2-naphthoate to naphthalene. If cell suspensions of strain NaphS6, which were grown on naphthalene and carefully separated from the growth substrate, were fed with 2-naphthoate as a substrate, net formation of naphthalene could be detected in sulfate-free conditions to prevent fast consumption of 2-naphthoate (Fig. 4). Addition of FCCP could slightly stimulate naphthalene production due to the inhibition of 2-naphthoate forward degradation (Fig. 4).

Reversibility of naphthalene carboxylation was also suggested by the observation of carbon radioisotope exchange reaction between CO_2/HCO_3^- and the carboxyl group of 2-naphthoate. When 2-naphthoate was supplied as a substrate to NaphS6 cells, with the

addition of radiolabeled bicarbonate in sulfate-free artificial seawater medium, due to the isotope exchange reaction the formation of radiolabeled 2-[carboxyl-^{14}C]-naphthoate could be measured (Fig. S3). A similar isotope exchange reaction was also reported in the naphthalene-degrading sulfate-reducing enrichment culture N47, where ^{13}C-bicarbonate was added along with 2-naphthoate as a substrate, and the formation of 2-[carboxyl-^{13}C]-naphthoate could be detected (Mouttaki *et al.*, 2012).
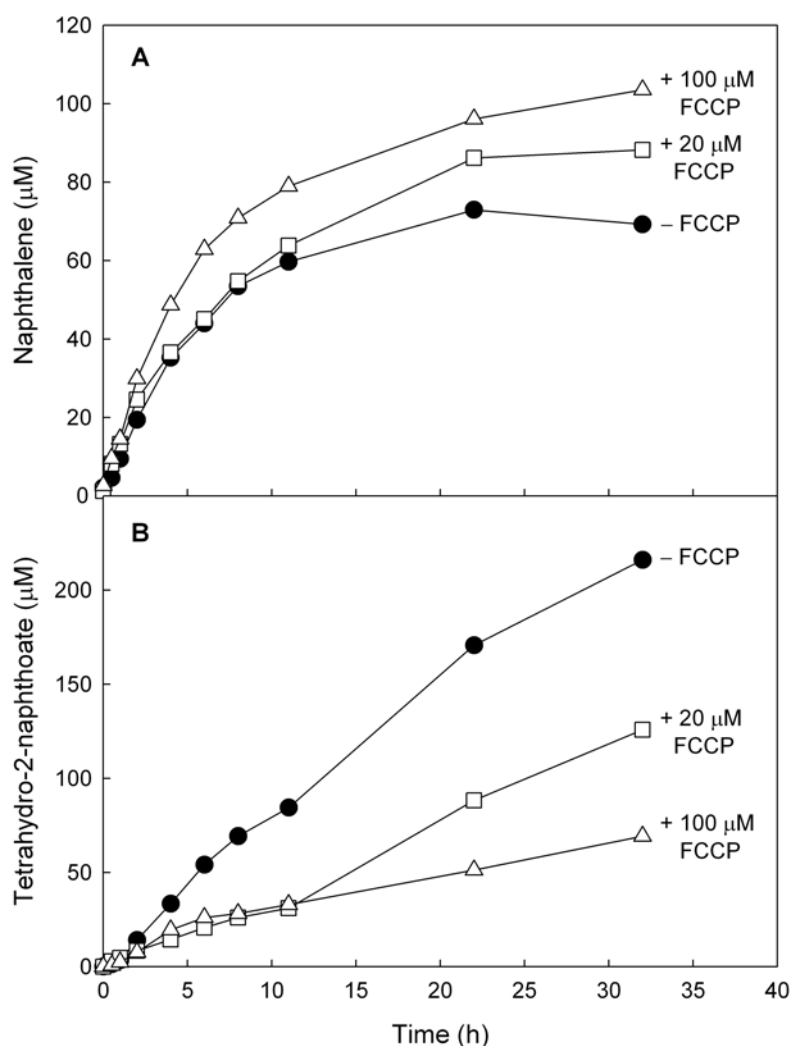

Fig. 4. Time course of naphthalene (A) and tetrahydro-2-naphthoate (B) formation by NaphS6 cell suspensions (OD$_{600}$ ≈ 126) incubated with 2-naphthoate as a substrate in sulfate-free conditions. FCCP could slightly stimulate naphthalene production from 2-naphthoate due to the inhibition of its forward degradation, in agreement with the inhibition effect of FCCP on tetrahydro-2-naphthoate formation (B).

Discussion and conclusions

Strains NaphS2, NaphS3 and NaphS6 are phylogenetically closely related and showed very similar metabolic characteristics (Musat *et al.*, 2009). Although results were selectively

presented above for the sake of simplicity, it was proven all three strains showed the same metabolic patterns. For example, a similar effect of FCCP was also observed in strain NaphS2; the net naphthalene production from 2-naphthoate was measured in strains NaphS2 and NaphS3 as well. The difference in concentration of 2-naphthoate production between whole-cell assays of NaphS3 and NaphS6 (Fig. 1) was explained to be due to a different cell density. It was observed that strain NaphS6 can grow to higher densities than strain NaphS3, thus processing of similar volumes would lead to higher cell densities in assays of strain NaphS6, and the 2-naphthoate concentration was correspondingly much higher than in assays of strain NaphS3 (Fig. 1).
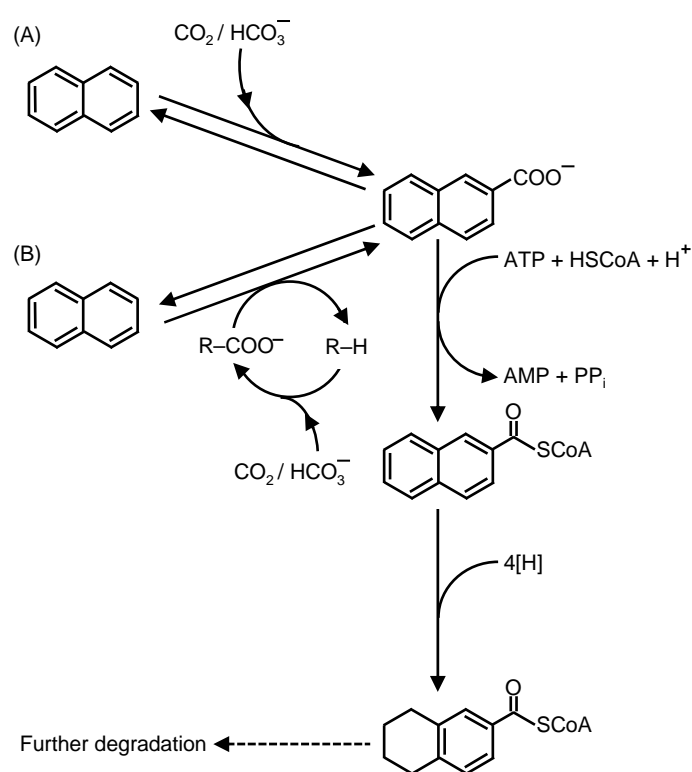
Fig. 5. Present hypotheses of initial steps involved in anaerobic naphthalene degradation. Because of the quite high energetic barrier of aromatic structure in naphthalene, it is difficult to imagine simple carboxylation by CO_2 or HCO_3^- (A). It is possible that CO_2/HCO_3^- is first activated to generate a carboxyl group donor R–COO$^-$ (presently hypothetical; B); then carboxylation occurs afterwards to form 2-naphthoate, which is subsequently subjected to an ATP-dependent CoA-ligation reaction, forming 2-naphthoyl-CoA. 2-Naphthoyl-CoA is further reduced to tetrahydro-2-naphthoyl-CoA, which might be an energy-independent reaction according to recent enzymatic study on freshwater enrichment culture N47 (Eberlein *et al.*, 2013a, b). Afterwards, it can be further completely degraded to CO_2.

Because of the high energetic barrier of the resonance structure, naphthalene is not expected to be directly carboxylated by CO_2 or HCO_3^- (Fig. 5A), as discussed elsewhere (Mouttaki *et al.*, 2012). Although there is no clear evidence to exclude direct carboxylation, it

is speculated that CO_2 or HCO_3^- may be first activated by unknown factor R, which might be a chemical carrier or an enzymatic cofactor (Fig. 5B).

The scenario of naphthalene functionalization was expected to be similar as the anaerobic activation of phenol. It has been proven that phenol was first phosphorylated to phenylphosphate, which was further subjected to carboxylation to form *p*-hydroxybenzoate, then subsequently underwent reductive dehydroxylation to the central intermediate benzoyl-CoA (Lack and Fuchs, 1992; Schmeling *et al*., 2004; Schuhle and Fuchs, 2004). The mechanism is expected to require a phenolate anion as nucleophilic substrate and the electrophilic CO_2 as second substrate, which is analogous with the Kolbe-Schmidt reactions. Phosphorylation prior to carboxylation in anaerobic phenol activation could have kinetic as well as thermodynamic reasons, i.e. speed up the reaction and shift the equilibrium towards *p*-hydroxybenzoate (Boll and Fuchs, 2005).

In case of naphthalene, lacking the strong electron donor hydroxyl group in the ring makes an electrophilic attack by the moderate electrophile CO_2 quite difficult. Therefore it is expected that, similar as in the anaerobic phenol activation, either naphthalene or CO_2 is firstly activated and carboxylation occurs afterwards to channel to intermediate 2-naphthoate or 2-naphthoyl-CoA (Fig. 5B). However, all the attempts to trap an earlier intermediate prior to 2-naphthoate in all three naphthalene-degrading sulfate-reducers as well as to measure the carboxylation-like reaction in cell-free extracts were unsuccessful. The observations of 2-naphthoate as the earliest detectable intermediate, together with the net naphthalene production from 2-naphthoate in whole-cell assays could lead to the speculation of a carboxylation-like reaction as the initial step for anaerobic naphthalene activation.

Whereas the mechanism is currently not fully resolved, the present investigations shed light on the energetics of net carboxylation of aryl species. Most cases of carboxylation reaction are thermodynamically unfavorable processes and require an energy input to drive the formation of new C–C bond (Glueck *et al*., 2010; Bar-Even *et al*., 2012). The most noticeable examples are acetone carboxylase discovered in the aerobic bacterium *Xanthobacter* strain Py2 (Sluis and Ensign, 1997), and the recently revealed acetophenone carboxylase in *Aromatoleum aromaticum* strain EbN1 (Jobst *et al*., 2010), in which carboxylation of acetone and acetophenone are directly coupled with ATP hydrolysis. Our study showed that 2-naphthoate formation in all three strains was independent on the presence of sulfate as the terminal electron acceptor, and the protonophore FCCP could not inhibit 2-naphthoate formation. These results indicated that naphthalene carboxylation to 2-naphthoate by all strains might not directly require ATP input. In view of the changes of

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Gibbs free energy of equations (1) and (2), direct naphthalene carboxylation is still a slightly endergonic reaction, which leaves open the possibilities that naphthalene carboxylation probably couples with a downstream exergonic reaction, or that the fast consumption of 2-naphthoate by subsequent reactions will keep the product in low concentrations, thus facilitating the thermodynamically unfavorable reaction. Because of the energetic constrains, strict anaerobic sulfate-reducing bacteria could not afford high ATP-demanding reactions, thus requiring them to employ alternative strategies to minimize direct energy consumption (Fuchs *et al.*, 2011).

In view of changes of Gibbs energy, carboxylation at an aromatic ring is rather feasible (eq. 1), in contrast to carboxylation of an aliphatic compound or side chain (i.e. of an sp^3-carbon), for instance (calculation in Appendix),

$$C_6H_5–CH_3 \text{ (l)} + CO_2 \text{ (g)} \rightleftarrows C_6H_5–CH_2–COO^- \text{ (aq)} + H^+ \quad \Delta G^{\circ\prime} = +47.8 \text{ kJ mol}^{-1} \quad (4)$$

Rather, eq. (4) could occur in the reverse direction, which was revealed by the observations of toluene production from phenylacetate by *Tolumonas auensis* in anaerobic condition (Fischer-Romero *et al.*, 1996). Another example of reversibility of anaerobic hydrocarbon degradation has recently been unveiled in anaerobic oxidation of methane (AOM), in which labeled methane and sulfate could be detected in different AOM enrichment cultures when labeled bicarbonate and sulfide were present, suggesting the reversibility of the entire AOM pathway (Holler *et al.*, 2011). Considering the free energy change of naphthalene carboxylation in sulfate-reducing bacteria, the reverse reaction of naphthalene degradation, in terms of naphthalene formation from 2-naphthoate could be expected. In fact, the decarboxylation processes are mostly exergonic and thermodynamically favorable reactions, and could possibly associate with energy conservation via coupling with Na^+ translocation across cell membranes (Dimroth and Schink, 1998).

Experimental procedures

Organisms and cultivation

The naphthalene-degrading sulfate-reducing bacterium NaphS2 was isolated from anoxic sediment of a North Sea harbor near Wilhelmshaven (Germany) (Galushko *et al.*, 1999). Strains NaphS3 and NaphS6 were isolated from marine sediment of a Mediterranean lagoon, Etang de Berre (France) (Musat *et al.*, 2009). All strains have been maintained in the laboratory as active cultures by repeated transfers in fresh culture medium, with naphthalene

as a substrate. Medium preparation and anaerobic cultivation techniques were as described elsewhere (Widdel and Bak 1992; Widdel *et al*., 2006). Cultivation for preparation of cell suspensions was routinely done in 2 l Duran bottles with a side arm, containing 1.6 l medium, 200 ml inoculum from a grown culture, and 100 ml 2,2,4,4,6,8,8-heptamethylnonane (HMN) as an inert carrier phase for naphthalene (20 mg ml^{-1}). Growth was monitored by photometric measurements of sulfide concentrations via formation of colloidal CuS (Cord-Ruwisch, 1985). Cultures were harvested under anoxic conditions when sulfide concentrations reached around 10 ~ 15 mM.

Whole-cell incubation assays

Cultures were handled inside an anoxic chamber under a N_2:CO_2 gas mixture (90:10, v/v) or under a continuous stream of N_2. Naphthalene-grown cultures (1.6 l) were transferred to a separatory funnel and separated from the overlying HMN carrier phase. Cells were collected by centrifugation at 9,500 rpm for 25 min, washed with 330 ml artificial seawater medium without sulfate, and then re-suspended in 5 ml of the same medium, which was used as a stock of cell suspensions for assays. The assays were performed in a volume of 1 ml and carried out in serum bottles closed with teflon-lined septa fixed by Al-crimps. FCCP was injected from 1 mM methanol stock solutions to a final concentration of 20 μM (unless otherwise indication). The reaction was started by addition of defined concentrations of naphthalene or 2-naphthoate as substrates. Since naphthalene was poorly soluble in H_2O, naphthalene was added from stock solutions in methanol (20 mM). Aliquots of 50 μl were withdrawn from the assays at different time points with a Hamilton syringe, mixed with 50 μl of 70% ACN and acidified by 1 μl of H_2SO_4 (1 M). The samples were centrifuged and filtered through 0.2 μm nylon filters to remove cells. Samples were analyzed with an UPLC (Acquity H-Class, Waters) to determine the concentrations of naphthalene, 2-naphthoate and tetrahydro-2-naphthoate.

Preparation of cell-free extracts and measurement of CO dehydrogenase and formate dehydrogenase

Volumes of 1.6 l of cultures of strains NaphS2, NaphS3 and NaphS6 grown with naphthalene were harvested anoxically (wet weight around 0.3 g). Cells were washed once with 400 ml of 100 mM anoxic Tris/HCl buffer (pH = 7.25, reduced with 5 mM DTT), and then re-suspended in 1 ml Tris/HCl buffer. Cells were lyzed by vigorous vortexing with fine glass beads (0.1 mm diameter, Roth, Germany) and cell-free extracts were collected after centrifugation at 14,680 rpm for 15 min.

CODH and FDH as marker enzymes were measured photometrically at 578 nm with benzylviologen as an electron acceptor. The reactions were started by the injection of 30 μg protein. Protein concentration was determined using the Bradford assay (Bradford, 1976). CODH and FDH activity was determined to be 86.7 nkat mg^{-1} protein and 59.4 nkat mg^{-1} protein respectively (Fig. S2).

Chemical analyses

Samples were analyzed by an Acquity UPLC H-Class (Waters) equipped with Acquity UPLC BEH shield reverse phase C_{18} column (1.7 μm; 2.1 x 50 mm) and Photo diode array (PDA) detector. The column temperature was set to 30 °C and a gradient of 10% to 70% ACN with 1 mM H_3PO_4 was delivered at a flow rate of 0.6 ml min^{-1} for 5 min. Naphthalene (detected at 210 nm), 2-naphthoate and tetrahydro-2-naphthoate (detected at 230 nm) were identified and quantified by comparison with peaks of authentic standards.

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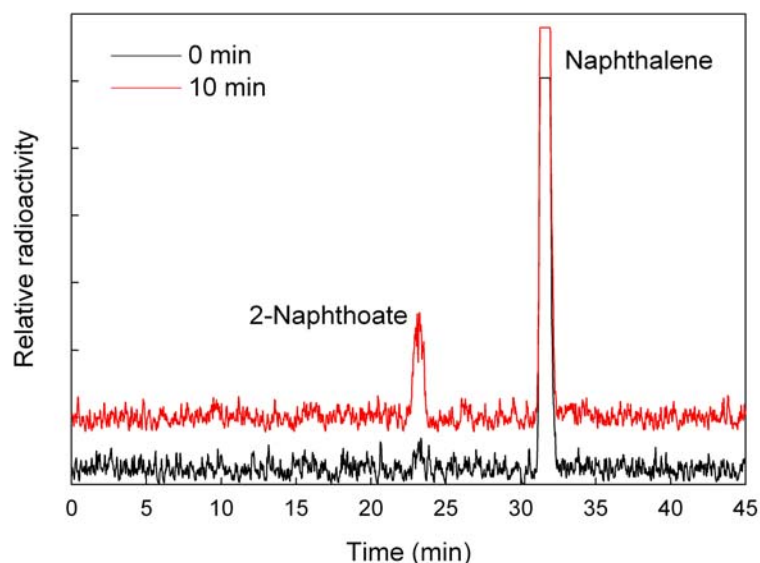
Supporting Information

Fig. S1. Formation of radiolabeled [1,4,5,8-^{14}C]-2-naphthoate from [1,4,5,8-^{14}C]-naphthalene (Hartmann, Germany) (26.67 μM, 14.8 kBq ml^{-1}) by NaphS6 cell suspensions in artificial seawater medium in the presence of 200 μM unlabeled 2-naphthoate as an intermediate trap. Sample was taken at 10 min by acidification with H$_2$SO$_4$ (pH around 3) and analyzed by HPLC (Sykam, Germany) coupled with UV and online radioflow detector (Berthold LB509, Germany). Radioactive peak was identified via the comparison with authentic standards co-eluted in the same condition. Radiolabeled 2-naphthoate could be detected as the first metabolite at the incubation time of 10 min and it was only detectable when unlabeled 2-naphthoate trap present, suggesting the faster downstream catabolism of 2-naphthoate.
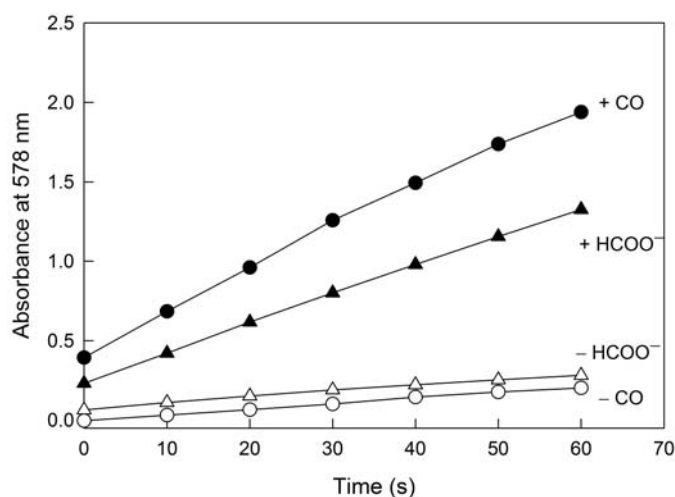
Fig. S2. CODH (●) and FDH (▲) activity of NaphS3 cell-free extracts. Cell-free extracts preparation was described in the experimental procedures. Enzyme assays were done in anoxic 100 mM Tris/HCl buffer (pH = 7.4, reduced with 5 mM DTT), with benzylviologen (5 mM) as artificial enelctron acceptor and reaction was started by injection of 30 μg protein. Reduction of benzylviologen was followed photometrically at 578 nm (ε = 8.65 mM^{-1} cm^{-1}). Open symbols represent controls without substrates.
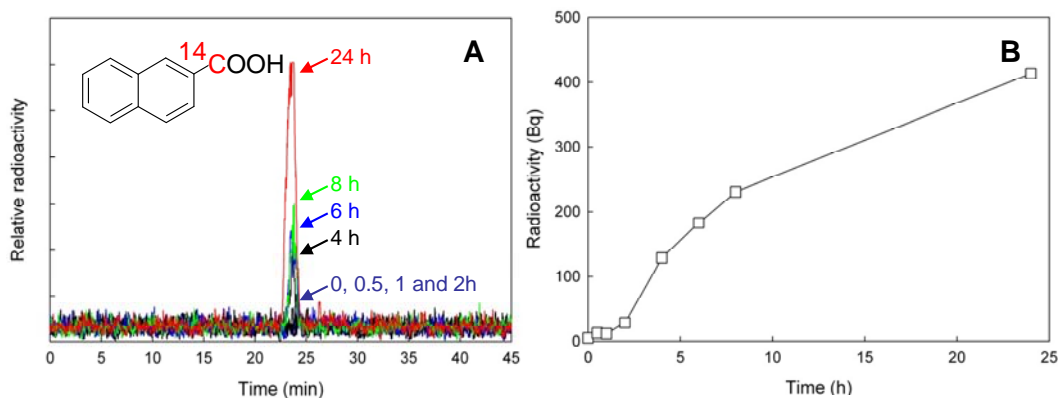
Fig. S3. Formation of radiolabeled 2-[carboxyl-^{14}C]-naphthoate in NaphS6 cell suspensions with unlabeled 2-napthoate (0.4 mM) and $H^{14}CO_3^-$ (20 µM, 42 kBq ml^{-1}) in sulfate-free Tris/HCl (30 mM) buffered artifical seawater medium. Samples were taken at different time points, acified by HCl, and then extracted with dichloromethane, which were then concentrated in H_2O. HPLC coupled with radioflow detector analysis of samples revealed the formation of single product ^{14}C-2-naphthoate after dichloromethane extraction, which was identified with co-elution of authentic standard (A). The formation of radiolabeled ^{14}C-2-naphthoate was due to the carbon isotope exchange between CO_2/HCO_3^- and carboxyl group in 2-naphthoate. Samples were analyzed with scintillation counter as well to quantify exact radioactivity in samples (B). The observation of carbon isotope exchange implied that the reaction of naphthalene functionalization to 2-naphthoate might be reversible.

9. Appendix

9.1 Measurements of CODH and FDH in cell-free extracts
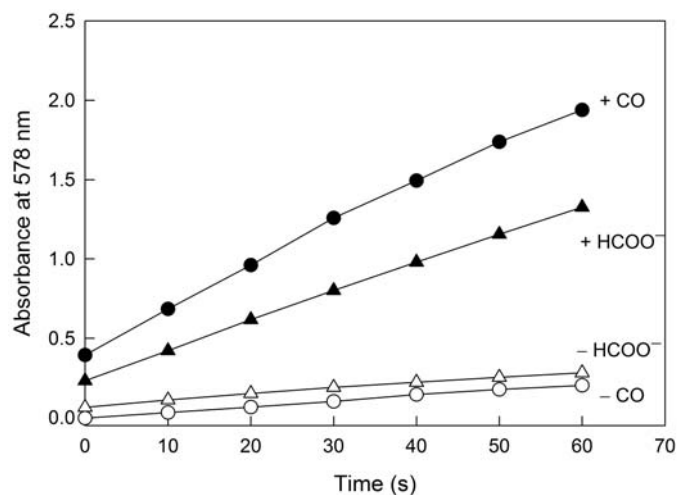
Fig. 9.1 Activity of CODH (●) and FDH (▲) in cell-free extracts of strain NaphS3. Enzyme assays were performed in anoxic 100 mM Tris/HCl buffer in closed cuvettes in a volume of 1 ml, with benzylviologen (5 mM) as an artificial electron acceptor. CO was directly saturated in Tris/HCl buffer, while formate was added at the concentration of 10 mM. Reactions were initiated by injection of 30 μg protein. Reduction of benzylviologen was followed photometrically at 578 nm ($\varepsilon = 8.65$ mM^{-1} cm^{-1}). Open symbols represent controls without CO or formate.

9.2 Thermodynamic calculation

(1). Calculations of Gibbs energy of formation ($\Delta G_f°$) of 2-naphthoate and phenylacetate based on group contribution method described by Mavrovouniotis (1991).
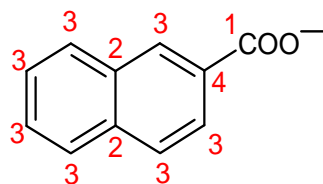
2-Naphthoate

$\Delta G_f° = -126.78$ kJ mol^{-1}
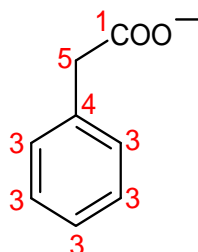
Phenylacetate

$\Delta G_f° = -192.46$ kJ mol^{-1}

Table 9.1. Contribution of groups and correction of standard Gibbs energies of formation[a]

ID	Group or correction	Number per molecule	Contributions (kJ mol^{-1})
0	Origin (a contribution which must be added to every compound)	1	−98.74
1	−COO$^-$	1	−301.25
2	>C= (participating in two fused benzene rings)	2	+10.46
3	−CH= (participating in a benzene ring)	7 (for 2-naphthoate) 5 (for phenylacetate)	+35.15
4	>C= (the formal double bond and a formal single bond participating in a benzene ring)	1	+6.28
5	−CH$_2$− (participating in a non-benzene ring)	1	+25.52

[a] Values given in kcal (Mavrovouniotis, 1991) were transformed to values in kJ (1 cal = 4.184 J)

(2). Calculation of the Gibbs energy ($\Delta G°$ or $\Delta G°'$) of naphthalene and toluene carboxylation

Table 9.2. Data and sources used for calculation

Substance	State	$\Delta G_f°$ (kJ mol^{-1})	Reference
$C_{10}H_8$	c	+201.00	Dean, 2004
$C_6H_5–CH_3$	l	+114.22	Thauer *et al*., 1977
CO_2	g	–394.359	Thauer *et al*., 1977
HCO_3^-	aq	–586.85	Thauer *et al*., 1977
H^+	aq, pH 7	–39.87	Thauer *et al*., 1977
H_2O	l	–237.178	Thauer *et al*., 1977
$C_{10}H_7–COO^-$	aq	–126.78	Group contribution, this study
$C_6H_5–CH_2–COO^-$	aq	–192.46	Group contribution, this study

$C_{10}H_8$ (c) + CO_2 (g) $\rightleftarrows$ $C_{10}H_7COO^-$ (aq) + H^+ (1)
$\Delta G°' = +26.71$ kJ mol^{-1}

$C_{10}H_8$ (c) + HCO_3^- (aq) $\rightleftarrows$ $C_{10}H_7COO^-$ (aq) + H_2O (2)
$\Delta G° = +21.89$ kJ mol^{-1}

$C_6H_5–CH_3$ (l) + CO_2 (g) $\rightleftarrows$ $C_6H_5–CH_2–COO^-$ (aq) + H^+ (3)
$\Delta G°' = +47.81$ kJ mol^{-1}
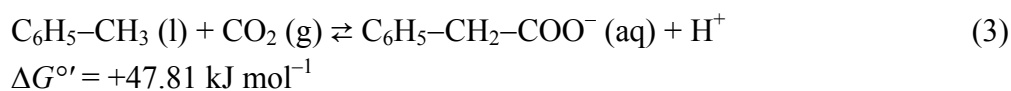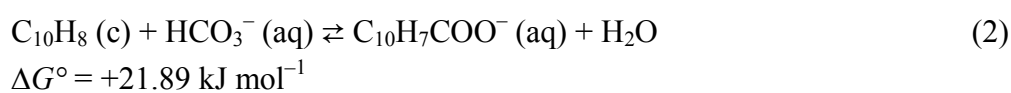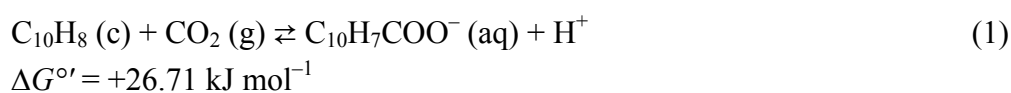
(3). Calculation of theoretical equilibrium concentration of 2-naphthoate formed according to eq. 1

The free energy for any concentrations or pressures (more precisely: activities, fugacities) is

$$\Delta G = \Delta G° + R\,T \ln \frac{\{C_{10}H_7COO^-\}\,\{H^+\}}{\{C_{10}H_8\}\,\{CO_2\}} \tag{4}$$

The standard Gibbs free energy change ($\Delta G°$, pH = 0) of eq. 1 is

$$\Delta G° = 66.58 \text{ kJ mol}^{-1} \tag{5}$$

In equilibrium (i.e. a special case of eq. 1), the actual free energy is

$$\Delta G = 0 \tag{6}$$

This converts eq. 4 to

$$0 = \Delta G° + R\,T \ln \left(\frac{\{C_{10}H_7COO^-\}\,\{H^+\}}{\{C_{10}H_8\}\,\{CO_2\}} \right)_{eq} \tag{7}$$

For the sake of shortness, we simply define

$$\left(\frac{\{C_{10}H_7COO^-\}\,\{H^+\}}{\{C_{10}H_8\}\,\{CO_2\}} \right)_{eq} = K_{eq} \tag{8}$$

so that

$$0 = \Delta G° + R\,T \ln K_{eq} \tag{9}$$

$$\ln K_{eq} = -\frac{\Delta G°}{RT} = -26.86 \tag{10}$$

$$K_{eq} = \exp(-\frac{\Delta G°}{RT}) \tag{11}$$

Thus, $K_{eq} = 2.16 \cdot 10^{-12}$,

$$K_{eq} = \frac{\{C_{10}H_7COO^-\}\,\{H^+\}}{\{C_{10}H_8\}\,\{CO_2\}} \tag{12}$$

Therefore, at equilibrium, the theoretical concentration is

$$[C_{10}H_7COO^-]_{eq} = 2.16 \text{ μmol l}^{-1}$$

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Acknowledgements

I would like to thank my former colleagues Alexander Galushko, Ulrike Jaekel, Johannes Zedelius, Frauke Lüddeke, and all the technicians in the department. Without them, nothing would have gone so smoothly.

Last but not least, many thanks go to my dear wife Ling and my family for their continuous support of my personal life and my academic studies.

Erklärung

Name: Gao Chen   Bremen, den 13.03.2014

Anschrift: Leobener Str. 4, App. 11-03, 28359 Bremen

Erklärung gemäß § 6 Abs. 5 der Promotionsordnung der Universität Bremen für die mathematischen, natur- und ingenieurwissenschaftlichen Fachbereiche

Hiermit erkläre ich, dass ich die Arbeit mit dem Titel:

"Physiology of the anaerobic degradation of naphthalene and benzene by marine sulfate-reducing bacteria"

selbstständig verfasst und geschrieben habe und außer den angegebenen Quellen keine weiteren Hilfsmittel verwendet habe.

Ebenfalls erkläre ich hiermit eidesstattlich, dass es sich bei den von mir abgegebenen Arbeiten um 3 identische Exemplare handelt.

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(Gao Chen)