Mikrobielle Diversität und neuartige Fähigkeiten beim anaeroben Abbau von Kohlenwasserstoffen

DISSERTATION

zur

Erlangung des Grades eines

Doktors der Naturwissenschaften

- Dr. rer. nat. -

dem Fachbereich Biologie/Chemie der

Universität Bremen

vorgelegt von

Karsten Zengler

aus Bad Harzburg

März 1999

Die vorliegende Doktorarbeit wurde in der Zeit von Mai 1996 bis Jannuar 1999 am Max-Planck Institut für marine Mikrobiologie in Bremen angefertigt.

1. Gutachter: Prof. Dr. Friedrich Widdel

2. Gutachter: Prof. Dr. Ulrich Fischer

Tag des Promotionskolloquiums: 12. Mai 1999

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Teil II: Publikationen

Abkürzungen

ALFA968	Oligonukleotidsonde, spezifisch für Bakterien der Alpha-Untergruppe der Proteobakterien
BET42a	Oligonukleotidsonde, spezifisch für Bakterien der Beta-Untergruppe der Proteobakterien
bp	Basenpaare
Bq	Becquerel
BSA	Rinderserumalbumin
BTEX	Benzol, Toluol, Ethylbenzol, Xylole
C_0-C_3-Alkylbenzole	Benzol und Alkylbenzole mit Seitenketten die 1-3 Kohlenstoffatome besitzen
cm	Zentimeter
CoA	Coenzym A
d	Tag
DAPI	4',6-Diamidino-2-phenylindol
DNA	Desoxyribonukleinsäure
DSMZ	Deutsche Sammlung von Mikroorgansimen und Zellkulturen GmbH
EDTA	Ethylendiamintetraessigsäure
EMBL	European Molecular Biology Laboratory
et al.	et alteri (und andere)
EUB338	Oligonukleotidsonde, spezifisch für Eubakterien
FID	Flammenionisationsdetektor
℃	Grad Celsius
g	Gramm
g	Erdbeschleunigung
G+C	Guanin und Cytosin
GAM42a	Oligonukleotidsonde, spezifisch für Bakterien der Gamma-Untergruppe der Proteobakterien
GC/MS	Gaschromatographie/Massenspektroskopie
h	Stunde
HMN	2,2,4,4,6,8,8-Heptamethylnonan
HPLC	High Performance Liquid Chromatography
IRM	Isotope ratio monitoring
k	Kilo
l	Liter
µ	Mikro
m	milli- oder Meter

M	molar oder Mega
m/z	Masse-Ladungs-Verhältnis
min	Minute
mol	Mol
MPN	most probable number
n	nano
OD	Optische Dichte
p	piko
Pa	Pascal
PAK	Polycyclische aromatische Kohlenwasserstoffe
%	Prozent
‰	Promille
ppm	Teile pro Million
PTFE	Polytetrafluorethylen (Teflon)
rpm	Umdrehungen pro Minute
rRNA	ribosomale Ribonukleinsäure
s(p)p	Spezies
SRB385	Oligonukleotidsonde, spezifisch für Bakterien der Delta-Untergruppe der Proteobakterien
T	Zeit
UV	Ultraviolett
Vis	Visuelles Licht
W	Watt

Zusammenfassung

In der vorliegenden Dissertation wurde die Diversität des anaeroben Kohlenwasserstoffabbaus durch neuartige Mikroorganismen unter verschiedenen Bedingungen für die Energiekonservierung untersucht. In die Untersuchungen wurden sowohl phototrophe als auch chemotrophe Mikroorganismen einbezogen; bei letzteren handelte es sich um nitratreduzierende, sulfatreduzierende und syntroph acetogene Bakterien. Die Fähigkeit dieser Organismen, bestimmte Kohlenwasserstoffe zu verwerten, ihr Vorkommen und ihre Verbreitung an natürlichen Standorten wurden bestimmt. Dadurch wurden neue Erkenntnisse über das Schicksal von Kohlenwasserstoffen, die eine wichtige Fraktion organischer Substanz in Sedimenten darstellen, unter anoxischen Bedingungen gewonnen.

1 Ein phototrophes Bakterium mit der Fähigkeit, den aromatischen Kohlenwasserstoff Toluol unter anoxischen Bedingungen in Gegenwart von CO_2 vollständig in Zellmasse umzuwandeln, wurde aus einer Anreicherungskultur mit Belebtschlamm isoliert. Das Bakterium, Stamm ToP1, wurde aufgrund von 16S rRNA Sequenzanalysen und DNA-DNA Hybridisierungen der Art *Blastochloris sulfoviridis* zugeordnet. Der bekannte Typstamm dieser Art war nicht in der Lage, aromatische Verbindungen und Kohlenwasserstoffe als Wachstumssubstrate zu nutzen. Benzylsuccinat, das erste Intermediat beim anaeroben Abbau von Toluol in chemotrophen Bakterien, wurde im wäßrigen Überstand von mit Toluol gewachsenen Zellen nachgewiesen. In zellfreien Extrakten mit ^{14}C-markiertem Toluol in Abhängigkeit von der Zugabe von Fumarat wurde ebenfalls Benzylsuccinat nachgewiesen. Zählreihen mit Standortproben ergaben einen Anteil von Toluol-verwertenden phototrophen Bakterien von fast 2% an der Gesamtpopulation photoheterotropher Bakterien. Stamm ToP1 ist das erste beschriebene phototrophe Bakterium, das unter anoxischen Bedingungen einen Kohlenwasserstoff verwertet.

2 In marinen Anreicherungen mit Erdöl oder Toluol unter nitratreduzierenden Bedingungen wurde, je nach Herkunft des Inokulums, ein Vorherrschen von Bakterien der α- oder γ-Untergruppe der Proteobakterien nachgewiesen. Die bis dahin bekannten Denitrifizierer, die zum Abbau aromatischer Kohlenwasserstoffe in der Lage waren, stammten alle aus Süßwasserhabitaten und gehörten zur β-Untergruppe der Proteobakterien. Die erhaltenen marinen Anreicherungen nutzten unterschiedliche Kohlenwasserstoffe aus dem Erdöl. Aus einer Anreicherung auf Erdöl mit Nordseesediment und einer Anreicherung auf Toluol mit Sediment aus dem Schwarzen Meer wurden zwei neue Bakterien, Stamm Col2 bzw. Stamm TH1, isoliert und charakterisiert. Beide Stämme waren zum anaeroben Abbau von Toluol mit Nitrat in der Lage. Stamm Col2 gehörte der γ-Untergruppe und Stamm TH1 der α-Untergruppe der Proteobakterien an. Bislang war kein chemotrophes Bakterium aus der α- oder der γ-Untergruppe der Proteobakterien beschrieben worden, das unter anoxischen Bedingungen aromatische Kohlenwasserstoffe abzubauen vermochte. Untersuchungen zur Salzabhängigkeit

des Wachstums zeigten, daß beide Isolate an NaCl-Konzentrationen ähnlich denen im Meerwasser angepaßt waren.

3 Ein neues sulfatreduzierendes Bakterium, Stamm oXyS1, wurde aus einer Anreicherungskultur aus der Wasserphase eines Ölseparators einer Erdölraffinerie isoliert. Das Bakterium baute *o*-Xylol mit Sulfat als Elektronenakzeptor vollständig ab. Neben *o*-Xylol dienten auch *o*-Ethyltoluol, *o*-Methylbenzylalkohol und *o*-Methylbenzoat als Wachstumssubstrate; *meta*- und *para*-substituierte aromatische Verbindungen wurden von Stamm oXyS1 nicht verwertet. Beim Wachstum auf Erdöl wurden spezifisch *ortho*-substituierte Verdingungen aus dem Öl verbraucht. Des weiteren wurden Metabolite nach dem Wachstum von Stamm oXyS1 auf Erdöl nachgewiesen. Bei den identifizierten Metaboliten handelte es sich um Benzoat, 2-Methylbenzoat, 3-Methylbenzoat, 4-Methylbenzoat, 2,3-Dimethylbenzoat, 2,4-Dimethylbenzoat, 2,5-Dimethylbenzoat und 3,5-Dimethylbenzoat, sowie um 2-Ethylbenzoat und 3-Ethylbenzoat. Während des Wachstums von Stamm oXyS1 mit Erdöl wurde eine Fraktionierung der Kohlenstoffisotope des *o*-Xylols und *o*-Ethyltoluols beobachtet. Der sulfatreduzierende Stamm oXyS1 stellt die erste Reinkultur dar, welche anaerob *o*-Xylol abbaut.

4 Der Abbau von langkettigen Alkanen unter methanogen Bedingungen wurde in einer Anreicherungskultur, die aus Grabensediment gewonnen wurde, untersucht. Diese Anreicherung setzte Hexadecan vollständig zu Methan und Kohlendioxid um. Untersuchungen mit ^{13}C-markiertem Hexadecan zeigten, daß das gebildete Methan tatsächlich aus dem Alkan stammte. Die Identifizierung der mikrobiellen Gemeinschaft aufgrund von 16S rRNA Sequenzanalysen ergab verwandtschaftliche Ähnlichkeiten sowohl mit syntrophen Bakterien der δ-Untergruppe der Proteobakterien als auch mit acetoclastischen und CO_2-reduzierenden methanogenen Archaeen. Dieses stimmt mit der Hypothese überein, daß Alkane zunächst von syntrophen Bakterien zu Acetat und Wasserstoff umgewandelt werden, welche dann methanogenen Mikroorganismen als Wachstumssubstrate dienen. Alkane könnten somit zu den Substraten zählen, die durch langsamen Umsatz in tiefen Sedimenten Anteil an der Erhaltung einer anaeroben mikrobiellen Gemeinschaft haben und zur Bildung von Methan in alten Sedimenten, Kohlelagerstätten und Erdölreservoirs beitragen.

5 Aus Süßwasserstandortproben wurden Anreicherungskulturen mit dem polycyclischen aromatischen Kohlenwasserstoff (PAK) Fluoren mit Nitrat und Sulfat als Elektronenakzeptor erhalten. In quantitativen Wachstumsversuchen wurde eine vollständige Oxidation von Fluoren zu CO_2 nachgewiesen. Durch Analysen der wäßrigen Überstände der Anreicherungskulturen mittels HPLC, GC und GC-MS wurden mögliche Intermediate des anaeroben Fluorenabbaus nachgewiesen. Der initiale Schritt beim anaeroben Abbau von Fluoren verläuft wahrscheinlich über eine Oxidation des Fluorens zu 9-Fluorenol und weiter zu 9-Fluorenon. Anschließend wird vermutlich eine weitere Hydroxylgruppe eingefügt, und es entsteht Hydroxy-9-Fluorenon. Die Ringspaltung könnte über eine thiolytische Spaltung des Hydroxy-9-Fluorenons (Position 9,10)

oder über eine in Analogie zum anaeroben Phenolstoffwechsel vorausgehende Carboxylierung und Dehydroxylierung (Position 4 und 1) mit sich anschließender Reduktion des aromatischen Ringsystems erfolgen.

Summary

1 A phototrophic bacterium, strain ToP1, with the capacity for anaerobic toluene utilization in the presence of CO_2 was isolated from activated sludge. Sequence analysis of the 16S rRNA gene and DNA-DNA hybridization indicated an affiliation of strain ToP1 with *Blastochloris sulfoviridis*, a member of the α-subclass of Proteobacteria. However, the type strain of *Blc. sulfoviridis* neither grew on toluene nor on benzoate. Benzylsuccinate, the first intermediate of the anaerobic toluene metabolism in chemotrophic bacteria, was detected in the supernatant of toluene-grown cultures of strain ToP1. Benzylsuccinate was also formed in cell-free extracts incubated with toluene and fumarate. The natural abundance of phototrophic bacteria with the ability to utilize toluene was examined in freshwater habitats. Counting series revealed that up to 2% of the photoheterotrophic population that was cultivable with acetate grew on toluene. Strain ToP1 represents the first phototrophic bacterium with the capacity for anaerobic hydrocarbon utilization.

2 Utilization of crude oil and toluene under nitrate-reducing conditions was investigated in marine enrichment cultures and counting series. So far, anaerobic oxidation of alkylbenzenes with nitrate has been studied only in freshwater environments and all pure cultures of nitrate-reducing bacteria obtained from theses studies affiliate with the β-subclass of Proteobacteria. The marine counting series and enrichment cultures were examined by 16S rRNA-targeted whole-cell hybridization with group-specific fluorescent oligonucleotide probes. Enrichment cultures and counting series from North Sea sediment were always dominated by members of the γ-subclass of Proteobacteria, whereas grown populations of Black Sea origin were dominated by members of the α-subclass of Proteobacteria. Oil analysis after growth of the enrichment cultures revealed consumption of toluene and other hydrocarbons from the crude oil. Two new denitrifying bacteria, strain Col2 and TH1, were isolated and characterized as representatives of the enriched γ- and α-Proteobacteria from North Sea and Black Sea sediment, respectively. Both strains were able to oxidize toluene and exhibited a pronounced requirement for NaCl, in accordance with their marine origin. Strain Col2 and TH1 are the first representatives of the γ- and α-subclass of Proteobacteria which are capable of anaerobic alkylbenzene utilization.

3 A new sulfate-reducing bacterium, strain oXyS1, was isolated from an anaerobic enrichment culture which originated from the water phase of an oil tank. Strain oXyS1 degraded toluene, *o*-xylene, and *o*-ethyltoluene with sulfate as electron acceptor. Complete mineralization of *o*-xylene was demonstrated in quantitative growth experiments. Hydrocarbon utilization was restricted to that of toluene and *o*-dialkylbenzenes. The strain was unable to grow on *m*- and *p*-dialkylbenzenes. When transferred to medium with crude oil, specific depletion of *o*-xylene and toluene was observed. Furthermore, benzoate, 2-methylbenzoate, 3-methylbenzoate, 4-methylbenzoate, 2,3-dimethylbenzoate, 2,4-dimethylbenzoate, 2,5-dimethylbenzoate and 3,5-dimethylbenzoate, as well as 2-ethylbenzoate and 3-ethylbenzoate were identified as metabolites

after growth of strain oXyS1 on crude oil. $^{13}C/^{12}C$-Isotope fractionation was detectable in remaining substrates when strain oXyS1 metabolized *o*-xylene and *o*-ethyltoluene from the oil. Strain oXyS1 represents the first pure culture that is able to oxidize *o*-xylene under strictly anoxic conditions completely to CO_2.

4 Formation of methane from long-chain alkanes was demonstrated in an anaerobic enrichment culture from freshwater sediment. Balance measurements revealed that consumed hexadecane was completely metabolized to methane and CO_2. Studies with ^{13}C labelled hexadecane confirmed that produced methane originated from the alkane. Identification of the microbial community by Sequence analysis of 16S rRNA genes retrieved from the microbial community revealed highest similarity of the enriched microorganisms with syntrophic acetogenic bacteria and with acetoclastic and CO_2-reducing methanogenic Archaea. These findings are in accordance with the hypotheses that alkanes are first metabolized by syntrophic bacteria yielding acetate and hydrogen. Acetate and hydrogen are further metabolized methanogenic Archea. The demonstrated process of alkane degradation may contribute to methane formation in deep, old sediments. Alkanes may be one of the substrates which by slow utilization contribute to the maintenance of microbial communities in deep subsurface environments in the absence of terminal electron acceptors other than CO_2.

5 Enrichment cultures capable of anaerobic degradation of fluorene, a polycyclic aromatic hydrocarbon, were established with nitrate and sulfate as electron acceptors. Complete mineralization of fluorene was demonstrated in quantitative growth experiments. Possible intermediates of anaerobic fluorene degradation were determined by HPLC, GC, and GC-MS analysis of the supernatant of the enrichment cultures. Activation of fluorene might occur by oxidation to 9-fluorenol and further to 9-fluorenone. Addition of a hydroxyl group may lead to hydroxy-9-fluorenone. Further metabolism of this compound may occur via a thiolytic cleavage of the tautomeric diketo form, or by a carboxylation-dehydroxylation mechanism followed by reductive dearomatization, analogous to reactions described for anaerobic phenol degradation.

Teil I: Darstellung der Ergebnisse im Gesamtzusammenhang

A Einleitung

1. Vorkommen und Bedeutung von Kohlenwasserstoffen

Kohlenwasserstoffe stellen eine industriell wichtige und auch in der Natur weit verbreitete Stoffklasse dar. Chemisch werden gesättigte, ungesättigte und aromatische Kohlenwasserstoffe unterschieden. Unter diesen sind die gesättigten (aliphatische und alicylische) Kohlenwasserstoffe am wenigsten reaktionsfähig. Ungesättigte Kohlenwasserstoffe sind nicht so reaktionsträge wie gesättigte Verbindungen und werden chemisch leichter angegriffen. Eine Sonderstellung unter den ungesättigten Kohlenwasserstoffen nehmen die aromatischen Kohlenwasserstoffe ein, die deshalb von den Alkenen unterschieden werden. Aufgrund des delokalisierten π-Elektronensystems ist diese Stoffklasse chemisch recht stabil (Sykes 1988).

Kohlenwasserstoffe kommen in der Umwelt sowohl in lebenden Pflanzen, Tieren und Bakterien als auch in toter, diagenetisch umgewandelter Biomasse in Sedimenten und fossilen Brennstoffen vor.

1.1 Kohlenwasserstoffe in lebenden Organismen

Gesättigte aliphatische Kohlenwasserstoffe (Alkane) sind in allen Böden und Sedimenten enthalten (Eglinton 1968; Giger et al. 1980; Wakeham 1990; Beier et al. 1991; Wakeham 1996). Sie stammen zum einen aus den Wachsschichten höherer Pflanzen, zum anderen werden Alkane von benthischen und planktonischen Organismen gebildet (Blumer et al. 1971). Untersuchungen an *Botryococcus braunii* zeigten, daß unverzweigte Alkane bis zu 32% der Trockenmasse dieser Grünalge ausmachen (Dennis und Kolattukudy 1992). Aufgrund der Art, Zusammensetzung und Verteilung der Alkane in Sedimenten können Rückschlüsse auf den Ursprung der Alkane gezogen werden (Elias et al. 1997; Lichtfouse et al. 1997; Santos Neto et al. 1998). Ungesättigte Kohlenwasserstoffe sind ein wichtiger Bestandteil biologischer Membranen und spielen (z.B. als Terpenoide) außerdem eine Rolle beim Sekundärstoffwechsel der Pflanzen (Zimmer 1997). Neben den ungesättigten Kohlenwasserstoffen sind auch aromatische Kohlenwasserstoffe Produkte des pflanzlichen Sekundärstoffwechsels (Gildemeister und Hoffmann 1960). Der aromatische Kohlenwasserstoff Toluol wird durch anaerobe Transformationsprozesse im Hypolimnion aus der Aminosäure Phenylalanin gebildet (Jüttner und Henatsch 1986). Ein hierfür wahrscheinlich verantwortliches, in Reinkultur isoliertes Bakterium ist ebenfalls zur Bildung von Toluol aus Phenylpyruvat und Phenylacetat in der Lage (Fischer-Romero et al. 1996). Eine weitere Bildungsweise aromatischer Kohlenwasserstoffe durch Bakterien ist die Umformung von Monoterpenen zu *p*-Cymol unter

methanogenen Bedingungen (Hylemon und Harder 1999). Obwohl der Anteil an biogen gebildeten gesättigten und aromatischen Kohlenwasserstoffen gegenüber der Gesamtmenge an biogenen Kohlenwasserstoffen gering ist, finden sich sowohl in tiefen Sedimenten als auch in fossilen Brennstoffen fast ausschließlich gesättigte und aromatische Kohlenwasserstoffe. Das liegt darin begründet, daß während der Diagenese und Katagenese aus ungesättigten Kohlenwasserstoffen vorwiegend chemisch stabilere Verbindungen, nämlich gesättigte und aromatische Kohlenwasserstoffe, gebildet werden (Eglinton 1968; Eglinton 1973).

1.2 Kohlenwasserstoffe in Sedimenten und Erdölen

Die weitaus größte Fraktion an Kohlenwasserstoffen auf der Erde ist diagenetischen Ursprungs und in fossilen Lagerstätten und in Sedimenten verschiedenen Alters verteilt. Von den jährlich durch Photosynthese gebildeten 100 Milliarden Tonnen organischer Substanz werden durchschnittlich zwischen 0,1 und 0,8% in Sedimente eingetragen und festgelegt (Bordenave 1993). Der Anteil an Kohlenwasserstoffen an der gesamten organischen Substanz in Sedimenten ist unbekannt; nach vorsichtigen Schätzungen umfaßt er weltweit etwa 4 Billionen Tonnen (Fukui et al. 1998).

Die geschätzte Menge der fossilen Brennstoffe Erdöl, Kohle und Erdgas beläuft sich weltweit auf 1,77 Billionen Tonnen (Schmidt und Romey 1981; Tissot und Welte 1984). Erdöl besteht durchschnittlich zu 86% aus Kohlenwasserstoffen, wobei Alkane und aromatische Kohlenwasserstoffe im Mittel 57% bzw. 29% ausmachen (Schmidt und Romey 1981; Tissot und Welte 1984). Erdöl ist ein komplexer fossiler Rohstoff, der aus bis zu 600 verschiedenen Einzelkomponenten besteht (Hunt 1979). Das geschätzte Erdöl-Vorkommen auf der Erde beträgt ca. 0,27 Billionen Tonnen (Schmidt und Romey 1981; Tissot und Welte 1984). Legt man den durchschnittlichen Gehalt an Alkanen und aromatischen Kohlenwasserstoffen in Erdölen zugrunde, so entspricht dieses 154,4 Milliarden Tonnen an *n*- und *iso*-Alkanen, sowie 78,3 Milliarden Tonnen an aromatischen Kohlenwasserstoffen. Die verbleibenden 37,3 Milliarden Tonnen entfallen auf hochmolekulare, polyheterocyclische Verbindungen, sogenannte Harze und Asphaltene.

Das Alter der Erdöle ist sehr unterschiedlich; es reicht von 4 Tausend bis zu 600 Millionen Jahren (Schmidt und Romey 1981). Es gibt allerdings Orte, an denen auch heute noch erdölähnliche Stoffe gebildet werden. Ein solcher Ort ist das Guaymas Becken im Golf von Kalifornien. An der Spreitzungszone zwischen der Pazifischen und Nordamerikanischen Platte lagert sich in 2000 Meter Wassertiefe aufgrund der hohen Primärproduktion organischer Detritus auf dem Meeresboden ab (Simoneit und Galimov 1984). Durch hohe Temperaturen infolge hydrothermaler Aktivität und durch hohen Druck kommt es zur Bildung von Kohlenwasserstoffen (Simoneit und Lonsdale 1982; Kawka und Simoneit 1987; Bazylinski et al. 1988).

Der fossile Brennstoff Erdgas besteht hautpsächlich aus Methan, dem weltweit häufigsten Alkan. Die gesamte Erdgas-Menge auf der Erde wird auf etwa 60 Milliarden Tonnen geschätzt. Zusätzlich werden jährlich zwischen 0,4 und 0,64 Milliarden Tonnen des Treibhausgases Methan neu gebildet. Davon sind annähernd zwei Drittel auf die Aktivität methanogener Bakterien zurückzuführen (Whitman et al. 1992). Das restliche Drittel ist thermogenen Ursprungs und entsteht bei der Pyrolyse organischen Materials (Scott et al. 1994; Sackett und Conkright 1997).

In rezenten Sedimenten finden sich Kohlenwasserstoffe unterschiedlichen Ursprungs. Hierbei handelt es sich zum einen um die oben erwähnten biogen gebildeten Kohlenwasserstoffe, zum anderen um Kohlenwasserstoffe fossilen Ursprungs. Letztere werden in erheblichem Umfang durch anthropogenen Einfluß in die Umwelt eingetragen (Vanloocke et al. 1975; US Environmental Protection Agency 1986; Radwan et al. 1995; Swannell et al. 1996; Lee und Page 1997; Al-Hasan et al. 1998). Die monoaromatischen Kohlenwasserstoffe Benzol, Toluol, Ethylbenzol und die Xylole (BTEX) sind dabei von besonderer Bedeutung. Mengenmäßig zählen sie zu den 50 wichtigsten chemischen Verbindungen (Fishbein 1985), sie finden Verwendung als Lösungsmittel und sind Bestandteil zahlreicher chemischer Synthesen (Koch und Wagner 1989). Aufgrund ihres toxischen Potentials (Kalberlah et al. 1995; Sikkema et al. 1995) ist ihr Abbau unter oxischen, als auch unter anoxischen Bedingungen von wachsender Bedeutung. Über das Schicksal dieser Verbindungen in unterschiedlichen Habitaten ist bislang nur wenig bekannt.

2. Übersicht zur mikrobiellen Verwertung von Kohlenwasserstoffen unter anoxischen Bedingungen

Der aerobe Abbau von Kohlenwasserstoffen ist seit vielen Jahren bekannt und wurde in einer Vielzahl von Bakterien eingehend untersucht (Gibson und Subramanian 1984; Cerniglia 1992), wohingegen zum Verbleib und Abbau von Kohlenwasserstoffen unter anoxischen Bedingungen noch viele Fragen ungeklärt sind. Hinweise auf eine Oxidation von Kohlenwasserstoffen durch anaerobe Bakterien gab es bereits in der ersten Hälfte diese Jahrhunderts (Bastin et al. 1926; ZoBell 1946; Rosenfeld 1947). Diese Befunde wurden jedoch lange Zeit in Frage gestellt, da aus den Arbeiten weder Bakterienstämme konserviert wurden noch die Techniken des anaeroben Arbeitens ausgereift waren. Das Interesse am mikrobiellen Abbau von Kohlenwasserstoffen unter anoxischen Bedingungen wuchs, nachdem die unerwünschte Bildung von Schwefelwasserstoff in Anlagen der Erdölförderung wiederholt auf die Aktivität sulfatreduzierender Bakterien zurückgeführt wurde (Shelton und Hunter 1975; Jobson und Westlake 1979; Ganahl und Kleinitz 1983; Cord-Ruwisch et al. 1987). Weiteres Intessse an einem anaeroben Abbau dieser toxischen Substanzklasse (Dean 1978) erwuchs aus der Tatsache, daß zunehmend Böden und Grundwasserleiter durch Kohlenwasserstoffe kontaminiert wurden (US Environmental Protection Agency 1986). Erst in den 80er konnte eine

anaerobe Verwertung von gesättigten (Widdel 1988), ungesättigten (Schink 1985a) und aromatischen Kohlenwasserstoffen (Vogel und Gribic-Galic 1986; Zeyer et al. 1986; Grbic-Galic und Vogel 1987; Kuhn et al. 1988) einwandfrei in Anreicherungskulturen nachgewiesen werden.

2.1 Gesättigte Kohlenwasserstoffe

Denitrifizierende Reinkulturen mit der Fähigkeit zur Verwertung von kurz- bzw. langkettigen Alkanen wurden von Ehrenreich (Ehrenreich 1996) und Behrends et al. (Behrends A, Harder J, Rainey F, Widdel F, unveröffentlicht) beschrieben. Unter sulfatreduzierenden Bedingungen wurde eine Oxidation von Alkanen sowohl in Sedimenten (Coates et al. 1997; Caldwell et al. 1998) als auch in Untersuchungen mit Reinkulturen (Aeckersberg et al. 1991; Ehrenreich 1996; Aeckersberg et al. 1998) gezeigt. Eine Mineralisierung von Pristan, einem verzweigten, gesättigten aliphatischen Kohlenwasserstoff, wurde für eine nitratreduzierende Anreicherungskultur gezeigt (Bregnard et al. 1997). Bislang gibt es in der Literatur jedoch keine Hinweise auf einen anaeroben Abbau von gesättigten alicyclischen Kohlenwasserstoffen wie z.B. Cyclohexan.

2.2 Ungesättigte Kohlenwasserstoffe

Die Verwertung ungesättigter Kohlenwasserstoffe unter Aussschluß molekularen Sauerstoffs wurde zuerst in methanogenen Anreicherungskulturen für das Alken 1-Hexadecen beobachtet (Schink 1985a). Gilewicz et al. zeigten die Oxidation von 1-Heptadecen durch eine marine nitratreduzierende Reinkultur (Gilewicz et al. 1991). Die sulfatreduzierenden Alkan-verwertenden Stämme Hxd1 und Pnd1 sind ebenfalls zur Oxidation von langkettigen Alkenen in der Lage (Aeckersberg et al. 1991; Aeckersberg et al. 1998). Acetylen, ein Kohlenwasserstoff mit einer C-C Dreifachbindung, wird gleichfalls bakteriell abgebaut (Schink 1985b). Ungesättigte alicyclische Verbindungen, wie z.B. Monoterpene, werden von nitrat-reduzierenden Süßwasserisolaten vollständig mineralisiert (Harder und Probian 1995; Foss et al. 1998; Heyen und Harder 1998; Hylemon und Harder 1999). Eine Verwertung ungesättigter nichtaromatischer Kohlenwasserstoffe unter Eisen(III)- oder Mangan(IV)-reduzierenden Bedingungen wurde bislang nicht beschrieben.

2.3 Aromatische Kohlenwasserstoffe

Im Vergleich zu Alkenen, Alkinen und Monoterpenen stellen aromatische Kohlenwasserstoffe aufgrund ihres delokalisierten π-Elektronensystems chemisch stabilere Verbindungen dar (Sykes 1988). Eine Verwertung des monoaromatischen Kohlenwasserstoffs Benzol unter

anoxischen Bedingungen wurde bislang nur in Anreicherungskulturen gezeigt (Vogel und Gribic-Galic 1986; Grbic-Galic und Vogel 1987; Major et al. 1988; Edwards und Grbic-Galic 1992; Lovley et al. 1994; Lovley et al. 1995; Lovley et al. 1996; Phelps et al. 1996; Chen und Taylor 1997; Kazumi et al. 1997; Phelps et al. 1998; Weiner und Lovley 1998a; Weiner und Lovley 1998b). Am besten wurde der anaerobe Abbau von aromatischen Kohlenwasserstoffen im Fall von Toluol untersucht. Reinkulturen von Eisen(III)-reduzierenden (Lovley et al. 1989; Lovley und Lonergan 1990), nitratreduzierenden (Dolfing et al. 1990; Altenschmidt und Fuchs 1991; Evans et al. 1991a; Schocher et al. 1991; Fries et al. 1994; Rabus und Widdel 1995) und sulfatreduzierenden (Rabus et al. 1993; Beller et al. 1996) Bakterien wurden isoliert. Obwohl wiederholt methanogene, Toluol-verwertende Anreicherungskulturen beschrieben wurden (Vogel und Gribic-Galic 1986; Grbic-Galic und Vogel 1987; Edwards und Grbic-Galic 1994; Langenhoff et al. 1996; Wang und Barlaz 1998), wurde bislang keine definierte Co-Kultur von Toluol-verwertenden Bakterien und methanogenen Archaeen erhalten. Auch mit Mangan(VI) als Elektronenakzeptor wurde bisher kein Toluol-oxidierendes Bakterium isoliert (Langenhoff et al. 1997). Des weiteren wurde eine Oxidation der Alkylbenzole Ethylbenzol (Rabus und Widdel 1995; Ball et al. 1996), Propylbenzol (Rabus und Widdel 1995), *m*-Xylol (Dolfing et al. 1990; Fries et al. 1994; Rabus und Widdel 1995; Hess et al. 1997) und *p*-Cymol (Harms et al. 1999) durch nitratreduzierende Bakterien beschrieben. Eine anaerobe Oxidation von *o*-Xylol und *p*-Xylol wurde bislang nicht in Reinkulturen beobachtet. Bei den beschriebenen Reinkulturen handelt es sich bis auf den sulfatreduzierenden Stamm Tol2 (Rabus et al. 1993) um Isolate aus dem Süßwasser. Ein Abbau von aromatischen Kohlenwasserstoffen unter nitratreduzierenden Bedingungen in marinen Habitaten wurde bisher nicht gezeigt.

Hinweise auf eine anaerobe Mineralisierung der polycyclischen aromatischen Kohlenwasserstoffe Naphthalin, Methylnaphthalin, Fluoren, Phenanthren und Fluoranthen stammen aus Inkubationsexperimenten mariner Sedimente mit radioaktiv markierten Substanzen (Coates et al. 1996; Coates et al. 1997; Zhang und Young 1997). Eine Verwertung von polycyclischen aromatischen Kohlenwasserstoffen in Reinkultur wurde bisher nur für Naphthalin (zwei Benzolringe) nachgewiesen. Der marine Stamm NaphS2 oxidiert Naphthalin mit Sulfat als Elektronenakzeptor zu CO_2 (Galushko et al. 1999). Eine vollständige Mineralisierung polycyclischer aromatischer Kohlenwasserstoffe unter strikt anoxischen Bedingungen in Süßwasserhabitaten wurde bisher nicht einwandfrei nachgewiesen.

3. Kenntnisstand zum anaeroben Abbau von Kohlenwasserstoffen und Zielsetzung der Arbeit

In den vergangenen zehn Jahren wurde intensiv am Abbau von aromatischen Kohlenwasserstoffen, insbesondere von Toluol, unter anoxischen Bedingungen in Gegenwart verschiedener Elektronenakzeptoren geforscht. Allerdings waren alle erhaltenen Anreicherungen und Isolate chemotrophe Mikroorganismen. Ein Ziel der vorliegenden Arbeit war es daher, die

Verwertung von aromatischen Kohlenwasserstoffen durch phototrophe anaerobe Bakterien zu zeigen.

Bis auf zwei marine sulfatreduzierende Bakterien (Rabus et al. 1993; Galushko et al. 1999) wurden alle bekannten Bakterien mit der Fähigkeit zum anaeroben Kohlenwasserstoffabbau aus Süßwasserstandorten isoliert. Alle denitrifizierenden Alkylbenzol-verwertenden Isolate gehörten dem *Azoarcus/Thauera* Cluster der β-Untergruppe der Proteobakterien an. Ein weiteres Ziel der Arbeit war es deshalb, nach marinen nitratreduzierenden Bakterien mit der Fähigkeit zum Abbau von Alkylbenzolen zu suchen und festzustellen, ob diese ebenfalls der β-Untergruppe der Proteobakterien angehören.

Der Abbau der monoaromatischen Kohlenwasserstoffe Benzol, Toluol, Ethylbenzol und der Xylole (BTEX) ist aufgrund des toxischen Potentials dieser Verbindungen von besonderer Bedeutung. Bisher existierten keine Reinkulturen anaerober Mikroorganismen mit der Fähigkeit zum Abbau von *ortho*-substituierten Alkylbenzolen. In der vorliegenden Arbeit sollten deshalb anaerobe Bakterien mit dieser Fähigkeit isoliert werden.

Sowohl in tiefen Sedimenten als auch in Erdöllagerstätten besteht der Hauptteil der Fraktion der Kohlenwasserstoffe aus gesättigten Verbindungen. Da in diesen Habitaten außer Kohlendioxid keine weiteren Elektronenakzeptoren zur Verfügung stehen, sollte untersucht werden, ob unter methanogenen Bedingungen ein Abbau von gesättigten Kohlenwasserstoffen möglich ist und welche Mikroorganismen an diesem Prozeß Anteil haben.

Ein Abbau von polycyclischen aromatischen Kohlenwasserstoffen unter strikt anoxischen Bedingungen wurde bislang nur in marinen Habitaten untersucht. Die Umsetzung des polycyclischen aromatischen Kohlenwasserstoffs Fluoren durch eine anaerobe Anreicherungskultur sollte daher für einen Süßwasserstandort gezeigt werden.

B Ergebnisse und Diskussion

1. Photoheterotrophe anaerobe Verwertung von Toluol

Der mikrobielle Abbau von Kohlenwasserstoffen wurde lange Zeit als ein rein aerober Prozeß angesehen. Erst in den letzten zehn Jahren konnte auch eine anaerobe Oxidation von Kohlenwasserstoffen nachgewiesen werden (siehe Übersichtsartikel Heider et al. 1998). Die meisten Untersuchungen zum anaeroben Kohlenwasserstoffabbau wurden an dem aromatischen Kohlenwasserstoff Toluol durchgeführt. Ein Abbau von Toluol unter Ausschluß von molekularem Sauerstoff wurde zuerst unter methanogenen (Grbic-Galic und Vogel 1987) und nitratreduzierenden Bedingungen (Kuhn et al. 1988) gezeigt. Wenig später wurden die ersten Reinkulturen von Eisen(III)-reduzierenden (Lovley et al. 1989), nitratreduzierenden (Dolfing et al. 1990; Altenschmidt und Fuchs 1991; Evans et al. 1991a; Schocher et al. 1991; Fries et al. 1994; Rabus und Widdel 1995) und sulfatreduzierenden (Rabus et al. 1993; Beller et al. 1996) Bakterien isoliert. Eine definierte Co-Kultur von Toluol-verwertenden Bakterien und methanogenen Archaen wurde bislang genauso wenig erhalten wie eine Reinkultur aus einer Anreicherung mit Mangan(IV) als Elektronenakzeptor (Langenhoff et al. 1997). Der einzige anaerobe bakterielle Prozeß, für den eine Verwertung von Kohlenwasserstoffen bislang nicht beschrieben wurde, ist die anoxygene Photosynthese. Zwar sind phototrophe Bakterien in der Lage, polare aromatische Verbindungen wie z.B. Benzoat zu verwerten (Pfennig et al. 1965; Whittle et al. 1976; Harwood und Gibson 1988; Wright und Madigan 1991), die Verwertung eines aromatischen Kohlenwasserstoffes wurde jedoch weder in Anreicherungen noch in Reinkulturen nachgewiesen. In dieser Arbeit sollte daher untersucht werden, ob es photoheterotrophe Bakterien mit der Fähigkeit zur Verwertung aromatischer Kohlenwasserstoffe gibt.

1.1 Isolierung des phototrophen Bakterienstammes ToP1 auf Toluol

Als Inokulum für photoheterotrophe Anreicherungen mit Toluol diente Belebtschlamm (Kläranlage Seehausen, Bremen), da schon in früheren Untersuchungen gezeigt wurde, daß Belebtschlamm eine große Anzahl phototropher Bakterien enthält (Siefert et al. 1978). Um das Wachstum von Sauerstoff bildenden Mikroorganismen, z.B. Cyanobakterien und Algen, zu unterdrücken, wurden alle Anreicherungen durch einen Infrarotfilter hindurch beleuchtet. Dieser Filter ließ nur Licht oberhalb einer Wellenlänge von 750 nm durch. Wurden die anoxischen phototrophen Anreicherungen ohne diesen Filter inkubiert, wurde innerhalb weniger Tage ein Wachstum von Cyanobakterien und Algen beobachtet.

Die nach wiederholter Übertragung erhaltene Toluol-verwertende Anreicherung zeigte eine rotbraune Färbung und bestand hauptsächlich aus zwei Zelltypen mit ovalen bzw. stäbchenförmigen Zellen. In anoxischen Agarverdünnungsreihen mit Toluol wuchsen zwei

unterschiedliche Kolonietypen. Der eine Kolonietyp im Agar bestand aus den ovalen Zellen und war glattrandig und gelb, der andere bestand aus den stäbchenförmigen Zellen und war glattrandig und rot gefärbt. Beide Zelltypen wurden in Reinkultur isoliert. Stamm ToP1 aus der gelben Kolonie war in der Lage, photoheterotroph mit Toluol als einziger organischer Kohlenstoffquelle zu wachsen. Hingegen wuchs Stamm BP1 aus der roten Kolonie nicht mit aromatischen Kohlenwasserstoffen, obwohl er aus einer Agarverdünnungsreihe mit Toluol isoliert wurde. Aromatische Verbindungen wie Benzoat wurden jedoch verwertet.

1.2 Phylogenetische Charakterisierung der Stämme ToP1 und BP1

Das 16S rRNA Gen des Toluol-verwertenden Stammes ToP1 wurde nahezu vollständig (1440 Basenpaare) sequenziert. Der Vergleich der 16S rRNA-Sequenz mit bekannten Sequenzen aus der Datenbank (Maidak et al. 1997) zeigte eine enge Verwandtschaft von Stamm ToP1 zu *Blastochloris sulfoviridis* (früher *Rhodopseudomonas sulfoviridis*) und *Blastochloris viridis* (früher *Rhodopseudomonas viridis*), und zwar mit einer Sequenzähnlichkeit von 99,9 bzw. 98,7%. Aufgrund der großen Sequenzähnlichkeit auf der Ebene des 16S rRNA-Gens wurde eine DNA-DNA Hybridisierung durchgeführt. Diese Methode erlaubt eine noch differenziertere Aussage über die phylogenetischen Verwandschaftsbeziehungen zweier Bakterien. Stamm ToP1 wies eine DNA-DNA-Ähnlichkeit von 78,4% zu *Blc. sulfoviridis* auf. Aufgrund dieser Befunde, des ähnlichen G+C-Gehalts der DNA und der großen morphologischen Ähnlichkeit zu *Blc. sulfoviridis*, wurde Stamm ToP1 als neuer Stamm dieser Art beschrieben.

Von dem Stamm BP1 wurde nur eine Teilsequenz (432 Basenpaare) der 16S rRNA erstellt. Ein Vergleich mit Sequenzen aus der Datenbank ergab eine 100%ige Ähnlichkeit von Stamm BP1 mit dem Typenstamm von *Rhodopseudomonas palustris.*

1.3 Morphologische und physiologische Charakterisierung der Stämme ToP1 und BP1

Zellen von Stamm ToP1 waren 1,3-2 µm lang und 0,7 µm im Durchmesser. Die für *Blc. sulfoviridis* beschriebene knospende Teilung war kaum ausgeprägt. Das in vivo Absorptionsspektrum von Stamm ToP1 zeigte ein kleines Maximum bei 833 nm und ein großes Maximum bei 1017 nm; diese Maxima sind typisch für Bakteriochlorophyll *b*. Stamm ToP1 wuchs in einem Temperaturbereich von 12 bis 36 °C mit einem Optimum bei 34 °C und in einem pH-Bereich von 6,5 bis 8,3 mit einem Optimum um 7,4. Die Verdopplungszeit beim Wachstum mit Toluol lag zwischen 33 und 35 Stunden. Das schnellste Wachstum wurde beobachtet, wenn sich die zur Anzucht verwendeten Röhrchen in einem Abstand von 10 bis 15 cm zu einer 25 W Wolframlampe befanden. Neben Toluol verwertete Stamm ToP1 ebenfalls die aromatischen Verbindungen Benzoat und Cinnamat. Auf Benzylsuccinat, einem Intermediat des anaeroben Toluolabbaus, wurde ein schwaches Wachstum festgestellt. Der Stamm war ebenfalls in der

Lage, photoheterotroph mit Rohöl zu wachsen. Dabei wurde eine spezifische Verwertung von Toluol aus dem Rohöl beobachtet. Des weiteren verwertete Stamm ToP1 eine Reihe aliphatischer Säuren, Sulfid, Thiosulfat und Wasserstoff.

Der Stamm BP1 wurde nicht vollständig charakterisiert. Die Zellen waren keulenförmig, 3 µm lang und besaßen einen Durchmesser von 0,6 µm. Neben aliphatischen Fettsäuren verwertete Stamm BP1 auch Benzoat und 4-Hydroxybenzoat. Benzylsuccinat und Toluol wurden von diesem Stamm nicht verwertet.

Wurden beide Stämme, ToP1 und BP1, zusammen über 15 Generationen mit Toluol als einzigem Wachstumssubstrat inkubiert, so besaß diese Co-Kultur die gleiche Färbung wie die ursprüngliche Anreicherung. Durch Zählen von Einzelkolonien in Agarverdünnungsreihen wurde das Verhältnis von Zellen der Stämme ToP1 und BP1 in der Co-Kultur mit 3:1 bestimmt. Weil Stamm BP1 nicht fähig war mit Toluol zu wachsen, wurde vermutet, daß er wahrscheinlich einen Metaboliten verwertete, welcher von Stamm ToP1 ausgeschieden wurde. Benzylsuccinat kommt hierfür allerdings nicht in Frage, da Stamm BP1 nicht zum Wachstum mit dieser Verbindung in der Lage war.

Die Fähigkeit zur Verwertung von Toluol wurde auch mit Reinkulturen bekannter phototropher Bakterien getestet. Die Typenstämme der Arten *Blastochloris sulfoviridis, Blastochloris viridis,* sowie die Benzoat-verwertenden Arten *Rhodocyclus purpureus, Rhodomicrobium vannielii* und *Rhodopseudomonas palustris* waren nicht in der Lage, photoheterotroph mit Toluol zu wachsen.

1.4 Bestimmung von Enzymaktivitäten des Toluolstoffwechsels von Stamm ToP1

In Kulturüberständen von Stamm ToP1 auf Toluol wurde Benzylsuccinat per HPLC nachgewiesen. In nitratreduzierenden sowie in sulfatreduzierenden Bakterien wird Benzylsuccinat beim initialen Schritt der Toluolaktivierung durch Addition von Fumarat an die Methylgruppe des Toluols gebildet (Evans et al. 1992; Biegert et al. 1996; Beller und Spormann 1997a; Beller und Spormann 1997b; Coschigano et al. 1998; Leuthner et al. 1998; Rabus und Heider 1998). Es erschien daher wahrscheinlich, daß die Aktivierung von Toluol in dem photoheterotrophen Stamm ToP1 über die gleiche Reaktion verläuft. In einem Enzymtest mit zellfreiem Extrakt von Stamm ToP1 und mit ^{14}C-Toluol und Fumarat wurde diese Annahme bestätigt. Die spezifische Aktivität der Benzylsuccinat Synthase im Extrakt war 33,8 $pmol \cdot min^{-1} \cdot mg^{-1}$ (bezogen auf Protein); ohne die Zugabe von Fumarat wurde eine 19-fach niedrigere Aktivität gemessen. Das gebildete Benzylsuccinat wurde mittels Dünnschicht-Chromatographie autoradiographisch nachgewiesen.

1.5 Natürliche Verbreitung von photoheterotrophen Bakterien mit der Fähigkeit zur Verwertung von Toluol

Die natürliche Häufigkeit von photoheterotrophen Bakterien mit der Fähigkeit zur Verwertung von Toluol wurde in flüssigen Verdünnungsreihen aus Standortproben bestimmt. Zum Vergleich wurden weitere Zählungen von photoheterotrophen Bakterien mit Acetat und Benzoat als Wachstumssubstrate durchgeführt. Als Inokulum für diese Untersuchungen dienten Belebtschlamm (Kläranlage Seehausen, Bremen), Sediment aus dem Uferbereich der Weser (Bremen) und Sediment eines eutrophierten Teiches (Bremen). Toluol-verwertende phototrophe Bakterien wurden an allen Standorten nachgewiesen. Der Anteil Toluol-verwertender Bakterien betrug fast 2%, hingegen lag der Anteil an Benzoat-verwertenden Bakterien an der erfaßbaren Gesamtpopulation phototropher Bakterien bei knapp 40%.

2. Neue marine denitrifizierende Bakterien mit der Fähigkeit zum anaeroben Abbau von Kohlenwasserstoffen

Der anaerobe Abbau von Kohlenwasserstoffen unter denitrifizierenden Bedingungen wurde in den letzten Jahren intensiv in Anreicherungskulturen (Zeyer et al. 1986; Kuhn et al. 1988; Major et al. 1988; Mihelcic und Luthy 1988b; Evans et al. 1991b; Hutchins 1991; Arcangeli und Arvin 1995a; Arcangeli und Arvin 1995b; Häner et al. 1995; Jørgensen et al. 1995; Langenhoff et al. 1996; Schmitt et al. 1996; Bregnard et al. 1997; Häner et al. 1997; Rabus et al. 1999) und Reinkulturen (Dolfing et al. 1990; Altenschmidt und Fuchs 1991; Evans et al. 1991a; Schocher et al. 1991; Fries et al. 1994; Su und Kafkewitz 1994; Biegert und Fuchs 1995; Rabus und Widdel 1995; Ball et al. 1996; Ehrenreich 1996; Beller und Spormann 1997a; Hess et al. 1997; Harms et al. 1999) untersucht. All diese Untersuchungen beschränkten sich ausschließlich auf Süßwasserstandorte. Die isolierten Bakterien wurden mit Ausnahme des Hexadecan-oxidierenden Stamms HdN1, welcher zu der γ-Untergruppe der Proteobakterien gehört (Ehrenreich 1996), dem *Azoarcus/Thauera* Cluster der β-Untergruppe der Proteobakterien (Macy et al. 1993; Anders et al. 1995) zugeordnet. Studien in mit Kohlenwasserstoffen kontaminierten Grundwasserleitern zeigten ebenfalls die Dominanz dieser Bakteriengruppe (Hess et al. 1997; Zhou et al. 1997; Zarda et al. 1998). Aus marinen Habitaten wurde bislang nur ein sulfatreduzierendes Bakterium (Rabus et al. 1993) mit Toluol isoliert, welches der δ-Untergruppe der Proteobakterien angehört.

Marine Habitate werden ebenso wie Süßwasserstandorte mit Kohlenwasserstoffen kontaminiert (Sauer 1980; Gundlach et al. 1983). Marine Denitrifizierer mit der Fähigkeit zum Abbau dieser Verbindungen unter anoxischen Bedingungen waren bislang nicht bekannt. Nitrat wird durch Oxidation von Ammonium aus dem Abbau von Proteinen und Nukleinsäuren gebildet (Jørgensen 1983). Nitrat im Meer stammt entweder aus dem Abbau von organischem Material oder gelangt über Zuflüsse ins Meer. Eine bakterielle Nitratreduktion findet hauptsächlich in den obersten Sedimentschichten statt (Sørensen et al. 1979; Thamdrup und Canfield 1996), wenngleich es auch Hinweise auf eine Nitratreduktion innerhalb von Partikeln in der Wassersäule gibt (Michotey und Bonin 1997). Obwohl marine Denitrifizierer schon Mitte der 60er Jahre beschrieben wurden (Rhodes et al. 1963; Barbaree und Payne 1967; Knowles 1982), ist bislang kein marines Bakterium bekannt, das unter denitrifizierenden Bedingungen Kohlenwasserstoffe verwerten kann (Bonin et al. 1987; Gauthier et al. 1992; Rontani et al. 1997). In der vorliegenden Arbeit sollte daher untersucht werden, ob marine nitratreduzierende Bakterien mit Erdöl-Kohlenwasserstoffen wachsen und ob diese Bakterien dem *Azoarcus/Thauera* Cluster der β-Untergruppe der Proteobakterien oder einer anderen Verwandtschaftsgruppe angehören.

2.1 Anzahl und Verbreitung mariner Denitrifizierer

Die Häufigkeit von marinen, nitratreduzierenden Bakterien mit der Fähigkeit zur Verwertung von Toluol wurde in flüssigen Verdünnungsreihen aus marinen Sedimentproben bestimmt. Zum Vergleich wurden weitere Zählungen von marinen, denitrifizierenden Bakterien mit Acetat und Benzoat als Wachstumssubstrate durchgeführt. Als Inokulum dienten Sedimente aus der Nordsee und dem Schwarzen Meer. Toluol verwertende Denitrifizierer wurden in beiden Sedimenten nachgewiesen, obwohl ihr Anteil an der Gesamtpopulation der mit Acetat kultivierbaren Nitratreduzierer in den jeweiligen Sedimenten stark variierte und teils sehr niedrig war. In Zählreihen mit Sediment aus der Nordsee betrug der Anteil der Kohlenwasserstoff verwertenden Bakterien an der kultivierbaren nitratreduzierenden Gesamtpopulation 1,2%, im Sediment aus dem Schwarzen Meer 0,03%.

2.2 Zusammensetzung der Bakterienpopulation in nitratreduzierenden Anreicherungen und Zählreihen mit Kohlenwasserstoffen

Da die meisten Studien im Süßwasser mit Toluol durchgeführt wurden, wurde dieser monoaromatische Kohlenwasserstoff ebenso in den folgenden Untersuchungen eingesetzt. Sedimente von zwei verschiedenen Standorten dienten als Inokulum für denitrifizierende Anreicherungen mit Erdöl bzw. Toluol. Die Sedimente stammten aus der Nordsee und dem Schwarzen Meer. Die Bakterienpopulation der einzelnen Anreicherungskulturen wurde durch in situ Hybridisierung mit Gruppen-spezifischen Oligonukleotidsonden analysiert. In den Anreicherungen aus der Nordsee mit Erdöl dominierten Bakterien der γ-Untergruppe der Proteobakterien. Das gleiche Bild zeigte sich für die jeweiligen Anreicherungskulturen von diesem Standort mit Toluol sowie für anaerobe Zählreihen auf Toluol. In den Anreicherungen aus dem Schwarzen Meer wurden allerdings mit Erdöl überwiegend Bakterien der β-Untergruppe der Proteobakterien nachgewiesen, wohingegen in Anreicherungen mit Toluol von diesem Standort Bakterien der α-Untergruppe der Proteobakterien vorherrschten. In den anaeroben Zählreihen mit Toluol aus dem Schwarzen Meer dominierten ebenfalls Bakterien der α-Untergruppe der Proteobakterien. Somit unterschieden sich die von diesen marinen Standorten erhaltenen denitrifizierenden Anreicherungen deutlich von entsprechenden Anreicherungen von Süßwasserstandorten; von diesen wurden mit aromatischen Kohlenwasserstoffen immer Bakterien der β-Untergruppe der Proteobakterien angereichert (Heider et al. 1999). In allen Anreicherungskulturen mit dem komplexen Substrat Erdöl wurde eine höhere Diversität als in den Anreicherungen auf Toluol festgestellt.

Neben der Populationsanalyse der Anreicherungskulturen mit Erdöl wurde die Zusammensetzung des Erdöls nach dem Wachstum bestimmt. Dadurch sollte das Abbaupotential der angereicherten Bakterienpopulation gegenüber verschiedenen Kohlenwasserstoffen ermittelt werden. Die Anreicherungskultur aus der Nordsee verwertete nur

Toluol aus dem Erdöl, wohingegen die Anreicherung aus dem Schwarzen Meer zusätzlich noch Octan, Nonan, Decan und verschiedene Xylole aus dem Erdöl verwertete.

2.3 Isolierung der nitratreduzierenden Stämme Col2 und TH1

Als Inokulum für anaerobe Agarverdünnungsreihen dienten eine nitratreduzierende Anreicherungskultur mit Nordseesediment auf Erdöl und eine nitratreduzierende Anreicherungskultur mit Sediment aus dem Schwarzen Meer auf Toluol. Das verfestigte anoxische Agarmedium wurde mit Toluol als einziger organischer Kohlenstoffquelle in einer inerten Trägerphase aus 2,2,4,4,6,8,8-Heptamethylnonan überschichtet. Nach wiederholter Übertragung von aus dem Agar isolierten, klar abgegrenzten Kolonien in neue Verdünnungsreihen wurde jeweils eine einzelne Kolonie in Flüssigmedium überführt. Die so erhaltenen Stämme, Col2 aus der Nordsee und TH1 aus dem Schwarzen Meer, wuchsen mit Toluol als einzigem organischen Substrat in Gegenwart von Nitrat.

2.4 Physiologische und phylogenetische Charakterisierung der Stämme Col2 und TH1

Stamm Col2, welcher aus einer Erdöl-Anreicherung mit Nordseesediment isoliert wurde, wuchs in einem Temperaturbereich von 5 bis 40 °C mit einem Optimum um 37 °C und im pH-Bereich von 6,4 bis 9,8 mit einem Optimum bei 7,6. Stamm Col2 wurde aufgrund von Sequenzanalysen der 16S rRNA Gene der γ-Untergruppe der Proteobakterien zugeordnet. Der nächste Verwandte war *Halomonas salina* (Valderrama et al. 1991; Dobson und Franzmann 1996) mit einer Sequenzähnlichkeit von 97,9%. Der G+C Gehalt der DNA von Stamm Col2 wurde mit 68 mol% bestimmt. Die Werte der G+C Gehalte der DNA für sieben verschiedene *Halomonas salina* Stämme sind mit 61 bis 64 mol% beschrieben.

Der aus Sediment vom Schwarzen Meer isolierte Stamm TH1 wuchs in einem Temperaturbereich von 15 bis 30 °C mit einem Optimum um 28 °C und in einem pH-Bereich von 6,4 bis 8,6 mit einem Optimum bei 7,2. Aufgrund von Sequenzanalysen der 16S rRNA Gene wurde Stamm TH1 der α-Untergruppe der Proteobakterien zugeordnet. Die nächsten Verwandten waren *Agrobacterium gelatinovorum* (Rüger und Höfle 1992) und *Roseobacter algicola* (Lafay et al. 1995) mit Sequenzähnlichkeiten von 96,0% bzw. 95,1%. Der G+C Gehalt der DNA von Stamm TH1 betrug 65 mol%. Der Wert des G+C Gehaltes für *Agrobacterium gelatinovorum* wurde mit 59 mol% angegeben; für *Roseobacter algicola* wurde kein G+C Gehalt bestimmt.

2.5 Vergleich des Einflusses von NaCl auf das Wachstum ausgesuchter nitratreduzierender Bakterienstämme mit der Fähigkeit zur anaeroben Kohlenwasserstoffverwertung

Die erwartete Anpassung der aus Meeressediment isolierten Toluol abbauenden Bakterien, Stamm Col2 und TH1, an erhöhte NaCl-Konzentrationen im Vergleich zu Vertretern der β-Untergruppe der Proteobakterien wurde in Wachstumsversuchen gezeigt. Stamm EbN1, ein Ethylbenzol-verwertendes Bakterium (Rabus und Widdel 1995), und *Alcaligenes defragrans* Stamm 54Pin, ein α-Pinen-oxidierendes Bakterium (Foss et al. 1998), wurden als Repräsentanten der β-Untergruppe der Proteobakterien ausgewählt (nicht im Manuskript beschrieben). Obwohl Stamm EbN1 und *Alcaligenes defragrans* Stamm 54Pin noch bei NaCl-Konzentrationen ähnlich wie im Meerwasser wuchsen, lag ihr Optimum bei niedrigeren NaCl-Konzentrationen. Die beiden Neuisolate hingegen zeigten ein optimales Wachstum bei Salzkonzentrationen zwischen 26 und 33 g NaCl/l, was etwa der durchschnittlichen Konzentration im Meerwasser entspricht (Dietrich et al. 1975).

Aufgrund der Ergebnisse wird vermutet, daß in marinen Habitaten hauptsächlich Vertreter der α- und γ-Untergruppe der Proteobakterien für den anaeroben Abbau von Kohlenwasserstoffen mit Nitrat verantwortlich sind. Vertreter der β-Untergruppe der Proteobakterien, welche an vielen Süßwasserstandorten vorherrschend sind, spielen bei diesem Prozeß an marinen Standorten wahrscheinlich nur eine untergeordnete Rolle.

3. Anaerober Abbau von Toluol und *o*-Xylol durch ein neues sulfatreduzierendes Bakterium, Stamm oXyS1

In marinen Sedimenten spielt die Reduktion von Sulfat zu Sulfid eine entscheidende Rolle bei der Mineralisierung organischen Kohlenstoffs. Dabei werden durch sulfatreduzierende Bakterien bis zu über 50% des Kohlenstoffs umgesetzt (Jørgensen 1982). In Erdöllagerstätten, bei der Erdölförderung und während des Transports bzw. der Lagerung von Erdölen kommt es ebenfalls zu einer Bildung von Sulfid durch die Aktivitäten von sulfatreduzierenden Bakterien, die hier allerdings Probleme wie Korrosion und „Souring" verursacht und daher unerwünscht ist (Cord-Ruwisch et al. 1987; Odom 1993). In einer sulfatreduzierenden Anreicherungskultur konnte erstmals gezeigt werden, daß diese Bakterien aliphatische und aromatische Kohlenwasserstoffe aus dem Erdöl zum Wachstum nutzen können und dabei Sulfat zu Sulfid reduzieren (Rueter et al. 1994). Während des anaeroben Wachstums der Anreicherungskultur wurde ein selektiver Abbau von Toluol, *o*-Xylol, *p*-Xylol, *o*-Ethyltoluol, *m*-Ethyltoluol, *m*-Propyltoluol und *m*-Cymol aus dem Erdöl nachgewiesen (Rabus et al. 1996). Der anaerobe Abbau von *o*-Xylol und anderen *ortho*-substituierten Alkylbenzolen war bislang noch nie für Reinkulturen beschrieben wurden, weshalb die Isolierung eines Bakteriums mit dieser katabolischen Fähigkeit von besonderem Interesse war.

3.1 Isolierung des sulfatreduzierenden Stammes oXyS1

Als Inokulum für anaerobe Agarverdünnungsreihen diente eine sulfatreduzierende Anreicherungskultur mit Erdöl als organischer Substratquelle. Das verfestigte Agarmedium wurde mit *o*-Xylol als einzigem organischen Substrat in einer inerten Trägerphase aus HMN überschichtet. Nach wiederholtem Transfer von aus dem Agar isolierten, klar abgegrenzten Kolonien wurde eine einzelne Kolonie in Flüssigmedium überführt. Der so erhaltene sulfatreduzierende Stamm oXyS1 wuchs mit o-Xylol als Elektronendonator und organischer Kohlenstoffquelle mit einer Verdopplungszeit von ungefähr 75 Stunden.

3.2 Phylogenetische Verwandtschaft des Stammes oXyS1

Der Stamm oXyS1 wurde aufgrund von Sequenzanalysen der 16S rRNA Gene als Angehöriger der δ-Untergruppe der Proteobakterien erkannt. Die nächsten Verwandten waren *Desulfobacterium cetonicum,* welcher aus einem Ölfeld isoliert wurde (Galushko und Rozanova 1991), und *Desulfosarcina variabilis* (Widdel und Bak 1992); die Sequenzähnlichkeiten betrugen 98,4% bzw. 98,7%. Der G+C Gehalt der DNA von Stamm oXyS1 wurde mit 51 mol% bestimmt. Die Werte der G+C Gehalte von *Desulfobacterium cetonicum* und *Desulfosarcina variabilis* wurden mit 59 bzw. 51 mol% angegeben (Galushko und Rozanova 1991; Widdel und Bak 1992). Aufgrund der hohen Ähnlichkeiten der 16S rRNA Gene kann

nicht ausgeschlossen werden, daß es sich bei den drei Stämmen um eine Art handelt. Um diese Frage zu klären, müßten weiterführende Untersuchungen, insbesondere eine DNA-DNA Hybridisierung, durchgeführt werden.

3.3 Morphologische und physiologische Charakterisierung von Stamm oXyS1

Die Zellen von Stamm oXyS1 waren stäbchenförmig (0,6-1,0 µm × 1-2 µm), unbeweglich, Gram-negativ und nicht in der Lage, Sporen auszubilden. Obwohl die Zellen während des Wachstums keine Aggregate bildeten, hefteten sie sich dennoch an Flocken von präzipitiertem Eisenmonosulfid im Medium. Der Stamm wuchs in einem Temperaturbereich von 15 °C bis 35 °C mit einem Optimum um 32 °C und in einem pH-Bereich von 6,2 bis 7,9 mit einem Optimum bei 7,5.

Stamm oXyS1 war in der Lage, neben den aromatischen Kohlenwasserstoffen Toluol, *o*-Xylol und *o*-Ethyltoluol auch polare aromatische Verbindungen wie *o*-Methylbenzylalkohol, Benzoat, *o*-Methylbenzoat und Benzylsuccinat zu verwerten. Die Fähigkeit, Benzylsuccinat zu oxidieren, deutet darauf hin, daß die Methylgruppe des Toluols (bzw. des *o*-Xylols) durch eine Addition von Fumarat zu Benzylsuccinat (bzw. zu *o*-Methylbenzylsuccinat) aktiviert wird. Für Toluol wurde diese Art der Aktivierung bereits in nitratreduzierenden und sulfatreduzierenden Bakterien nachgewiesen (Biegert et al. 1996; Beller und Spormann 1997b; Leuthner et al. 1998; Rabus und Heider 1998). Auffällig war, daß Dialkylbenzole nur dann von Stamm oXyS1 abgebaut wurden, wenn einer der beiden Substituenten eine Methylgruppe war und die Substituenten in *ortho*-Position zueinander standen; Dialkylbenzole mit Substituenten in *meta*- oder *para*-Position zueinander wurden nicht verwertet. Während des anaeroben Wachstums von Stamm oXyS1 auf Erdöl wurde ebenfalls eine spezifische Verwertung von *ortho*-substituierten Alkylbenzolen aus dem Erdöl festgestellt (siehe Wilkes et al., II.B.6). Neben Stamm oXyS1 wurde aus der oben erwähnten Erdölanreicherung ein weiteres sulfatreduzierendes Bakterium, Stamm mXyS1, isoliert. Dieser Stamm wuchs mit Toulol, *m*-Xylol, *m*-Ethyltoluol und m-Cymol. Das Verschwinden der Alkylbenzole aus dem Erdöl in der Anreichungskultur (Rabus et al. 1996) ließe sich größtenteils durch die Aktivitäten sulfatreduzierender Bakterien von der Art der isolierten Stämme oXyS1 und mXyS1 erklären.

Aufgrund der engen Verwandtschaft von *Desulfobacterium cetonicum* und *Desulfosarcina variabilis* mit Stamm oXyS1 wurden auch diese Arten auf die Fähigkeit zum anaeroben Abbau von Alkylbenzolen getestet. Von beiden Stämmen war bereits bekannt, daß sie Benzoat mit Sulfat als Elektronenakzeptor vollständig oxidieren (Galushko und Rozanova 1991; Widdel und Bak 1992). Tatsächlich verwertete *D. cetonicum* anaerob auch Toluol, jedoch keine Xylole. Im Gegensatz dazu war *D. variabilis* nicht in der Lage, Alkylbenzole abzubauen.

In quantitativen Wachstumsexperimenten wurde gezeigt, daß Stamm oXyS1 zur vollständigen Oxidation von *o*-Xylol zu CO_2 fähig ist. Durch die Bestimmung von

Enzymaktivitäten, insbesondere der Kohlenmonoxid-Dehydrogenase (CO-Dehydrogenase), wurden weitere Hinweise auf die Fähigkeit zur vollständigen Oxidation organischer Substrate zu Kohlendioxid erhalten. In zellfreien Extrakten von Stamm oXyS1 wurden spezifische Aktivitäten der Kohlenmonoxid-Dehydrogenase von 0,12 µmol · min^{-1} · mg^{-1} und der Formiat-Dehydrogenase von 0,23 µmol · min^{-1} · mg^{-1} (jeweils bezogen auf Protein) gemessen. Diese Ergebnisse bestätigen, daß die terminale Oxidation über den CO-Dehydrogenase-Weg, auch C_1-Weg genannt, verläuft, der in den meisten Arten vollständig oxidierender sulfatreduzierender Bakterien vorkommt (Widdel und Hansen 1992).

Bislang war der anaerobe Abbau von *o*-Xylol nur in Anreicherungskulturen beobachtet worden (Edwards et al. 1992; Edwards und Grbic-Galic 1994; Rueter et al. 1994; Chen und Taylor 1997). Stamm oXyS1 ist somit die erste Reinkultur, die unter anoxischen Bedingungen *o*-Xylol verwertet und vollständig zu CO_2 oxidiert.

4. Methanbildung durch syntrophen Abbau von Alkanen unter anoxischen Bedingungen

Rezente Sedimente enthalten unterschiedliche Mengen organischer Substanz, deren Anteil zwischen 1 und 8% der Trockenmasse der Sedimente variiert. Eine wichtige Fraktion der organischen Verbindungen in Meeres- und Süßwassersedimenten bilden gesättigte Kohlenwasserstoffe, und zwar sowohl aliphatische als auch alicylische Verbindungen. Aliphatische Kohlenwasserstoffe in Sedimenten sind unterschiedlicher Herkunft. Zum einen stammen sie aus den Wachsschichten höherer Pflanzen, zum anderen werden sie sowohl von benthischen als auch von planktonischen Organismen gebildet (Blumer et al. 1971). Durch diagenetische Umwandlungen werden Alkene größtenteils zu Alkanen reduziert (Eglinton 1968), so daß letztere die vorherrschenden aliphatischen Kohlenwasserstoffe in alten Sedimenten sind. Des weiteren gelangen aliphatische Kohlenwasserstoffe durch den Eintrag von Erdöl in Sedimente (Simoneit und Lonsdale 1982; Gundlach et al. 1983).

Sauerstoff dringt in den meisten Meeres- und Süßwassersedimenten nur wenige Millimeter ein; unterhalb dieser dünnen oxischen Schicht sind die Sedimente anoxisch (Revsbech et al. 1980; Canfield 1993). Während der mikrobielle Abbau von Alkanen unter oxischen Bedingungen in einer Vielzahl von Bakterien nachgewiesen und intensiv untersucht wurde (Britton 1984; Morgan und Watkinson 1994), wurde der Abbau unter anoxischen Bedingungen bislang nur für wenige nitratreduzierende (Ehrenreich 1996; Behrends A, Harder J, Rainey F, Widdel F, unveröffentlicht) und sulfatreduzierende (Aeckersberg et al. 1991) Bakterien gezeigt.

Ein Hinweis, daß gesättigte Kohlenwasserstoffe auch in anoxischen Sedimenten umgesetzt werden, wurde Anfang der 80er Jahre erbracht (Giger et al. 1980). In rezenten Sedimenten des Greifensees (Schweiz) wurde eine Abnahme von *n*-Heptadecan innerhalb eines Versuchszeitraums von 80 Wochen mit einhergehender Gasbildung beobachtet. Obwohl die Autoren für die Abnahme des Alkans mikrobiologische Prozesse verantwortlich machten, wurde die Gasbildung nicht weiter im Detail untersucht.

Das Hauptvorkommen von Alkanen stellen fossile Brennstoffe wie Erdgas, Erdöl und Kohle dar. Erdöl besteht zu über 50% aus gesättigten Kohlenwasserstoffen (Tissot und Welte 1984). In Erdöllagerstätten wird eine Bildung von Methan nicht nur auf thermische (Mango 1991; Sackett und Conkright 1997), sondern zum Teil auch auf rezente biologische Prozesse zurückgeführt. Anhand der stabilen Isotope des Methans ($^{12}C/^{13}C$) und deren Fraktionierung kann zwischen den beiden Prozessen unterschieden werden (Schoell 1983; Alperin et al. 1992; Hayes 1993), da Mikroorganismen bei der Methanogenese Verbindungen des leichteren Kohlenstoffatoms (^{12}C) bevorzugen (Fuchs et al. 1979; Krzycki et al. 1987; Gelwicks et al. 1994; Botz et al. 1996). Aus Erdölfeldern wurden wiederholt methanogene Archaeen isoliert (Ekzertsev 1960; Belyaev et al. 1977; Nazina und Rozanova 1980; Berdichevskaya 1982; Ivanov et al. 1982; Nazina 1984; Obraztsova et al. 1984; Nazina et al. 1985; Rozanova et al. 1985; Belyaev et al. 1986; Davydova-Charakhck´yan et al. 1993; Nazina et al. 1995; Nilsen und

Torsvik 1996; Ollivier et al. 1998), wobei über deren Bedeutung im Erdölfeld immer noch Unklarheit herrscht. Die Wachstumssubstrate dieser Methanogenen in Laborversuchen waren Wasserstoff, Formiat und Acetat. Wasserstoff und Formiat wurden in Erdölfeldern bislang noch nicht nachgewiesen. Acetat wird in Erdölfeldern abiotisch nur bei Temperaturen zwischen 80 und 160 °C gebildet (Carothers und Kharaka 1987), obwohl es auch in Bereichen mit Temperaturen unter 80 °C gefunden wird (Means und Hubbard 1987). Eine mögliche Erklärung für die Bildung von Acetat bei gemäßigten Temperaturen wäre eine unvollständige aerobe mikrobielle Oxidation von Erdöl-Kohlenwasserstoffen aufgrund des unbeabsichtigten Eintrags von molekularem Sauerstoff während der sekundären Erdölförderung (Rozanova et al. 1985). In der Literatur finden sich allerdings auch indirekte Hinweise auf eine Bildung von Wachstumssubstraten methanogener Mikroorganismen in Erdöl- und Kohlelagerstätten unter anoxischen Bedingungen. So wurde in einem mit Erdöl kontaminiertem Grundwasserleiter eine sekundäre biologische Methanbildung mit einhergehendem partiellen Abbau des Erdöls beobachtet (Baedecker et al. 1993; Bennett et al. 1993; Eganhouse et al. 1993). In Kohlelagerstätten fand eine sekundäre mikrobielle Methanbildung mit gleichzeitigem Abbau von Alkanen mit Kettenlängen $\leq C_{32}$ statt (Scott et al. 1994). Sowohl in tieferen Meeres- und Süßwassersedimenten als auch in Erdöl- und Kohlereservoirs stehen für einen mikrobiellen Alkanabbau außer CO_2 keine weiteren Elektronenakzeptoren zur Verfügung. Das Ziel der vorliegenden Arbeit war es daher, einen Abbau von gesättigten Kohlenwasserstoffen unter methanogenen Bedingungen nachzuweisen. Sollte ein solcher Prozeß existieren, hätte er nicht nur eine große Bedeutung für die Methanbildung in tiefen Sedimentschichten, sondern auch für Umwandlungsprozesse in Erdölen.

4.1 Wachstumsverlauf syntropher Anreicherungen mit Alkanen

Methanogene Anreicherungen mit Sedimentproben eines Grabens (Seehausen, Bremen) wurden mit *n*-Hexadecan als einzigem organischen Substrat angesetzt. Um die Bakterien auch im Medium in unmittelbaren Kontakt mit dem hydrophoben Kohlenwasserstoff zu bringen, wurde das Hexadecan auf Siedesteine oder Filter aus Polytetrafluorethylen (PTFE) aufgebracht, die spezifisch schwerer als Wasser sind (siehe auch I.B.5). Nach einer Inkubationszeit von vier Monaten wurde eine Zunahme der Gasbildung im Vergleich zur Kontrolle beobachtet. Durch wiederholtes Übertragen wurde eine Anreicherungskultur erhalten, die Hexadecan unter Gasproduktion verwertete. Das Wachstum der Anreicherungskultur wurde anhand der Gasbildung über mehrere Jahre verfolgt. Innerhalb von 812 Tagen wurden aus 175 µl Hexadecan in 45 ml Medium 102 ml Gas im Vergleich zu einer Kontrolle ohne Hexadecan gebildet. Das produzierte Gas wurde als Methan identifiziert. Die Anreicherung verwertete ebenfalls *n*-Pentadecan, jedoch kein Hexan oder Decan.

4.2 Zusammensetzung der syntrophen Anreicherungskultur

Die mikroskopische Betrachtung zeigte vier Morphotypen in der Anreicherungskultur. Drei von ihnen, ovale Zellen, lange dünne Filamente und lange schlanke Stäbchen kamen überwiegend in lockeren Aggregaten vor; der vierte Morphotyp, vibrioide Zellen, war gleichmäßig im Medium verteilt.

Durch Klonierung und Sequenzierung von 16S rRNA Genen aus der Anreicherungskultur wurden Mikroorganismen von vier verschiedenen Verwandtschaftsgruppen identifiziert. Drei Typen der erhaltenen Klone hatten die höchste Sequenzähnlichkeit zu Organismen aus dem Reich der Archaea, diese waren *Methanosaeta soehngenii* (91,2%), *Methanospirillum hungatei* (97,1%) und *Methanoculleus marisnigri* (95,5%). Alle weiteren erhaltenen Klontypen gehörten der δ-Untergruppe der Proteobakterien an. Ein Klontyp zeigte die höchste Sequenzähnlichkeit mit der Gattung *Desulfovibrio* (98,4%). Die meisten der erhaltenen Klontypen (90%) gehörten jedoch der Gattung *Syntrophus* an, die Sequenzähnlichkeit betrug 95.0%.

Aufgrund der langen Inkubationszeiträume von mehreren Jahren wäre es theoretisch möglich, daß molekularer Sauerstoff durch den Butylstopfen diffundiert und die Alkane somit durch ein aerobes oder mikroaerophiles Bakterium aktiviert werden. Um diese Möglichkeit auszuschließen, wurde beimpftes Medium mit Hexadecan sowohl unter oxischen als auch unter mikrooxischen Bedingungen (1% Sauerstoff) inkubiert. In diesen Ansätzen wurde innerhalb von vier Wochen kein Wachstum beobachtet, was ebenfalls für einen rein anaeroben Prozeß der Methanbildung aus Alkanen spricht.

4.3 Methanbildung aus ^{13}C-markiertem Hexadecan

Der Nachweis, daß das gebildete Methan tatsächlich aus Hexadecan stammt, wurde in Versuchen mit ^{13}C-markiertem Hexadecan erbracht. Hierzu wurden Wachstumsversuche mit uniform markiertem ^{13}C-Hexadecan im Vergleich zu Ansätzen mit ^{12}C-Hexadecan durchgeführt. Während des Versuchs wurden Gasproben entnommen, in denen das ^{13}C/^{12}C-Verhältnis im Methan und Kohlendioxid bestimmt wurde. Methan und Kohlendioxid aus dem Ansatz mit ^{12}C-Hexadecan hatten während des Versuchs einen konstanten δ^{13}C-Wert von -40,7 bzw. -19,5‰. Das produzierte Methan aus dem Ansatz mit ^{13}C-Hexadecan und ^{12}C-Hexadecan (1 : 9, mit einem δ^{13}C-Wert für das Gemisch von +9963‰) zeigte nach 158 Tagen Inkubation einen δ^{13}C-Wert von +8975‰. Zusätzlich wurde eine Zunahme des δ^{13}C-Werts von Kohlendioxid in dem Ansatz auf +680‰ gemessen. Im Ansatz mit ausschließlich ^{13}C-Hexadecan stiegen die δ^{13}C-Wert von Methan und Kohlendioxid auf +6334 bzw. -2,5‰, die gemessene Gasbildung war allerdings gering. Vermutlich wurden die Organismen durch das reine ^{13}C-Hexadecan stark in ihrem Wachstum beeinträchtigt, da sie den überwiegenden Teil ihrer Zellmasse aus ^{13}C-

Verbindungen aufbauen mußten. Insgesamt zeigte der Versuch eindeutig, daß Methan und Kohlendioxid aus dem Hexadecan gebildet werden.

In quantitativen Wachstumsexperimenten wurde gezeigt, daß die Anreicherungskultur zur vollständigen Oxidation von *n*-Hexadecan zu CO_2 fähig war. Die Umsetzung von Hexadecan zu Methan durch die drei verschiedenen Haupttypen an Organismen der Anreicherungskultur verläuft nach unserem heutigen Verständnis der Methanogenese gemäß folgenden Gleichungen:

Syntrophes Bakterium, in der Anreicherungskultur wahrscheinlich ein *Syntrophus*-ähnlicher Organismus

$$4\ C_{16}H_{34} + 64\ H_2O \rightarrow 32\ CH_3COO^- + 32\ H^+ + 68\ H_2$$

Methanogenes Archaeon, in der Anreicherungskultur wahrscheinlich ein *Methanospirillum*-ähnlicher Organismus

$$68\ H_2 + 17\ CO_2 \rightarrow 17\ CH_4 + 34\ H_2O$$

Methanogenes Archaeon, in der Anreicherungskultur wahrscheinlich ein *Methanosaeta/Methanothrix*-ähnlicher Organismus

$$32\ CH_3COO^- + 32\ H^+ \rightarrow 32\ CH_4 + 32\ CO_2$$

Gesamtgleichung

$$4\ C_{16}H_{34} + 30\ H_2O \rightarrow 49\ CH_4 + 15\ CO_2$$

5. Verwertung des polycyclischen aromatischen Kohlenwasserstoffes Fluoren durch nitratreduzierende und sulfatreduzierende Anreicherungskulturen

Diese Arbeit wurde bislang nicht in Form eines Manuskriptes verfaßt.

Polycyclische aromatische Kohlenwasserstoffe (PAK) sind eine in der Natur weit verbreitete Stoffgruppe. PAKs gelangen durch Pyrolyse und unvollständige Verbrennung von fossilen Brennstoffen wie z.B. Kohle und Erdöl und durch die Freisetzung von Erdölen und dessen Produkten in die Umwelt (Wakeham et al. 1980a). Neben diesem anthropogenen Eintrag werden PAKs auch auf natürliche Weise durch einige Insekten (Chen et al. 1998), durch Busch- und Waldbrände und durch Prozesse der Diagenese gebildet (Wakeham et al. 1980b; Simoneit und Lonsdale 1982; Kawka und Simoneit 1987; Tan et al. 1996). Aufgrund ihres delokalisierten π-Elektronensystems stellen PAKs eine ausgesprochen stabile Stoffklasse dar. Im Gegensatz zu monoaromatischen Kohlenwasserstoffen spielt beim mikrobiellen Abbau von PAKs außerdem die Verfügbarkeit eine entscheidende Rolle. PAKs sind fast nicht in Wasser löslich (May 1980) und adsorbieren an Böden und Sedimente (Means et al. 1980).

Mehrere PAKs haben für den Menschen mutagene und karzinogene Eigenschaften, worin das Interesse an Untersuchungen zum Verbleib dieser Substanzklasse in der Natur begründet liegt (Kalberlah et al. 1995; Sikkema et al. 1995). Eine akute Toxizität von PAKs auf Prokaryoten wurde bisher nicht festgestellt (Reid et al. 1998). Während der aerobe Abbau von PAKs seit vielen Jahren bekannt ist und eingehend untersucht wurde (Gibson und Subramanian 1984; Cerniglia 1992), gibt es nur wenig Information über den Abbau von PAKs unter anoxischen Bedingungen. Es ist zu vermuten, daß der Abbau von PAKs unter Ausschluß von molekularem Sauerstoff nur sehr langsam verläuft. Anhand der Verteilung von PAKs in verschiedenen anoxischen Habitaten läßt sich z.B. das Zeitalter der industriellen Revolution anhand des erhöhten anthropogenen Eintrags dieser Verbindungen genau datieren (Wakeham 1996; Yunker et al. 1996). Dennoch gibt es in den letzten Jahren vermehrt Hinweise auf einen Abbau dieser Verbindungen in anoxischen Habitaten. In marinen Sedimenten wurde unter sulfatreduzierenden Bedingungen eine Umsetzung von ^{14}C-markiertem Naphthalin, Methylnaphthalin, Fluoren, Phenanthren und Fluoranthen zu $^{14}CO_2$ gefunden (Coates et al. 1996; Coates et al. 1997). Ebenfalls in marinen sulfidischen Sedimenten wurde eine Carboxylierung als mögliche initiale Aktivierungsreaktion beim Abbau von Naphthalin und Phenanthren nachgewiesen (Zhang und Young 1997). In Böden wurde ein Abbau von Naphthalin und Acenaphthen unter denitrifizierenden Bedingungen beobachtet (Mihelcic und Luthy 1988b; Mihelcic und Luthy 1988a; McNally et al. 1998). Allerdings wurden diese Versuche ohne ein Reduktionsmittel durchgeführt, so daß noch genügend molekularer Sauerstoff für die initiale Aktivierung der PAKs zur Verfügung gestanden haben könnte. Untersuchungen an Reinkulturen zeigten eine Verwertung von Anthracen, Phenanthren und Pyren unter denitrifizierenden Bedingungen (McNally et al. 1998). Bei den Reinkulturen handelte es sich

um Pseudomonaden; diese Bakterien sind bekannt, aerob mit PAKs zu wachsen. Da in den Versuchen ebenfalls kein Reduktionsmittel verwendet wurde, ist auch hier eine Aktivierung durch molekularen Sauerstoff wahrscheinlich. Die Umsetzungsraten der jeweiligen PAKs unterschieden sich kaum unter anoxischen und oxischen Bedingungen, welches ebenfalls für eine aerobe Aktivierung der PAKs spricht. In der vorliegenden Arbeit sollte daher eine anaerobe Anreicherungskultur etabliert werden, die zur vollständigen Mineralisation eines polycyclischen aromatischen Kohlenwasserstoffs in der Lage war.

5.1 Anreicherung und Wachstum von nitrat- und sulfatreduzierenden Bakterien mit Fluoren

Als Inokulum für PAK-abbauende Anreicherungen wurde Bodenmaterial vom Gelände einer ehemaligen Metallhütte (Lübeck, Deutschland) verwendet. Der Boden enthielt nicht nur Schwermetalle, sondern war auch stark mit PAKs kontaminiert, welche aus dem Betrieb einer Kokerei auf dem Gelände stammten. Bei der Verkokung von Steinkohle entsteht Steinkohlenteer, welcher ca. 1,6% Fluoren enthält (Willmes 1990).

Anreicherungen auf Fluoren wurden sowohl mit Nitrat als auch mit Sulfat als Elektronenakzeptor durchgeführt. Um eine möglichst große Verteilung der hydrophoben PAKs im wäßrigen Medium zu erhalten, wurden die Kohlenwasserstoffe auf kleine Siedesteine aus Polytetrafluorethylen (PTFE, Teflon) aufgebracht (Abb. 1). Dieses hatte den Vorteil, daß zum einen die Oberfläche stark vergrößert wurde und zum anderen die Bakterien aus dem Bodenmaterial in direkten Kontakt mit den PAKs kamen. Nach 60 bzw. 16 Wochen wurden positive Anreicherungen mit Sulfat und Nitrat als Elektronenakzeptoren erhalten.

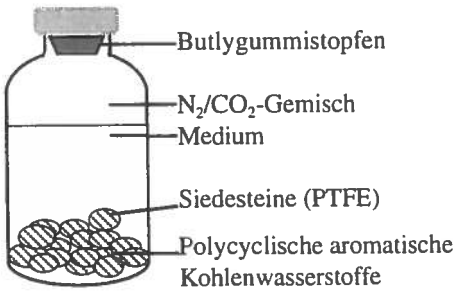

Abb. 1 Serumflasche (100 ml) mit Siedesteinen aus Polytetrafluorethylen (PTFE), die mit polycyclischen aromatischen Kohlenwasserstoffen (Fluoren bzw. Phenanthren) beschichtet wurden.

5.2 Bilanzierung des anaeroben Abbaus von Fluoren

Die Fähigkeit der anaeroben Anreicherungskulturen, Fluoren vollständig zu Kohlendioxid zu oxidieren, wurde in quantitativen Wachstumsexperimenten mit Nitrat (Tabelle 1) bzw. Sulfat als Elektronenakzeptor gezeigt (II.B.1 und II.B.3). Das Wachstum der nitratreduzierenden Anreicherung wurde anhand der Nitratreduktion verfolgt (Abb. 2). Um einen toxischen Effekt von ausgeschiedenem Nitrit auf die Anreicherungskultur zu verhindern, wurde die Kultur mit einer Anfangskonzentration von nicht mehr als 10 mM Nitrat angezogen; Nitrat wurde anschließend, nachdem es durch HPLC-Analyse nicht mehr nachweisbar war, nachgefüttert.

Tabelle 1 Bilanzierung des Abbaus von Fluoren durch die nitratreduzierende Anreicherung.

Experiment[a]	Fluoren eingesetzt (mmol)	Fluoren verbraucht[b] (mmol)	Nitrat reduziert[c] (mmol)	Elektronen aus verbrauchtem Fluoren[d] (mmol)	Elektronen verbraucht durch NO_3^--Reduktion[e] (mmol)
Anreicherung mit Fluoren	0,4	0,388	5,62	24,1	28,1
Kontrolle ohne Fluoren	0,0	0,0	0,096	0,0	0,48
Kontrolle ohne Nitrat	0,4	0,002	0,0	0,124	0,0

[a] Experimente wurden in 250 ml Flachflaschen mit 200 ml Medium unter anoxischen Bedingungen durchgeführt.
[b] Differenz zwischen der eingesetzten und der wiedergefundenen Fluorenmenge am Ende des Versuchs.
[c] Nitrat wurde vollständig zu N_2 reduziert. Nitrit oder Stickstoffdioxid waren am Versuchsende nicht nachweisbar.
[d] Stöchiometrisch werden bei der vollständigen Oxidation von 1 mmol Fluoren zu CO_2 62 mmol Elektronen erhalten.
[e] Stöchiometrisch werden für die Reduktion von 1 mmol Nitrat zu 0,5 mmol N_2 5 mmol Elektronen benötigt.

Das Defizit von Elektronen, die in die Nitratreduktion eingeflossen sind, zu den Elektronen, die aus dem Fluoren stammten, beruht wahrscheinlich auf einer unvollständigen Extraktion des Fluorens. Während der mehrmaligen Extraktion der gewachsenen Kultur mit Dichlormethan bildete sich eine starke Interphase aus; aufgrund von Absorption an Zellmasse könnte ein bestimmter Teil des Fluorens nicht extrahiert worden sein.

Eine Bilanzierung der Oxidation von Fluoren wurde ebenfalls mit der sulfatreduzierenden Anreicherungskultur durchgeführt (Daten nicht gezeigt). Das Verhältnis der Elektronen aus der Oxidation des Fluorens zu den Elektronen für die Reduktion von Sulfat zu Sulfid betrug 1 : 0,83. Aufgrund der Bilanzierung des Abbaus kann auf eine vollständige Oxidation von Fluoren zu CO_2 sowohl unter nitrat- als auch unter sulfatreduzierenden Bedingungen geschlossen werden.

5.3 Bildung von möglichen Intermediaten während des Abbaus von Fluoren unter anoxischen Bedingungen

In HPLC-Analysen (II.B.1) des wäßrigen Überstandes der nitratreduzierenden Anreicherungskultur auf Fluoren konnte 9-Fluorenol aufgrund der Retentionszeit, anhand des Absorptionsspektrums und durch Standardaddition nachgewiesen werden. Eine Abnahme von 9-Fluorenol bei längerer Inkubation der Kultur deutete darauf hin, daß 9-Fluorenol ein Intermediat beim anaeroben Fluorenabbau war. In Wachstumsversuchen wurde gezeigt, daß sowohl 9-Fluorenol als auch das entsprechende Keton, 9-Fluorenon, als Wachstumssubstrate für die nitratreduzierende Anreicherung dienten.

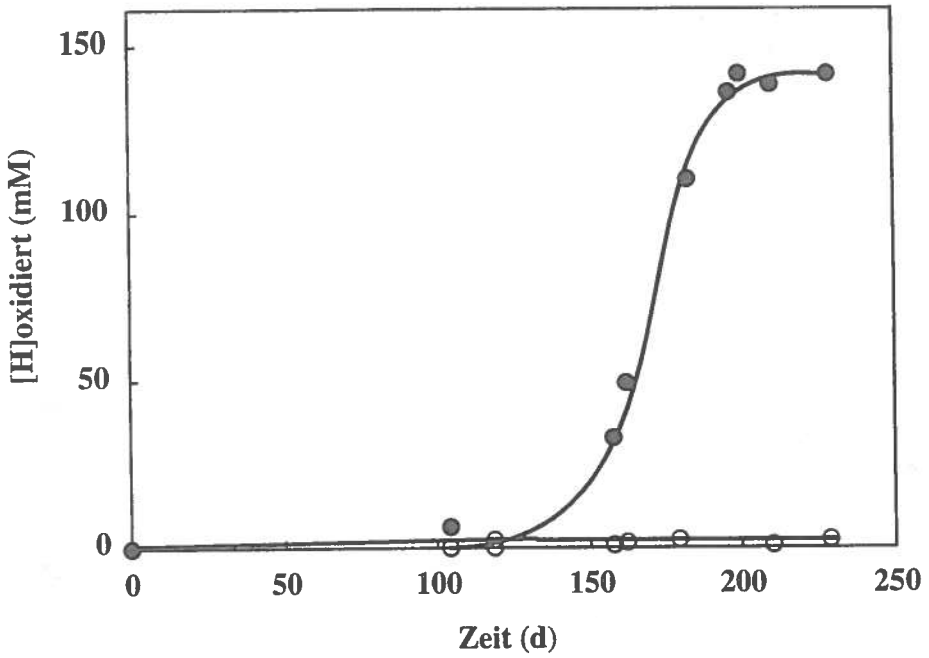

Abb. 2 Substratoxidation durch die nitratreduzierende Anreicherungskultur auf dem polycyclischen aromatischen Kohlenwasserstoff Fluoren (●) im Vergleich zur Kontrolle ohne organisches Substrat (○). Fluoren (0,4 mmol) wurde auf Siedesteine aus PTFE aufgebracht und vorsichtig mit 200 ml anoxischem Medium überschichtet. Als Kulturgefäße dienten 250 ml Flachflaschen.
[H] oxidiert = 5 · [Nitrat zugegeben - (Nitrat bestimmt + Nitrit bestimmt)] + 2 · (Nitrit bestimmt).

Um die Bildung der durch HPLC nachgewiesenen Intermediate zu bestätigen, wurden zusätzlich GC- und GC-MS-Analysen der wäßrigen Überstände der nitrat- und sulfatreduzierenden Anreicherungskulturen durchgeführt. In beiden Anreicherungskulturen wurden wiederum 9-Fluorenol und 9-Fluorenon aufgrund von Retentionszeiten und Massenspektren nachgewiesen (Abb. 3; Abb. 4A und B). In der nitratreduzierenden Anreicherung wurden außerdem zwei weitere Verbindungen gefunden, deren exakte Struktur bislang nicht aufgeklärt werden konnte. Eine dieser Verbindungen könnte aufgrund des

Massenspektrums ein Hydroxy-9-Fluorenon sein (Abb. 4C). Um welches Strukturisomer von Hydroxy-9-Fluorenon (1-, 2-, 3- bzw. 4-) es sich handelt, konnte bislang nicht aufgeklärt werden; allerdings konnte 2-Hydroxy-9-Fluorenon anhand von Coinjektionen ausgeschlossen werden. Für die andere Verbindung wurde aufgrund des Massenspektrums auf ein 9-Fluorenonderivat mit einer Säuregruppe geschlossen (Abb. 4 D).

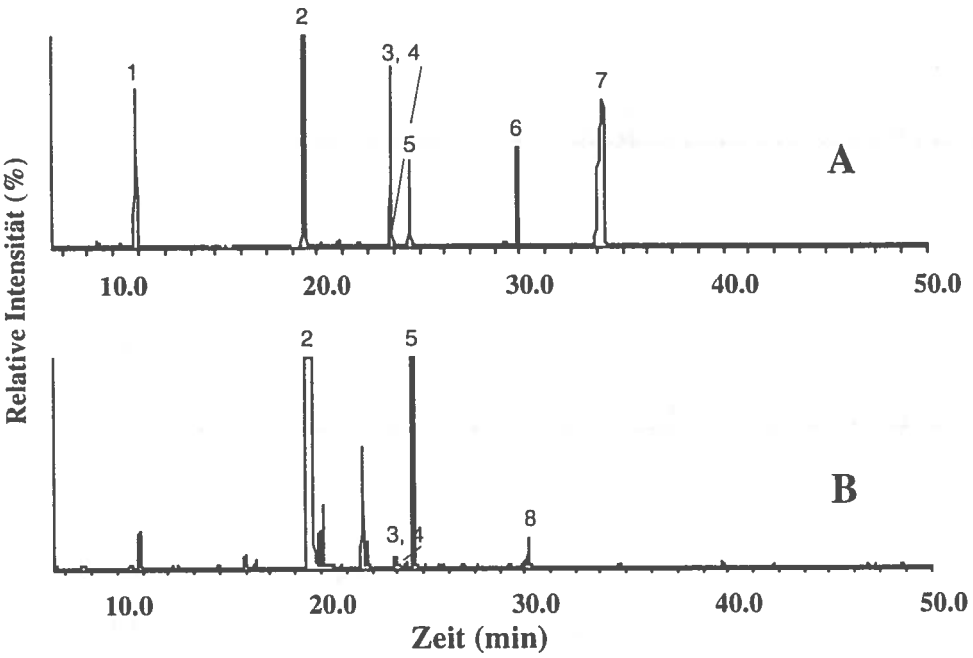

Abb. 3 Gaschromatogramm der wäßrigen Überstände der nitratreduzierenden (**A**) und der sulfatreduzierenden Anreicherungskultur (**B**) mit Fluoren. Peaks wurden anhand der Retentionszeiten und der Massenspektren (Abb. 4) folgenden Verbindungen zugeordnet: 1, Heptamethylnonan (wurde in diesem Ansatz als inerte Trägerphase verwendet); 2, Fluoren; 3, 9-Fluorenon; 4, 9-Fluorenol; 5, interner Standard (Phenanthren); 6, unbekannte Verbindung; 7; Hydroxy-9-Fluorenon; 8, elementarer Schwefel.

Aufgrund der gefundenen Intermediate wurde folgender Weg für den anaeroben Abbau von Fluoren in den Anreicherungskulturen vorgeschlagen (Abb. 5). Zunächst wird Fluoren durch eine Hydroxylierung der Methylengruppe zu 9-Fluorenol oxidiert. Eine weitere Oxidation führt zur Bildung von 9-Fluorenon. Anschließend wird eine weitere Hydroxylgruppe (vermutlich an Position 1 oder 3) eingefügt, und es entsteht Hydroxy-9-Fluorenon. Alle drei potentiellen Intermediate wurden nachgewiesen. Eine anschließende Spaltung des aromatischen Ringsystems wäre, ausgehend vom 1-Hydroxy-9-Fluorenon, durch zwei verschiedene Mechanismen möglich. Zum einen wäre eine thiolytische Spaltung des Ringes denkbar, zum anderen könnte der Abbau über eine vorausgehende Carboxylierung und einer Spaltung analog

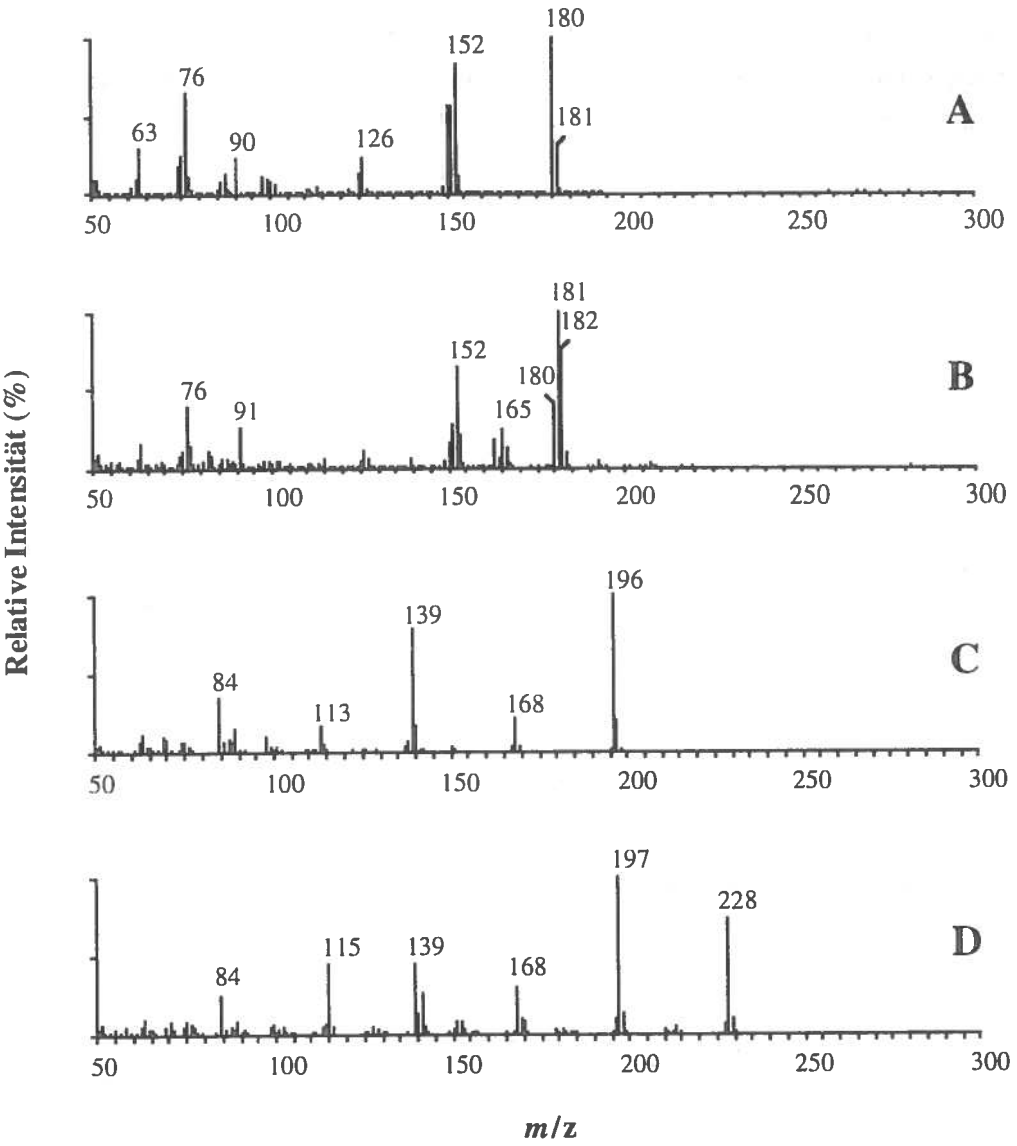

Abb. 4 Massenspektren von 9-Fluorenon (**A**), 9-Fluorenol (**B**), Hydroxy-9-Fluorenon (**C**) und einem nicht identifizierten 9-Fluorenonderivat (**D**). Bei letzterem handelt es sich um Peak Nr. 6 in Abb. 3A.

zum anaeroben Phenolabbau (Tschech und Fuchs 1989) ablaufen. In Analogie zum anaeroben Phenolstoffwechsel könnte sich nach einer Carboxylierung eine Dehydroxylierung anschließen (Biegert et al. 1993; Breese und Fuchs 1998). Bei der nicht identifizierten Verbindung (Abb. 4D) handelt es sich anhand des Massenspektrums um ein solches Derivat des 9-Fluorenons mit einer Säuregruppe. Es ist daher denkbar, daß der anaerobe Abbau von Fluoren über diesen Weg verläuft.

Der aerobe Abbau von Fluoren wurde außer in verschiedenen Pilzen auch in Bakterien der Gattungen *Arthrobacter* (Grifoll et al. 1992; Casellas et al. 1997), *Brevibacterium* (Engesser et al. 1989; Trenz et al. 1994), *Mycobacterium* (Boldrin et al. 1993), *Pseudomonas*

(Weissenfels et al. 1990; Grifoll et al. 1994; Trenz et al. 1994; Yang et al. 1994; Grifoll et al. 1995a; Grifoll et al. 1995b; Stringfellow und Aitken 1995; Bouchez et al. 1996; Resnick und Gibson 1996; Selifonov et al. 1996) und *Staphylococcus* (Monna et al. 1993) nachgewiesen. Auch beim aeroben Abbau von Fluoren durch den *Arthrobacter* Stamm F101 wurden 9-Fluorenol, 9-Fluorenon und 4-Hydroxy-9-Fluorenon nachgewiesen. Diese Verbindungen wurden allerdings in einer Nebenreaktion als Dead-End-Metabolite gebildet. Der Stamm war nicht in der Lage, mit 9-Fluorenol bzw. 9-Fluorenon zu wachsen. Der eigentliche Abbau verläuft über eine Dioxygenasereaktion, die den Ring in Position 1,2 oder 3,4 spaltet (Casellas et al. 1997). Ähnliches wurde auch für *Staphylococcus auriculans* (Monna et al. 1993) beschrieben. Im Gegensatz dazu wurde während des aeroben Abbaus von Fluoren durch *Brevibacterium* sp. (Engesser et al. 1989; Trenz et al. 1994) und *Pseudomonas cepacia* (Grifoll et al. 1995a; Grifoll et al. 1995b) der Ring in Position 1,10 gespalten. Fluoren wurde in diesen Bakterien über 9-Fluorenol zu 9-Fluorenon oxidiert, welches anschließend durch eine Dioxygenase gespalten wurde. 9-Fluorenol und 9-Fluorenon stellten für beide Bakterien ein Wachstumssubstrat dar. Die Bildung eines carboxylierten Derivats des 9-Fluorenons wurde allerdings in keinem der aeroben Bakterien beobachtet und scheint daher ein Intermediat zu sein, welches ausschließlich beim anaeroben Abbau gebildet wird.

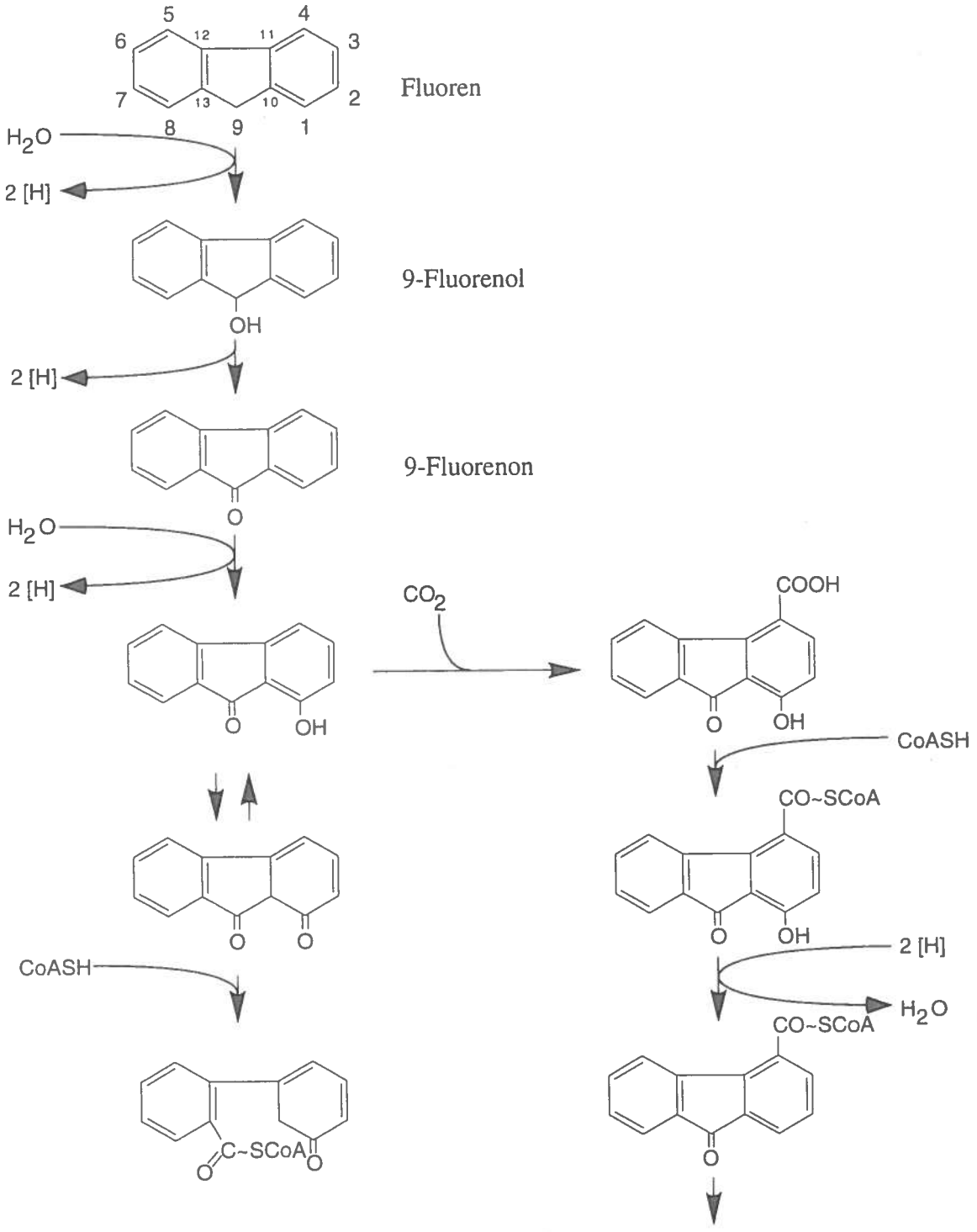

Abb. 5 Hypothetischer Weg des anaeroben Abbaus von Fluoren.

6. Anaerober Abbau und Fraktionierung der Kohlenstoffisotope von Alkylbenzolen aus Erdöl durch sulfatreduzierende Bakterien

Der Abbau von Erdölen in Reservoirs und Erdölfeldern wurde lange Zeit als ein rein aerober Prozeß infolge eines Eintrags oxischen Wassers angesehen (Connan 1984). Obwohl die Existenz von sulfatreduzierenden Bakterien in Ölreservoirs schon Mitte der 20er Jahre beschrieben (Bastin et al. 1926) und später wiederholt bestätigt wurde (Cord-Ruwisch et al. 1987; Rosnes et al. 1991; Voordouw et al. 1992; Stetter et al. 1993; Beeder et al. 1994; Beeder et al. 1995; Beeder et al. 1996; Mueller und Nielsen 1996; Nilsen et al. 1996; Voordouw et al. 1996), wurde dabei die Möglichkeit eines anaeroben Kohlenwasserstoffabbaus durch diese Bakterien nicht untersucht. Erst in den letzten Jahren wurden sulfatreduzierende Bakterien isoliert, die in der Lage waren, Erdölkohlenwasserstoffe zu verwerten (Aeckersberg et al. 1991; Rabus et al. 1993; Rueter et al. 1994; Beller et al. 1996; Harms et al. 1999). Die beiden sulfatreduzierenden Stämme oXyS1 und mXyS1 (Harms et al. 1999), welche mit *m*-Xylol bzw. *o*-Xylol wuchsen, wurden dabei aus einer Anreicherungskultur mit Sulfat und Erdöl als einziger Quelle organischer Substrate (Rabus et al. 1996) isoliert. Während des Wachstums der Anreicherungskultur wurden aus dem Erdöl die Alkylbenzole Toluol, *o*- und *m*-Xylol, *o*- und *m*-Ethyltoluol, *m*-Propyltoluol und *m*-Cymol verwertet.

6.1 Anaerobe Verwertung von Alkylbenzolen aus Erdölen

In Wachstumsexperimenten wurde die Verwertung von C_0-C_3-Alkylbenzolen aus einem ausgesuchten Nordsee-Erdöl sowohl durch die Anreicherungskultur (siehe oben) als auch durch die beiden Reinkulturen oXyS1 und mXyS1 untersucht. Stamm oXyS1 verwertete Toluol, *o*-Xylol, *o*-Ethyltoluol und kleinere Mengen an *m*-Xylol und 1,2,4-Trimethylbenzol aus dem Öl. Stamm mXyS1 verwertet Toluol, *m*-Xylol, *m*-Ethyltoluol und kleinere Mengen an *p*-Xylol, 1,3,5-Trimethylbenzol und 1,2,4-Trimethylbenzol. Neben der Verwertung von Alkylbenzolen aus dem Nordseeöl wurden auch noch weitere Erdöle hinsichtlich des Abbaus durch die Anreicherungskultur untersucht. Die ausgesuchten Erdöle unterschieden sich in Herkunft, Schwefelgehalt, Viskosität und Zusammensetzung der Kohlenwasserstoffe. Nach dem Wachstum der Anreicherungskultur mit den unterschiedlichen Erdölen wurden die einzelnen Alkylbenzole analysiert. Obwohl auch eine Verwertung all der genannten Alkylbenzole durch die Anreicherungskultur stattfand, variierten deren relative Mengen stark in den einzelnen Erdölen. Der Abbau der Alkylbenzole schien mit der Verfügbarkeit der Kohlenwasserstoffe aus den Erdölen zu korrelieren.

6.2 Bildung von Metaboliten

Der initiale Schritt beim anaeroben Abbau von Toluol ist die Addition von Fumarat an die Methylgruppe des Toluols, welches zur Bildung von Benzylsuccinat führt (Evans et al. 1992; Biegert et al. 1996; Beller und Spormann 1997b; Beller und Spormann 1997a; Coschigano et al. 1998; Leuthner et al. 1998; Rabus und Heider 1998). Benzylsuccinat wird anschließend zu Benzoyl-CoA, dem zentralen Intermediat beim anaeroben Abbau aromatischer Verbindungen, oxidiert. In Wachstumsansätzen mit der Anreicherungskultur und den Stämmen oXyS1 und mXyS1 wurde daher nach der Bildung von analogen Metaboliten beim Abbau von C_0-C_3-Alkybenzolen aus dem Erdöl gesucht. Stamm oXyS1 reicherte beim Wachstum mit Erdöl Benzoat, 2-Methylbenzoat, 3-Methylbenzoat, 4-Methylbenzoat und 2,4-Dimethylbenzoat im Medium an. Des weiteren wurden Spuren von 2-Ethylbenzoat, 3-Ethylbenzoat, 2,3-Dimethylbenzoat, 2,5-Dimethylbenzoat und 3,5-Dimethylbenzoat gefunden. Stamm mXyS1 bildete beim Wachstum mit Erdöl Benzoat, 4-Methylbenzoat, 3,4-Dimethylbenzoat und 3,5-Dimethylbenzoat und Spuren von 3-Methylbenzoat. Mit Ausnahme von 3-Ethylbenzoat wurden all diese aromatischen Säuren auch beim Wachstum der Anreicherungskultur auf Erdöl nachgewiesen.

Die Bildung von Benzylsuccinat-Homologen wurde nur in Wachstumsansätzen von Stamm mXyS1 nachgewiesen. Vergleiche der Massenspektren mit Werten aus der Literatur (Evans et al. 1992; Beller et al. 1995; Beller et al. 1996; Beller und Spormann 1997a) deuteten auf die Bildung von 2-Methylbenzylsuccinat, 3-Methylbenzylsuccinat und 4-Methylbenzylsuccinat hin. Ebenso wurde anhand der Massenspektren auf die Bildung von Dimethylbenzylsuccinat oder Ethylbenzylsuccinat geschlossen. Die Bildung von allen drei Methylbenzylsuccinat-Isomeren durch Stamm mXyS1, der nur mit *m*-Xylol wuchs, deutet darauf hin, daß die Xylol-Isomere durch Addition von Fumarat unspezifisch aktiviert werden.

6.3 Fraktionierung der Kohlenstoffisotope von Alkylbenzolen

Die δ^{13}C-Werte für die einzelnen Alkylbenzole in dem Nordsee-Erdöl variierten zwischen -29 und -25‰. Nach dem Wachstum der Anreicherungskultur mit Erdöl wurden die δ^{13}C-Werte der verbliebenen Alkylbenzole erneut bestimmt. Eine ^{13}C-Anreicherung wurde besonders für *o*-Xylol (δ^{13}C um 4‰) und *o*-Ethyltoluol (δ^{13}C um 6‰), aber auch für *m*- und *p*-Xylol, sowie für *m*- und *p*-Ethyltoluol beobachtet. Diese Isotopenfraktionierung könnte in zukünftigen in situ Studien als ein Indikator für einen Abbau von Alkylbenzolen durch sulfatreduzierende Bakterien in Erdölreservoirs und anderen aquatischen Habitaten verwendet werden.

C Literaturverzeichnis

Aeckersberg F, Bak F, Widdel F 1991. Anaerobic oxidation of saturated hydrocarbons to CO_2 by a new type of sulfate-reducing bacterium. Archives of Microbiology. 156:5-14.

Aeckersberg F, Rainey FA, Widdel F 1998. Growth, natural relationships, cellular fatty acids and metabolic adaptation of sulfate-reducing bacteria that utilize long-chain alkanes under anoxic conditions. Archives of Microbiology. 170:361-369.

Al-Hasan RH, Al-Bader DA, Sorkhoh NA, Radwan SS 1998. Evidence for *n*-alkane consumption and oxidation by filamentous cyanobacteria from oil-contaminated coasts of the Arabian Gulf. Marine Biology. 130:521-527.

Alperin MJ, Blair NE, Albert DB, Hoehler TM, Martens CS 1992. Factors that control the stable carbon isotopic composition of methane produced in an anoxic marine sediment. Global Biogeochemical Cycles. 6:271-291.

Altenschmidt U, Fuchs G 1991. Anaerobic degradation of toluene in denitrifying *Pseudomonas* sp.: indication for toluene methylhydroxylation and benzoyl-CoA as central aromatic intermediate. Archives of Microbiology. 156:152-158.

Anders H-J, Kaetzke A, Kämpfer P, Ludwig W, Fuchs G 1995. Taxonomic position of aromatic-degrading denitrifying pseudomonad strain K 172 and KB 740 and their description as new members of the genera *Thauera aromatica* sp. nov., and *Azoarcus*, as *Azoarcus evansii* sp. nov., respectively, members of the beta subclass of the *Proteobacteria*. International Journal of Systematic Bacteriology. 45:327-333.

Arcangeli J-P, Arvin E 1995a. Cometabolic transformation of *o*-xylene in a biofilm system under nitrate reducing conditions. Biodegradation. 6:19-27.

Arcangeli J-P, Arvin E 1995b. Modelling of cometabolic transformation of *ortho*-xylene in a denitrifying biofilm system. Biodegradation. 6:29-38.

Baedecker MJ, Cozzarelli IM, Eganhouse RP, Siegel DI, Bennett PC 1993. Crude oil in a shallow sand and gravel aquifer-III. Biogeochemical reactions and mass balance modeling in anoxic groudwater. Applied Geochemistry. 8:569-586.

Ball HA, Johnson HA, Reinhard M, Spormann AM 1996. Initial reactions in anaerobic ethylbenzene oxidation by a denitrifying bacterium, strain EB1. Journal of Bacteriology. 178:5755-5761.

Barbaree JM, Payne WJ 1967. Products of denitrification by a marine bacterium as revealed by gas chromatography. Marine Biology. 1:136-139.

Bastin ES, Greer FE, Merritt CA, Moulton G 1926. The presence of sulphate reducing bacteria in oil field waters. Science. 63:21-24.

Bazylinski DA, Farrington JW, Jannasch HW 1988. Hydrocarbons in surface sediments from Guaymas Basin hydrothermal vent site. Organic Geochemistry. 12:547-558.

Beeder J, Nilsen RK, J.T. R, Torsvik T, Lien T 1994. *Archaeoglobus fulgidus* isolated from hot North Sea oil field waters. Applied and Environmental Microbiology. 60:1227-1231.

Beeder J, Nilsen RK, Thorstenson T, Torsvik T 1996. Penetration of sulfate reducers through a porous North Sea oil reservoir. Applied and Environmental Microbiology. 62:3551-3553.

Beeder J, Torsvik T, Lien T 1995. *Thermodesulforhabdus norvegicus* gen. nov., sp. nov., a novel thermophilic sulfate-reducing bacterium from oil field water. Archives of Microbiology. 164:331-336.

Beier JA, Wakeham SG, Pilskaln CH, Honjo S 1991. Enrichment in saturated compounds of Black Sea interfacial sediment. Nature. 351:642-644.

Beller HR, Ding W-H, Reinhard M 1995. Byproducts of anaerobic alkylbenzene metabolism useful as indicators of in situ bioremediation. Environmental Science and Technology. 29:2864-2870.

Beller HR, Spormann AM 1997a. Anaerobic activation of toluene and *o*-xylene by addition to fumarate in denitrifying strain T. Applied and Environmental Microbiology. 179:670-676.

Beller HR, Spormann AM 1997b. Benzylsuccinate formation as a means of anaerobic toluene activation by sulfate-reducing strain PRTOL1. Applied and Environmental Microbiology. 63:3729-3731.

Beller HR, Spormann AM, Sharma PK, Cole JR, Reinhard M 1996. Isolation and characterization of a novel toluene-degrading, sulfate-reducing bacterium. Applied and Environmental Microbiology. 62:1188-1196.

Belyaev SS, Laurinavicus KS, Gaitan VI 1977. Recent microbiological formation of methane in quarternary and pliocene deposits of the trough in the caspian region. Geochimija. 8:1251-1256.

Belyaev SS, Obraztsova AY, Laurinavichus KS, Bezrukova LV 1986. Characteristics of rod-shaped methane-producing bacteria from an oil pool and description of *Methanobacterium ivanovii* sp. nov. Microbiology. 55:821-826.

Bennett PC, Siegel DE, Baedecker MJ, Hult MF 1993. Crude oil in a shallow sand and gravel aquifer-I. Hydrogeology and inorganic geochemistry. Applied Geochemistry. 8:529-549.

Berdichevskaya MV 1982. Effects of prolonged flooding of an oil pool on development of a biocenosis and activity of stratal microflora. Microbiology. 51:133-137.

Biegert T, Altenschmidt U, Eckerskron C, Fuchs G 1993. Enzymes of anaerobic metabolism of phenolic compounds. European Journal of Biochemistry. 213:555-561.

Biegert T, Fuchs G 1995. Anaerobic oxidation of toluene (analogues) to benzoate (analogues) by whole cells and by cell extracts of a denitrifying *Thauera* sp. Archives of Microbiology. 163:407-417.

Biegert T, Fuchs G, Heider J 1996. Evidence that anaerobic oxidation of toluene in the denitrifying bacterium *Thauera aromatica* is initiated by formation of benzylsuccinate from toluene and fumarate. European Journal of Biochemistry. 238:661-668.

Blumer M, Guillard R, Chase T 1971. Hydrocarbons of marine phytoplankton. Marine Biology. 8:183-189.

Boldrin B, Tiehm A, Fritzsche C 1993. Degradation of phenanthrene, fluorene, fluoranthene, and pyrene by a *Mycobacterium* sp. Applied and Environmental Microbiology. 59:1927-1930.

Bonin P, Gilewicz M, Bertrand JC 1987. Denitrification by a marine bacterium *Pseudomonas nautica* strain 617. Annales de Institut Pasteur / Microbiologie. 138:371-383.

Bordenave ML. 1993. Applied Petroleum Geochemistry. Éditions Technip, Paris.

Botz R, Pokojski H-D, Schmitt M, Thomm M 1996. Carbon isotope fractionation during bacterial methanogenesis by CO_2 reduction. Organic Geochemistry. 25:255-262.

Bouchez M, Blanchet D, Vandecasteele J-P 1996. The microbiological fate of polycyclic aromatic hydrocarbons: carbon and oxygen balances for bacterial degradation of model compounds. Applied Microbiology and Biotechnology. 45:556-561.

Breese K, Fuchs G 1998. 4-hydroxybenzoyl-CoA reductase (dehydroxylating) from the denitrifying bacterium *Thauera aromatica*. Prosthetic groups, electron donor, and genes of a member of the moylbdenum-flavin-iron-sulfur proteins. European Journal of Biochemistry. 251:916-923.

Bregnard TP-A, Häner A, Höhener P, Zeyer J 1997. Anaerobic degradation of pristane in nitrate-reducing microcosms amd enrichment cultures. Applied and Environmental Microbiology. 63:2077-2081.

Britton LN. 1984. Microbial degradation of aliphatic hydrocarbons, p. 89-129. *In* Gibson DT (ed.), Microbial degradation of organic compounds. Marcel Dekker, New York, Basel.

Caldwell ME, Garrett RM, Prince RC, Suflita JM 1998. Anaerobic biodegradation of long-chain *n*-alkanes under sulfate-reducing conditions. Environmental Science and Technology. 32:2191-2195.

Canfield DE. 1993. Organic matter oxidation in marine sediments, p. 333-363. *In* Wollast RM, F.T.; Chou, L. (ed.), Interactions of C, N, P and S Biogeochemical Cycle and Global Change, vol. I. Springer-Verlag, Berlin.

Carothers WW, Kharaka YK 1987. Aliphatic acid anions in oil-field waters - implication for origin of natural gas. The American Association of Petroleum Geologists Bulletin. 62:2441-2453.

Casellas M, Griffol M, Bayona JM, Solanas AM 1997. New metabolites in the degradation of fluorene by *Arthrobacter* sp. strain F101. Applied and Environmental Microbiology. 63:819-826.

Cerniglia CE 1992. Biodegradation of polycyclic aromatic hydrocarbons. Biodegradation. 3:351-368.

Chen CI, Taylor RT 1997. Thermophilic biodegradation of BTEX by two consortia of anaerobic bacteria. Applied Microbiology and Biotechnology. 48:121-128.

Chen J, Henderson G, Grimm CC, Lloyd SW, Laine RA 1998. Termites fumigate their nests with naphthalene. Nature. 392:558-559.

Coates JD, Anderson RT, Lovley DR 1996. Oxidation of polycyclic aromatic hydrocarbons under sulfate-reducing conditions. Applied and Environmental Microbiology. 62:1099-1101.

Coates JD, Woodward J, Allen J, Philp P, Lovley DR 1997. Anaerobic degradation of polycyclic aromatic hydrocarbons and alkanes in petroleum-contaminated marine harbor sediments. Applied and Environmental Microbiology. 63:3589-3593.

Connan J 1984. Biodegradation of crude oils in reservoirs. Advances in Petroleum Geochemistry. 1:299-333.

Cord-Ruwisch R, Kleinitz W, Widdel F 1987. Sulfate-reducing bacteria and their activities in oil production. Journal of Petroleum Technology. January 1987:97-106.

Davydova-Charakhck´yan IA, Kuznetsova VG, Mityushina LL, Belyaev SS 1993. Methane-froming bacilli from oil fields of Tataria and western Siberia. Microbiology. 61:202-207.

Dean BJ 1978. Genetic toxicology of benzene, toluene, xylene and phenols. Mutation Research. 47:75-97.

Dennis M, Kolattukudy PE 1992. A cobalt-porphyrin enzyme converts a fatty aldehyde to a hydrocarbon and CO. Proceedings of the National Academy of Science, U.S.A. 89:5306-5310.

Dietrich G, Kalle K, Kraus W, Siedler G 1975. Allgemeine Meereskunde, 3. Auflage ed. Gebrüder Borntraeger, Berlin, Stuttgart.

Dobson SJ, Franzmann PD 1996. Unification of the genera *Deleya* (Baumann et al. 1983), *Halomonas* (Vreeland et al. 1980), and *Halovibrio* (Fendrich 1988) and the species *Paracoccus halodenitrificans* (Robinson and Gibbons 1952) into a single genus, *Halomonas*, and placement of the genus *Zymobacter* in the family *Halomonadaceae*. International Journal of Systematic Bacteriology. 46:550-558.

Dolfing J, Zeyer J, Binder-Eicher P, Schwarzenbach RP 1990. Isolation and characterization of a bacterium that mineralizes toluene in the abscene of molecular oxygen. Archives of Microbiology. 154:336-341.

Edwards EA, Grbic-Galic D 1992. Complete mineralization of benzene by aquifer microorganisms under strictly anaerobic conditions. Applied and Environmental Microbiology. 58:2663-2666.

Edwards EA, Grbic-Galic D 1994. Anaerobic degradation of toluene and *o*-xylene by a methanogenic consortium. Applied and Environmental Microbiology. 60:313-322.

Edwards EA, Wills LE, Reinhard M, Grbic-Galic D 1992. Anaerobic degradation of toluene and xylene by aquifer microorganisms under sulfate-reducing conditions. Applied and Environmental Microbiology. 58:794-800.

Eganhouse RP, Baedecker MJ, Cozzarelli IM, Aiken GR, Thorn KA, Dorsey TF 1993. Crude oil in a shallow sand and gravel aquifer-II. Organic geochemistry. Applied Geochemistry. 8:551-567.

Eglinton G 1968. Hydrocarbons and fatty acids in living organisms and recent and ancient sediments, p. 1-24. *In* Schenck PA and Hayenaar I (ed.), Advances in Organic Geochemistry, vol. 31. Pergamon, New York.

Eglinton G 1973. Chemical fossils: a combined organic geochemical and environmental approach. Pure Applied Chemistry. 34:611-632.

Ehrenreich P. 1996. Dissertation. University of Bremen, Shaker Verlag, Aachen.

Ekzertsev VA 1960. Production of methane by mircoorganisms in petroleum deposits. Geochemistry. 4:432-442.

Elias VO, Simoneit BRT, Cardoso JN 1997. Even *n*-alkane predominances on the Amazon shelf and a northeast Pacific hydrothermal system. Naturwissenschaften. 84:415-420.

Engesser KH, Strubel V, Christoglou K, Fischer P, Rast HG 1989. Dioxygenolytic cleavage of aryl ether bonds: 1,10-dihydro-1,10-dihydroxyfluoren-9-one, a novel arene dihydrodiol as evidence for angular dioxygenation of dibenzofuran. FEMS Microbioly Letters. 65:205-210.

Evans PJ, Ling W, Goldschmidt B, Ritter ER, Young LY 1992. Metabolites formed during anaerobic transformation of toluene and *o*-xylene and their proposed relationship to the initial steps of toluene mineralization. Applied and Environmental Microbiology. 58:496-501.

Evans PJ, Mang DT, Kim KS, Young LY 1991a. Anaerobic degradation of toluene by a denitrifying bacterium. Applied and Environmental Microbiology. 57:1139-1145.

Evans PJ, Mang DT, Young LY 1991b. Degradation of toluene and *m*-xylene and transformation of *o*-xylene by denitrifying enrichment cultures. Applied and Environmental Microbiology. 57:450-454.

Fischer-Romero C, Tindall BJ, Jüttner F 1996. *Tolumonas auensis* gen. nov., sp. nov., a toluene-producing bacterium from anoxic sediments of a freswater lake. International Journal of Systematic Bacteriology. 46:183-188.

Fishbein L 1985. An overview of environmental and toxicological aspects of aromatic hydrocarbons II. Toluene. The Science of the Total Environment. 42:267-288.

Foss S, Heyen U, Harder J 1998. *Alcaligenes defragrans* sp. nov., description of four strains isolated on alkenoic monoterpenes ((+)-menthene, α-pinene, 2-carene, and α-phellandrene) and nitrate. Systematic and Applied Microbiology. 21:237-244.

Fries MR, Zhou J, Chee-Sanford J, Tiedje JM 1994. Isolation, characterization, and distribution of denitrifying toluene degraders from a variety of habitats. Applied and Environmental Microbiology. 60:2802-2810.

Fuchs G, Thsuer R, Ziegler H, Stichler W 1979. Carbon isotope fractionation by *Methanobacterium thermoautotrophicum*. Archives of Microbiology. 120:135-139.

Fukui M, Boreham C, Harms G, Rabus R, Schramm A, Widdel W, Wilkes H, Zengler K 1998. Presented at the Eighth International Symposium on Microbial Ecology (ISME), Halifax, Canada.

Galushko A, Minz D, Schink B, Widdel F 1999. Anaerobic degradation of naphthalene by a pure culture of a novel type of marine sulfate-reducing bacterium. Environmental Microbiology:submitted.

Galushko AS, Rozanova EP 1991. *Desulfobacterium cetonicum* sp. nov.: a sulphate-reducing bacterium which oxidizes fatty acids and ketones. Microbiology. 60:102-107.

Ganahl P, Kleinitz W 1983. Sulfatreduzierende Bakterien in der Rohölförderung. Erdoel Erdgas. 99. Jahrg.:241-251.

Gauthier MJ, Lafay B, Christen R, Fernandez L, Acquaviva M, Bonin P, Bertrand J-C 1992. *Marinobacter hydrocarbonoclasticus* gen. nov., sp. nov., a new, extremly halotolerant, hydrocarbon-degrading marine bacterium. International Journal of Systematic Bacteriology. 42:568-576.

Gelwicks JT, Risatti JB, Hayes JM 1994. Carbon isotope effects associated with acetoclastic methanogenesis. Applied and Environmental Microbiology. 60:467-472.

Gibson DT, Subramanian V 1984. Microbial degradation of aromatic hydrocarbons, p. 181-252. *In* Gibson DT (ed.), Microbial degradation of aromatic compounds. Marcel Dekker, Inc., New York.

Giger W, Schaffner C, Wakeham SG 1980. Aliphatic and olefinic hydrocarbons in recent sediments of Greifensee, Switzerland. Geochimica et Cosmochimica Acta. 44:119-129.

Gildemeister E, Hoffmann F 1960. Aromatische Kohlenwasserstoffe: (a) Benzoide Aromaten, *p*-Cymol, p. 402-411, Die ätherischen Öle, vol. IIIa. Akademischer Verlag, Berlin.

Gilewicz M, Monpert G, Acquaviva M, Mille G, Bertrand J-C 1991. Anaerobic oxidation of 1-*n*-heptadecene by a marine denitrifying bacterium. Applied Microbiology and Biotechnology. 36:252-256.

Grbic-Galic D, Vogel TM 1987. Transformation of toluene and benzene by mixed methanogenic cultures. Applied and Environmental Microbiology. 53:254-260.

Grifoll M, Casellas M, Bayona JM, Solanas AM 1992. Isolation and characterization of a fluorene-degrading bacterium: identification of ring oxidation and ring fission products. Applied and Environmental Microbiology. 58:2910-2917.

Grifoll M, Selifonov SA, Chapman PJ 1994. Evidence for a novel pathway in the degradation of fluorene by *Pseudomonas* sp. strain F274. Applied and Environmental Microbiology. 60:2438-2449.

Grifoll M, Selifonov SA, Chapman PJ 1995a. Transformation of substituted fluorenes and fluorene analogy by *Pseudomonas* sp. strain F274. Applied and Environmental Microbiology. 61:3490-3493.

Grifoll M, Selifonov SA, Gatlin CV, Chapman PJ 1995b. Actions of a versatile fluorene-degrading bacterial isolate on polycyclic aromatic compounds. Applied and Environmental Microbiology. 1:3711-3723.

Gundlach ER, Boehm PD, Marchand M, Atlas RM, Ward DM, Wolfe DA 1983. The fate of *Amico Cadiz* oil. Science. 221:122-129.

Häner A, Höhener P, Zeyer J 1995. Degradation of *p*-xylene by a denitrifying enrichment culture. Applied and Environmental Microbiology. 61:3185-3188.

Häner A, Höhener P, Zeyer J 1997. Degradation of trimethylbenzene isomers by an enrichment culture under N_2O-reducing conditions. Applied and Environmental Microbiology. 63:1171-1174.

Harms G, Rabus R, Widdel F 1999a. Anaerobic oxidation of the aromatic hydrocarbon, *p*-isopropyltoluene (*p*-cymene) by two new types of denitrifying bacteria. Archives of Microbiology:in press.

Harms G, Zengler K, Rabus R, Aeckersberg F, Minz D, Roselló-Mora R, Widdel F 1999b. Anaerobic oxidation of *o*-xylene, *m*-xylene, and homologous alkybenzenes by new types of sulfate-reducing bacteria. Applied and Environmental Microbiology. 65:999-1004.

Harwood C, Gibson J 1988. Anaerobic and aerobic metabolism of diverse aromatic compounds by the photosynthetic bacterium *Rhodopseudomonas palustris*. Applied and Environmental Microbiology. 54:712-717.

Hayes JM 1993. Factors controlling ^{13}C contents of sedimentary organic compounds: principles and evidence. Marine Geology. 113:111-115.

Heider J, Spormann AM, Beller HR, Widdel F 1999. Anaerobic bacterial metabolism of hydrocarbons. FEMS Microbiology Reviews. 22:459-473.

Hess A, Zarda B, Hahn D, Häner A, Stax D, Höhener P, Zeyer J 1997. In situ analysis of denitrifying toluene- and *m*-xylene-degrading bacteria in a diesel fuel-contaminated laboratory aquifer column. Applied and Environmental Microbiology. 63:2136-2141.

Hunt JM 1979. Petroleum Geochemistry and Geology. W.H. Freeman and Company, San Francisco.

Hutchins SR 1991. Biodegradation of monoaromatic hydrocarbons by aquifer microorganisms using oxygen, nitrate, or nitrous oxide as terminal electron acceptor. Applied and Environmental Microbiology. 57:2403-2407.

Hylemon PB, Harder J 1999. Biotransformation of monoterpenes, bile acids, and other isoprenoids in anaerobic ecosystems. FEMS Microbiology Reviews. 22:475-488.

Ivanov MV, Belyaev SS, Laurinavichus KS, Obraztsova AY, Gorlatov SN 1982. Distribution and geochemical activity of microorganisms in a flooded oil field. Microbiology. 51:282-286.

Jobson AM, Westlake DWS 1979. Interaction of aerobic and anaerobic bacteria in petroleum biodegradation. Chemical Geology. 24:355-365.

Jørgensen BB 1982. Mineralization of organic matter in the sea bed - the role of sulphate reduction. Nature. 296:643-645.

Jørgensen BB 1983. Processes at the sediment-water interface, p. 477-509. *In* Bolin B and Cook RB (ed.), The Major Biogeochemical Cycles and Their Interactions. J. Wiley & Sons, New York.

Jørgensen C, Nielsen B, Jensen BK, Mortensen E 1995. Transformation of *o*-xylene to *o*-methyl benzoic acid by a denitrifying enrichment culture using toluene as the primary substrate. Biodegradation. 6:141-146.

Jüttner F, Henatsch JJ 1986. Anoxic hypolimnion is a significant source of biogenic toluene. Nature. 323:797-798.

Kalberlah F, Frijus-Plessen N, Hassauer M 1995. Toxikologische Kriterien für die Gefährdungsabschätzung von polyzyklischen aromatischen Kohlenwasserstoffen (PAK) in Altlasten. Altlasten Spektrum. 5:231-237.

Kawka OE, Simoneit BRT 1987. Survey of hydrothermally-generated petroleums from the Guaymas Basin spreading center. Organic Geochemistry. 11:311-328.

Kazumi J, Caldwell ME, Suflita JM, Lovley DR, Young LY 1997. Anaerobic degradation of benzene in diverse anoxic environments. Environmental Science and Technology. 31:813-818.

Knowles R 1982. Denitrification. Microbiological Reviews. 46:43-70.

Koch R, Wagner BO 1989. Umweltchemikalien: Physikalische-chemische Daten, Toxizitäten, Grenz- und Richtwerte, Umweltverhalten. VCH Verlagsgesellschaft, Weinheim.

Krzycki JA, Kenealy WR, DeNiro MJ, Zeikus JG 1987. Stable carbon isotope fractionation by *Methanosarcina barkeri* during methnaogenesis from acetate, methanol, or carbon dioxide-hydrogen. Applied and Environmental Microbiology. 53:2597-2599.

Kuhn EP, Zeyer J, Eicher P, Schwarzenbach RP 1988. Anaerobic degradation of alkylated benzenes in denitrifying laboratory aquifer colomns. Applied and Environmental Microbiology. 54:490-496.

Lafay B, Ruimy R, Rausch de Traubenberg C, Breitmayer V, Gauthier MJ, Christen R 1995. *Roseobacter algicola* sp. nov., a new marine bacterium isoalted from the phycosphere of the toxin-producing dinoflagellate *Prorocentrum lima*. International Journal of Systematic Bacteriology. 45:290-296.

Langenhoff AAM, Nijenhuis I, Tan NCG, Briglia M, Zehnder AJB, Schraa G 1997. Characterisation of a manganese-reducing, toluene-degrading enrichment culture. FEMS Microbiology Ecology. 24:113-125.

Langenhoff AAM, Zehnder AJB, Schraa G 1996. Behavior of toluene, benzene and naphthalene under anaerobic conditions in sediment columns. Biodegradation. 7:267-274.

Lee RF, Page DS 1997. Petroleum hydrocarbons and their effects in subtidal regions after major oil spills. Marine Pollution Bulletin. 34:928-940.

Leuthner B, Leutwein C, Schulz H, Hörth P, Haehnel W, Schiltz E, Schäffer H, Heider J 1998. Biochemical and genetic characterization of benzylsuccinate synthase from *Thauera aromatica*: a new glycyl radical enzyme catalysing the frist step in anaerobic toluene metabolism. Molecular Microbiology. 28:615-628.

Lichtfouse É, Bardoux G, Mariotti A, Balesdent J, Ballentine DC, Macko SA 1997. Molucular, ^{13}C, and ^{14}C evidence for the allochthonous and ancient origin of C_{16}-C_{18} *n*-alkanes in modern soils. Geochimica et Cosmochimica Acta. 61:1891-1898.

Lovley DR, Baedecker MJ, Lonergan DJ, Cozzarelli IM, Phillips EJP, Siegel DI 1989. Oxidation of aromatic contaminants coupled to microbial iron reduction. Nature. 339:297-299.

Lovley DR, Coates JD, Woodward JC, Phillips EJP 1995. Benzene oxidation coupled to sulfate reduction. Applied and Environmental Microbiology. 61:953-958.

Lovley DR, Lonergan DJ 1990. Anaerobic oxidation of toluene, phenol, and *p*-cresol by the dissimilatory iron-reducing organism, GS-15. Applied and Environmental Microbiology. 56:1858-1864.

Lovley DR, Woodward JC, Chapelle FH 1994. Stimulated anoxic biodegradation of aromatic hydrocarbons using Fe(III) ligands. Nature. 370:128-131.

Lovley DR, Woodward JC, Chapelle FH 1996. Rapid anaerobic benzene oxidation with a variety of chelated Fe(III) forms. Applied and Environmental Microbiology. 62:288-291.

Macy JM, Rech S, Auling G, Dorsch M, Stackebrandt E, Sly LI 1993. *Thauera selenatis* gen. nov., sp. nov., a member of the beta subclass of *Proteobacteria* with a novel type of anaerobic respiration. International Journal of Systematic Bacteriology. 43:135-142.

Maidak BL, Olsen GJ, Larsen N, Overbeek R, McCaughey MJ, Woese CR 1997. The RDP (Ribosomal Database Project). Nucleic Acids Research. 25:109-110.

Major DW, Mayfield CI, Barker JF 1988. Biotransformation of benzene by denitrification in aquifer sand. Ground Water. 26:8-14.

Mango FD 1991. The stability of hydrocarbons under the time-temperature conditions of petroleum genesis. Nature. 352:146-148.

May WE 1980. The solubility behavior of polycyclic aromatic hydrocarbons in aqueous systems, p. 143-192. *In* Petrakis LaFTW (ed.), Petroleum in the marine environment. American Chemical Society, Washington, D.C.

McNally DL, Mihelcic JR, Lueking DR 1998. Biodegradation of three- and four-ring polycyclic aromatic hydrocabons under aerobic and denitrifying conditions. Environmental Science and Technology. 32:2633-2639.

Means JC, Wood SG, Hasset JJ, Banwart WL 1980. Sorption of polynuclear aromatic hydrocarbons by sediments and soils. Environmental Science and Technology. 14:1524-1528.

Means JL, Hubbard N 1987. Short-chain aliphatic acid anions in deep subsurface brines: A review of their origin, occuuence, properties, and importance and new data on their distribution and geochemical implications in the Palo Duro Basin, Texas. Organic Geochemistry. 11:177-191.

Michotey V, Bonin P 1997. Evidence for anaerobic bacterial processes in the water column: denitrification and dissimilatory nitrate ammonification in the northwestern Mediterranean Sea. Marine Ecology Progress Series. 160:47-56.

Mihelcic JR, Luthy RG 1988a. Degradation of polycyclic aromatic hydrocarbon compounds under various redox conditions in soil-water systems. Applied and Environmental Microbiology. 54:1182-1187.

Mihelcic JR, Luthy RG 1988b. Microbial degradation of acenaphthene and naphthalene under denitrification conditions in soil-water systems. Applied and Environmental Microbiology. 54:1188-1198.

Monna L, Omori T, Kodama T 1993. Microbial degradation of dibenzofuran, fluorene, and dibenzo-*p*-dioxin by *Staphylococcus auriculans* DBF63. Applied and Environmental Microbiology. 59:285-289.

Morgan P, Watkinson RJ 1994. Biodegradation of components of petroleum, p. 1-32. *In* Ratledge C (ed.), Biochemistry of Microbial Degradation. Kluwer Academic Publishers, Dordrecht.

Mueller RF, Nielsen PH 1996. Charaterization of thermophilic consortia from two souring oil reservoirs. Applied and Environmental Microbiology. 62:3083-3087.

Nazina TN 1984. Communities of methane-producing bacteria from Apsheron oil formations. Microbiology. 53:122-127.

Nazina TN, Ivanova AE, Borzenkov IA, Belyaev SS, Ivanov MV 1995. Occurrence and geochemical activity of microorganisms in high-temperature, water-flooded oil fields of Kazakhstan and Western Siberia. Geomicrobiology Journal. 13:181-192.

Nazina TN, Rosanova EP, Kuznetsov SI 1985. Microbial oil transformation processes accompained with methane and hydrogen-sulfide formation. Geomicrobiology Journal. 4:103-130.

Nazina TN, Rozanova EP 1980. Ecological conditions for the occurrence of methane-producing bacteria in oil-bearing strata in Apsheron. Microbiology. 49:104-109.

Nilsen RK, Beeder J, Thorstenson T, Torsvik T 1996. Distribution of thermophilic marine sulfate reducers in North Sea oid field waters and oil reservoirs. Applied and Environmental Microbiology. 62.

Nilsen RK, Torsvik T 1996. *Methanococcus thermolithotrophicus* isolated from North Sea oil field reservoir water. Applied and Environmental Microbiology. 62:728-731.

Obraztsova AY, Ratner EN, Nelyaev SS, Ivmnov MV 1984. Fine structure of methane-producing baoteria isolated from an oil deposit. Microbiology. 53:374-378.

Odom JM. 1993. Industrial and environmental activities of sulfate-reducing bacteria, p. 189-210. *In* Odom JM and Singleton RJr (eds.), The sulfate-reducing bacteria: contemporary perspectives. Springer-Verlag, New York.

Ollivier B, Fardeau M-L, Cayol J-L, Magot M, Patel BKC, Prensier G, Garcia J-L 1998. *Methanocalculus halotolerans* gen. nov., sp. nov., isolated from an oil-producing well. International Journal of Systematic Bacteriology. 48:821-828.

Pfennig N, Eimhjellen KE, Liaaen Jensen S 1965. A new isolate of the *Rhodospirillum fulvum* group and its photosynthetic pigments. Archiv für Mikrobiologie. 51:258-266.

Phelps CD, Kazumi J, Young LY 1996. Anaerobic degradation of benzene in BTX mixtures dependent on sulfate reduction. FEMS Microbiology Letters. 145:433-437.

Phelps CD, Kerkhof LJ, Young LY 1998. Molecular characterization of a sulfate-reducing consortium which mineralizes benzene. FEMS Microbiology Ecology. 27:269-279.

Rabus R, Fukui M, Wilkes H, Widdel F 1996. Degradative capacities and 16S rRNA-targeted whole-cell hydridization of sulfate-reducing bacteria in an anaerobic enrichment culture utilizing alkylbenzenes from crude oil. Applied and Environmental Microbiology. 62:3605-3613.

Rabus R, Heider J 1998. Initial reactions of anaerobic metabolism of alkylbenzenes in denitrifying and sulfate-reducing bacteria. Archives of Microbiology. 170:377-384.

Rabus R, Nordhaus R, Ludwig W, Widdel F 1993. Complete oxidation of toluene under strictly anoxic conditions by a new sulfate-reducing bacterium. Applied and Environmental Microbiology. 59:1444-1451.

Rabus R, Widdel F 1995. Anaerobic degradation of ethylbenzene and other aromatic hydrocarbons by new denitrifying bacteria. Archives of Microbiology. 163:96-103.

Rabus R, Wilkes H, Schramm A, Harms G, Behrends A, Amann R, Widdel F 1999. Anaerobic utilisation of alkylbenzenes and *n*-alkanes from crude oil in an enrichment culture of denitrifying bacteria affiliated with *Azoarcus/Thauera* cluster. Environmental Microbiology. in press.

Radwan S, Sorkhoh N, El-Nemir I 1995. Oil biodegradation around roots. Nature. 376:302.

Reid BJ, Semple KT, Macleod CJ, Weitz HJ, Paton GI 1998. Feasibility of using prokaryote biosensors to assess acute toxicity of polycyclic aromatic hydrocarbons. FEMS Microbiology Letters. 169:227-233.

Resnick SM, Gibson DT 1996. Regio- and stereospecific oxidation of fluorene, dibenzofuran, and dibenzothiophene by naphthalene dioxygenase from *Pseudomonas* sp. strain NCIB 9816-4. Applied and Environmental Microbiology. 62:4073-4080.

Revsbech NP, Jørgensen BB, Blackburn PH 1980. Oxygen in the sea bottom measured with a microelectrode. Science. 207:1355-1356.

Rhodes ME, Best A, Payne WJ 1963. Electron donors and cofactors fro denitrification by *Pseudomonas perfectomarinus*. Canadian Journal of Microbiology. 9:799-807.

Rontani J-F, Gilewicz MJ, Michotey VD, Zheng TL, Bonin PC, Bertrand J-C 1997. Aerobic and anaerobic metabolism of 6,10,14-trimethylpentadecan-2-one by a denitrifying bacterium isolated from marine sediments. Applied and Environmental Microbiology. 63:636-643.

Rosenfeld WD 1947. Anaerobic oxidation of hydrocarbons by sulfate-reducing bacteria. Journal of Bacteriology. 54:664-665.

Rosnes JT, Torsvik T, Lien T 1991. Spore-forming thermophilic sulfate-reducing bacteria isolated from North Sea oil field waters. Applied and Environmental Microbiology. 57:2302-2307.

Rozanova EP, Nazina TN, Kulik ES, Somov YP 1985. Methane production from hexadecane by microorganisms. Mikrobiologiya. 54:555-559.

Rueter P, Rabus R, Wilkes H, Aeckersberg F, Rainey FA, Jannasch H, Widdel F 1994. Anaerobic oxidation of hydrocarbons in crude oil by new types of sulphate-reducing bactera. Nature. 372:455-458.

Rüger H-J, Höfle MG 1992. Marine star-shaped-aggregate-forming bacteria: *Agrobacterium atlanticum* sp. nov.; *Agrobacterium meteori* sp. nov.; *Agrobacterium ferrugineum* sp. nov., nom. rev.; *Agrobacterium gelatinovorum* sp. nov., nom. rev.; and *Agrobacterium stellulatum* sp. nov., nom. rev. International Journal of Systematic Bacteriology. 42:133-143.

Sackett WM, Conkright ME 1997. Summary and re-evaluation of the high-temperature isotope geochemistry of methane. Geochimica et Cosmochimica Acta. 61:1941-1952.

Santos Neto EV, Hayes JM, Takaki T 1998. Isotopic biogeochemistry of the neocomian lacustrine and upper aptian marine-evaporitic sediments of the Potiguar Basin, Northeastern Brazil. Organic Geochemistry. 28:361-381.

Sauer TCJ 1980. Volatile liquid hydrocarbons in waters of the Gulf of Mexico and Caribian Sea. Limnology and Oceanography. 25:338-351.

Schink B 1985a. Degradation of unsaturated hydrocarbons by methanogenic enrichment cultures. FEMS Microbiology Ecology. 31:69-77.

Schink B 1985b. Fermentation of acetylene by an obligate anaerobic *Pelobacter acetylenicus* sp. nov. Archives of Microbiology. 142:295-301.

Schmidt K-H, Romey I. 1981. Kohle - Erdöl - Erdgas. Vogel-Verlag, Würzburg.

Schmitt R, Langguth H-R, Püttmann W, Rohns HP, Eckert P, Schubert J 1996. Biodegradation of aromatic hydrocarbons under anoxic conditions in a shallow sand and gravel aquifer of the Lower Rhine Valley, Germany. Organic Geochemistry. 25:41-50.

Schocher RJ, Seyfried B, Vazquez F, Zeyer J 1991. Anaerobic degradation of toluene by pure cultures of denitrifying bactera. Archives of Microbiology. 157:7-12.

Schoell M 1983. Genetic characterization of natural gas. The American Association of Petroleum Geologists Bulletin. 67:2225-2238.

Scott AR, Kaiser WR, Ayers WD, Jr. 1994. Thermogenic and secondary biogenic gases, San Juan Basin, Colorado and New Mexico - Implications for coalbed gas producibility. The American Association of Petroleum Geologists Bulletin. 78:1186-1209.

Selifonov SA, Grifoll M, Eaton RW, Chapman PJ 1996. Oxidation of naphthenoaromatic and methyl-substituted aromatic compounds by naphthalene 1,2-dioxygenase. Applied and Environmental Microbiology. 62:507-514.

Shelton TB, Hunter JH 1975. Anaerobic decomposition of oil in bottom sediments. Journal of the Water Pollution Control Federation. 47:2256-2270.

Siefert E, Irgens RL, Pfennig N 1978. Phototrophic purple and green bacteria in a sewage treatment plant. Applied and Environmental Microbiology. 35:38-44.

Sikkema J, De Bont JAM, Poolman B 1995. Mechanisms of membrane toxicity of hydrocarbons. Microbiological Reviews. 59:201-222.

Simoneit BRT, Galimov EM 1984. Geochemistry of interstitial gases in quanternary sediments of the Gulf of California. Chemical Geology. 43:151-166.

Simoneit BRT, Lonsdale PF 1982. Hydrothermal petroleum in mineralized mounds at the seabed of Guaymas Basin. Nature. 295:198-202.

Sørensen J, Jørgensen BB, Revsbech NP 1979. A comparison of oxygen, nitrate, and sulfate respiration in coastal marine sediments. Microbial Ecology. 5:105-115.

Stetter KO, Huber R, Blöchl E, Kurr M, Eden RD, Fleider M, Cash H, Vance I 1993. Hyperthermophilic archaea are thriving in deep North Sea and Alaskan oil reservoirs. Nature. 365:743-745.

Stringfellow WT, Aitken MD 1995. Competitive metabolism of naphthalene, methylnaphthalenes, and fluoren by phenanthrene-degrading pseudomonads. Applied and Environmental Microbiology. 61:357-362.

Su J-J, Kafkewitz D 1994. Utilization of toluene and xylene by a nitrate-reducing strain of *Pseudomonas maltophilia* under low oxygen and anoxic conditions. FEMS Microbiology Ecology. 15:249-258.

Swannell RPJ, Lee K, McDonagh M 1996. Field evaluations of marine oil spill bioremediation. Microbiological Reviews. 60:342-365.

Sykes P 1988. Reaktionsmechanismen der Organischen Chemie, 9 ed. VCH, Weinheim.

Tan YL, Kong A, Monetti MA 1996. Biogenic polycyclic aromatic hydrocarbons in an Alaskan artic lake sediment. Polycyclic Aromatic Compounds. 9:185-192.

Thamdrup B, Canfield DE 1996. Pathways of carbon oxidation in continental margin sediments off central Chile. Limnology and Oceanography. 41:1629-1650.

Tissot B, Welte D. 1984. Petroleum Formation and Occurence, 2nd edn. Springer-Verlag, New York.

Trenz SP, Engesser KH, Fischer P, Knackmuss H-J 1994. Degradation of fluorene by *Brevibacterium* sp. strain DPO 1361: a novel C-C bond cleavage mechanism via 1,10-dihydro-1,10-dihydroxyfluoren-9-one. Journal of Bacteriology. 176:789-795.

Tschech A, Fuchs G 1989. Anaerobic degradation of phenol via carboxylation to 4-hydroxybenzoate: in vitro study of isotope exchange between $^{14}CO_2$ and 4-hydroxybenzoate. Archives of Microbiology. 152:594-599.

US Environmental Protection Agency 1986. Underground motor fuel storage tanks: a national survey. Publication NTIS PB 86-216512. U.S. Environmental Protection Agency, Washington, D.C.

Valderrama MJ, Quesada E, Bejar V, Ventosa A, Gutierrez MC, Ruiz-Berraquero F, Ramos-Cormenzana A 1991. *Deleya salina* sp. nov., a moderately halophilic gram-negative bacterium. International Journal of Systematic Bacteriology. 41:377-384.

Vanloocke R, De Borger R, Voets JP, Verstraete W 1975. Soil and groundwater contamination by oil spills, problems and remedies. International Journal of Environmental Studies. 8:99-111.

Vogel TM, Gribic-Galic D 1986. Incorporation of oxygen from water into toluene and benzene during anaerobic fermentative transformation. Applied and Environmental Microbiology. 52:200-202.

Voordouw G, Armstrong SM, Reimer MF, Fouts B, Telang AJ, Shen Y, Gevertz D 1996. Characterization of 16S rRNA genes from oil field microbial communities indicates the presence of a variety of sulfate-reducing, fermentative, and sulfide-oxidizing bacteria. Applied and Environmental Microbiology. 62:1623-1629.

Voordouw G, Voordouw JK, Jack TR, Foght J, Fedorak PM, Westlake DWS 1992. Identification of distinct communities of sulfate-reducing bacteria in oil fields by reverse sample genome probing. Applied and Environmental Microbiology. 58:3542-3552.

Wakeham SG 1990. Algal and bacterial hydrocarbons in particulate matter and interfacial sediment of the Cariaco Trench. Geochimica et Cosmochimica Acta. 54:1325-1336.

Wakeham SG 1996. Aliphatic and polycyclic aromatic hydrocarbons in Black Sea sediments. Marine Chemistry. 53:187-205.

Wakeham SG, Schaffner C, Giger W 1980a. Polycyclic aromatic hydrocarbons in recent lake sediments - I. Compounds having anthropogenic origins. Geochimica et Cosmochimica Acta. 44:403-413.

Wakeham SG, Schaffner C, Giger W 1980b. Polycyclic aromatic hydrocarbons in recent lake sediments - II. Compounds derived from biogenic precursors during early diagenesis. Geochimica et Cosmochimica Acta. 44:415-429.

Wang Y-S, Barlaz MA 1998. Anaerobic biodegradability of alkylbenzenes and phenol by landfill derived microorganisms. FEMS Microbiology Ecology. 25:405-418.

Weiner JM, Lovley DR 1998a. Anaerobic benzene degradation in petroleum-contaminated aquifer sediments after inoculation with a benzene-oxidizing enrichment. Applied and Environmental Microbiology. 64:775-778.

Weiner JM, Lovley DR 1998b. Rapid benzene degradation in methanogenic sediments from a petroleum-contaminated aquifer. Applied and Environmental Microbiology. 64:1937-1939.

Weissenfels WD, Beyer M, Klein J 1990. Degradation of phenanthrene, fluorene and fluoranthene by pure bacterial cultures. Applied Microbiology and Biotechnology. 32:479-484.

Whitman WB, Bowen TC, Boone DR. 1992. The methanogenic bacteria, p. 719-767. *In* Balows AT, Trüper HG, Dworkin, M, Harder W, Schleifer KH (ed.), The Prokaryotes, 2 ed, vol. 1. Springer-Verlag, Berlin Heidelberg New York.

Whittle PJ, Lunt DO, Evans WC 1976. Anaerobic photometabolism of aromatic compounds by *Rhodopseudomonas* sp. Biochemical Society Transactions. 4:490-491.

Widdel F 1988. Microbiology and ecology of sulfate- and sulfur-reducing bacteria, p. 469-585. *In* Zehnder AJB (ed.), Biology of Anaerobic Microorganisms. John Wiley & Sons, New York.

Widdel F, Bak F 1992. Gram-negative mesophilic sulfate-reducing bacteria, p. 3352-3392. *In* Balows AT, Trüper HG, Dworkin, M, Harder W, Schleifer KH (ed.), The Prokaryotes, 2 ed, vol. 4. Springer-Verlag, Berlin Heidelberg New York.

Widdel F, Hansen TA. 1992. The dissimilatory sulfate- and sulfur-reducing bacteria, p. 583-624. *In* Balows AT, Trüper HG, Dworkin, M, Harder W, Schleifer KH (ed.), The Prokaryotes, 2 ed, vol. 4. Springer-Verlag, Berlin Heidelberg New York.

Willmes A 1990. Textbuch chemische Substanzen, vol. 1. Aufl. Harri Deutsch, Frankfurt a. Main

GE, Madigan MT 1991. Photocatabolism of aromatic compounds by the phototrophic purple bacterium *Rhodomicrobium vannielii.* Applied and Environmental Microbiology. 57:2069-2073.

Yang Y, Chen RF, Shiaris MP 1994. Metabolism of naphthalene, fluorene, and phenanthrene: preliminary characterization of a cloned gene cluster from *Pseudomonas putida* NCIB 9816. Journal of Bacteriology. 176:2158-2164.

Yunker MB, Snowdon LR, Macdonald, Smith JN, Fowler MG, Skibo DN, McLaughlin FA, Danyushevskaya AI, Petrova VI, Ivanov GI 1996. Polycyclic aromatic hydrocarbon composition and potential sources for sediment samples from Beaufort and Barents Seas. Environmental Science and Technology. 30:1310-1320.

Zarda B, Mattison G, Hess A, Hahn D, Höhener P, Zeyer J 1998. Analysis of bacterial and protozoan communities in an aquifer contaminated with monoaromatic hydrocarbons. FEMS Microbiology Ecology. 27:141-152.

Zeyer J, Kuhn EP, Schwarzenbach RP 1986. Rapid microbial mineralization of toluene and 1,3-dimethylbenzene in the absence of molecular oxygen. Applied and Environmental Microbiology. 52:944-947.

Zhang X, Young LY 1997. Carboxylation as an initial reaction in the anaerobic metabolism of naphthalene and phenanthrene by sulfidogenic consortia. Applied and Environmental Microbiology. 63:4759-4764.

Zhou J, Palumbo AV, Tiedje JM 1997. Sensitive detection of a novel class of toluene-degrading denitrifiers, *Azoarcus tolulyticus*, with small-subunit rRNA primers and probes. Applied and Environmental Microbiology. 63:2384-2390.

Zimmer W 1997. Funktion und Bedeutung der Isoprenabgabe durch Pflanzen. Biologie in unserer Zeit. 27:34-39.

ZoBell CE 1946. Action of microorganisms on hydrocarbons. Bacteriological Reviews. 10:1-49.

Teil II: Publikationen

A Publikationsliste mit Erläuterungen

Die Dissertation beruht zum großen Teil auf den folgenden sechs Publikationen. Die angefügten Erläuterungen sollen meinen Beitrag an der jeweiligen Arbeit aufzeigen.

1. Phototrophic utilization of toluene under anoxic conditions by a new strain of *Blastochloris sulfoviridis*

Karsten Zengler, Johann Heider, Ramon Rosselló-Mora, and Friedrich Widdel

Entwicklung des Konzepts und Durchführung aller mikrobiologischen Arbeiten. Versuche mit zellfreien Extrakten in Zusammenarbeit mit J. Heider. Erstellung des Manuskripts unter redaktioneller Mitwirkung von F. Widdel.

2. Anaerobic, nitrate-dependent utilization of hydrocarbons by marine members of the alpha and gamma subclass of Proteobacteria

Karsten Zengler, Enric Llobet-Brossa, Thuy Hang Dinh, Gerda Harms, Thierry Nadalig, Hans Hermann Richnow, and Friedrich Widdel

Entwicklung des Konzepts. Durchführung der meisten mikrobiologischen Arbeiten und Beratung der übrigen Arbeiten. Erstellung des Manuskripts unter redaktioneller Mitwirkung von F. Widdel.

3. Anaerobic oxidation of *o*-xylene, *m*-xylene, and homologous alkylbenzenes by new types of sulfate-reducing bacteria

Gerda Harms, Karsten Zengler, Ralf Rabus, Frank Aeckersberg, Dror Minz, Ramon Rosselló-Mora, and Friedrich Widdel

Gemeinsame Entwicklung des Konzepts mit G. Harms und R. Rabus. Durchführung aller mikrobiologischen Versuche mit Stamm oXyS1. Gemeinsame Erstellung des Manuskripts mit G. Harms unter redaktioneller Mitwirkung von F. Widdel.

4. Methane formation from long-chain saturated hydrocarbons in an anaerobic microbial community

Karsten Zengler, Hans Hermann Richnow, Ramon Rosselló-Mora, Walter Michaelis, and Friedrich Widdel

Entwicklung des Konzepts. Durchführung aller mikrobiologischen Versuche. Erstellung des Manuskripts unter redaktioneller Mitwirkung von F. Widdel.

5. Anaerobic degradation of oil hydrocarbons by sulfate-reducing and nitrate-reducing bacteria

Manabu Fukui, Chris Boreham, Gerda Harms, Ralf Rabus, Andreas Schramm, Friedrich Widdel, Heinz Wilkes, and Karsten Zengler

Durchführung von mikrobiologischen Arbeiten. Redaktionelle Mitarbeit am Manuskript.

6. Anaerobic degradation and carbon isotopic fractionation of alkylbenzenes in crude oil by sulphate-reducing bacteria

Heinz Wilkes, Chris Boreham, Gerda Harms, Karsten Zengler, and Ralf Rabus

Gemeinsame Entwicklung des Konzepts mit H. Wilkes und R. Rabus. Gemeinsame Durchführung der mikrobiologischen Arbeiten mit G. Harms. Redaktionelle Mitarbeit am Manuskript.

B Publikationen

1

Phototrophic utilization of toluene under anoxic conditions by a new strain of *Blastochloris sulfoviridis*

Karsten Zengler[1*], Johann Heider[2], Ramon Rosselló-Mora[1], and Friedrich Widdel[1]

Archives of Microbiology, in press

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[1] Max-Planck-Institute for Marine Microbiology, Celsiusstr. 1, D-28359 Bremen, Germany

[2] Universität Freiburg, Institut für Biologie II, Mikrobiologie, Schänzlestrasse 1, D-79104 Freiburg, Germany

* Corresponding author

Abstract

The capacity of anoxygenic phototrophic bacteria to utilize aromatic hydrocarbons was investigated in enrichment cultures with toluene. When mineral medium with toluene (provided in an inert carrier phase) was inoculated with activated sludge and incubated under infrared illumination (> 750 nm), a red to brownish culture developed. Agar dilution series indicated the dominance of two types of phototrophic bacteria. One type formed red colonies, had rod-shaped cells with budding division, and grew on benzoate but not on toluene. The other type formed yellow to brown colonies, had oval cells, and utilized toluene and benzoate. One strain of the latter type, ToP1, was studied in detail. Sequence analysis of the 16S rRNA gene and DNA-DNA hybridization indicated an affiliation of strain ToP1 with the species *Blastochloris sulfoviridis*, a member of the α-subclass of Proteobacteria. However, the type strain (DSM 729) of *Blc. sulfoviridis* neither grew on toluene nor on benzoate. Light-dependent consumption of toluene in the presence of carbon dioxide and formation of cell mass by strain ToP1 were demonstrated in quantitative growth experiments. Strain ToP1 is the first phototrophic bacterium shown to utilize an aromatic hydrocarbon. In the supernatant of toluene-grown cultures and in cell-free extracts incubated with toluene and fumarate, the formation of benzylsuccinate was detected. These findings indicate that the phototrophic bacterium activates toluene anaerobically by the same mechanism that has been reported for denitrifying and sulfate-reducing bacteria. The natural abundance of phototrophic bacteria with the capacity for toluene utilization was examined in freshwater habitats. Counting series revealed that up to nearly 2% ($1.8 \cdot 10^5$ cells per g dry mass of sample) of the photoheterotrophic population cultivable with acetate grew on toluene.

Introduction

Microbial utilization of hydrocarbons has for a long time been thought to occur only in the presence of molecular oxygen. However, during the past ten years bacterial utilization of aromatic and aliphatic hydrocarbons has been demonstrated also under anoxic conditions (for review see Heider et al. 1999). Most studies have focused on the aromatic hydrocarbon toluene. Toluene degradation without oxygen was initially demonstrated in enriched bacterial communities under conditions of methanogenesis (Grbic-Galic and Vogel 1987) and nitrate reduction (Kuhn et al. 1988). Later, anaerobic toluene oxidation was repeatedly found in pure cultures of iron-(III)-reducing (Lovley et al. 1989), denitrifying (Dolfing et al. 1990; Altenschmidt and Fuchs 1991; Evans et al. 1991; Schocher et al. 1991; Fries et al. 1994; Rabus and Widdel 1995), and sulfate-reducing (Rabus et al. 1993; Beller et al. 1996) bacteria. Defined co-cultures of anaerobic toluene degraders and methanogens have not yet been obtained. Anaerobic toluene degradation was also demonstrated under conditions of manganese(IV) reduction (Langenhoff et al. 1997), but a pure culture with this metabolic capacity has not been described. A principal process of anaerobic energy conservation in which a connection to toluene utilization has not been shown is anoxygenic photosynthesis. The goal of the present study was to search for toluene-utilizing phototrophic bacteria. If such bacteria exist, it would be of interest to investigate whether they activate toluene by the same mechanism as chemotrophic bacteria. In denitrifying and sulfate-reducing bacteria, toluene is activated by addition of fumarate to the methyl group yielding benzylsuccinate (Biegert et al. 1996; Beller and Spormann 1997; Coschigano et al. 1998; Leuthner et al. 1998; Rabus and Heider 1998).

Materials and Methods

Sources of bacteria. The phototrophic strains ToP1 and BP1 originated from an activated sludge sample taken at the local sewage plant (Seehausen/Bremen, Germany). The same sludge was used for counting of phototrophic bacteria. Further counting series were performed with sediment samples taken from the tidal zone of Weser river (freshwater) and from a freshwater pond, both in Bremen.

Blastochloris (formerly *Rhodopseudomonas*) *sulfoviridis* (DSM 729^T), *Blastochloris* (formerly *Rhodopseudomonas*) *viridis* (DSM 133^T), *Rhodocyclus purpureus* (DSM 168^T), *Rhodomicrobium vannielii* (DSM 162^T), and *Rhodopseudomonas palustris* (DSM 123^T) were obtained from the Deutsche Sammlung von Mikroorganismen und Zellkulturen (Braunschweig, Germany).

Media, cultivation techniques, and quantitative growth experiments. Techniques for preparation of media and for cultivation of phototrophic bacteria under anoxic conditions were as described (Widdel and Bak 1992). Cultures were grown in defined,

bicarbonate-buffered medium as used for cultivation of freshwater strains of sulfate-reducing bacteria (Widdel and Bak 1992) with an increased KH_2PO_4 concentration of 0.5 $g \cdot l^{-1}$. A non-chelated trace element solution was added (Widdel and Bak 1992). The pH of the medium for pure cultures of *Blc. sulfoviridis* was adjusted to 7.5, for all other cultures to 7.0. Media for enrichment cultures and most probable number (MPN) series were always prepared anaerobically and kept under N_2/CO_2 (90:10, v/v), but did not contain a reductant. Media for pure cultures were reduced with 0.5 mM sodium sulfide (from sterile 1 M stock solution; Widdel and Bak 1992), or with 4 mM sodium ascorbate (prepared by neutralization of the free acid; Rabus and Widdel 1995). Eventual slight growth on sulfide as electron donor was taken into consideration in controls without further addition of substrate. Ascorbate was added only if preceding experiments had verified that it did not serve as a growth substrate. For routine cultivation, butyl-rubber-sealed, flat 100-ml bottles or 20-ml tubes with 80 or 15 ml medium, respectively, under a head space of N_2/CO_2 (90:10, v/v) were used.

Filter-sterilized aromatic hydrocarbons, alcohols, aldehydes, and ketones were diluted (0.5-2%, v/v) in a carrier phase (0.5 ml per tube) of sterile, deaerated 2,2,4,4,6,8,8-heptamethylnonane to avoid toxic effects of the pure substances (Rabus et al. 1993). Crude oil (0.5-1 ml per tube) was added without dilution in a carrier phase. Tubes with overlying hydrocarbon phases were incubated nearly horizontally to facilitate diffusion of substances into the aqueous medium. The orifices sealed with a black rubber stoppers were kept somewhat below the surface level of the medium to avoid adsorption of hydrophobic compounds by the rubber (Aeckersberg et al. 1991; Rabus et al. 1993). Anoxic heptamethylnonane and crude oil were prepared and stored in special flasks under N_2 as described (Rabus and Widdel 1996). Water-soluble substrates were added from autoclaved or filter-sterilized aqueous stock solutions to yield the indicated concentrations. Growth tests with molecular hydrogen were carried out in stopper- or septum-sealed tubes incubated horizontally under an anoxic gas phase of H_2/CO_2 mixture (80:20, v/v). Aerobic growth tests were carried out by streaking on agar (10 $g \cdot l^{-1}$) plates prepared from the described medium containing 10 mM acetate, but no bicarbonate; plates were incubated under air in the dark.

All cultures were incubated in temperature-controlled incubators and illuminated with a 25-W tungsten light bulb. Optimal growth of pure cultures was observed when their distance to the light bulb was between 10 and 15 cm. The temperatures inside the illuminated cultures were inferred from measurements by thermometers in separate bottles kept at the same distances from the light source. Enrichment cultures and tubes for determination of most probable numbers (MPN) were kept inside a box equipped with an infrared filter (840E, Göttinger Farbfilter, Göttingen, Germany). Only wavelengths higher than 750 nm passed through this filter into the box.

For maintenance, strain ToP1 was grown on toluene, stored at 4 °C, and transferred every 6-8 weeks.

Quantitative growth experiments were carried out in flat 500-ml bottles with 450 ml medium under a head space of N_2/CO_2 (90:10, v/v). Two different amounts of toluene, 75 and 150 µl (0.7 and 1.4 mmol, respectively) were used; each amount was dissolved in 15 ml heptamethylnonane as carrier phase. Bottles were incubated horizontally, and contact of the carrier phase with the stoppers was avoided, as described for cultivation in tubes. Dry mass of cells was determined after washing with ammonium acetate (20 mM) and drying of the centrifuged cell pellet at 60 °C to constant weight.

Isolation of strains and enumeration of viable cells. Toluene-utilizing strains were isolated via repeated dilution in agar tubes (Widdel and Bak 1992), each overlaid with 0.5 ml heptamethylnonane containing 1% (v/v) toluene. Other phototrophs were isolated in agar with 1 mM benzoate. Agar dilutions with acetate were used to determine cell numbers of the newly isolated strains ToP1 and BP1, that could be distinguished by their color, in mixed cultures.

Sludge and sediment samples for enumeration of viable cells were sieved (mesh size, 1 mm) and homogenized by shaking under nitrogen. The most probable number (MPN) technique was used in three replicate series in liquid medium with subsequent tenfold dilution steps. Applied substrates were 10 mM acetate, 1 mM benzoate, and toluene (1%, v/v) in heptamethylnonane. MPN tubes were incubated for 14 weeks. The dry mass of the original samples was determined by drying at 60 °C to constant weight. Numbers of bacteria per ml of the respective sample were calculated by standard procedures (de Man 1975).

16S rRNA gene sequence and data analysis. The 16S rRNA gene was amplified in vitro and was directly sequenced as previously described (Springer et al. 1993). The Taq Dye-Deoxy™ Terminator Cycle Sequencing Kit (Applied Biosystems, Foster City, CA, USA) and a 373S DNA sequencer (Applied Biosystems) were used. The new sequence was added to an alignment of about 5300 homologous primary structures of 16S rRNA genes (Maidak et al. 1997) by using the aligning tool of the ARB program package (Strunk and Ludwig 1998). Overall similarity values, distance matrix, maximum parsimony, and maximum-likelihood were analyzed as implemented in the ARB software package. Phylogenetic trees were constructed using subsets of data that included sequences from representative members of the α-subclass of Proteobacteria as well as from outgroup species (Maidak et al. 1997). Topologies in the tree were evaluated by using the different approaches to elaborate a consensus tree (Ludwig et al. 1998). Sequence data of strain ToP1 are deposited under EMBL accession number AJ012089.

Determination G+C content of DNA and DNA-DNA similarity. DNA-DNA similarities were measured spectrophotometrically by following the reassociation kinetics (Escara and Hutton 1980; Huss et al. 1983; Jahnke, 1992). The G+C content of the DNA was determined by HPLC (Mesbah et al. 1989). Both analyses were performed at the Deutsche Sammlung von Mikroorganismen und Zellkulturen.

Measurement of *in vivo* absorption spectra. Absorption spectra of whole cells were recorded between 400 and 1100 nm using a Lambda 20 spectrophotometer (Perkin Elmer, Überlingen, Germany). Cells were collected on a glass microfiber filter (2.5 cm diameter) that was placed directly into the beam of the spectrophotometer (Amesz et al. 1961). A wet filter without cells was used as reference.

Preparation of cell-free extracts. All steps for preparation of cell-free extracts were performed under anoxic conditions at 4 °C. Cells of strain ToP1 cultivated on toluene for at least 20 generations were grown in volumes of 2 l. Cells were harvested before reaching the stationary phase and frozen at -70 °C under anoxic conditions before further use. Frozen cells (5 g of wet weight) were resuspended in two volumes of anoxic buffer containing 200 mM Tris (pH 8.0), 1 mM $MgCl_2$ and 0.5 mM sodium dithionite. Cells were broken by one passage through a French pressure cell (American Instruments, Silver Spring, MD, USA) at 137 MPa. The extracts were centrifuged at 100,000 × *g* for 90 min in steel centrifuge tubes. The supernatants (cell-free extracts) were used for enzyme assays with radiolabelled toluene.

Enzyme assays. Activity of benzylsuccinate synthase was assayed at 30 °C in cell extracts by determining the formation of [*phenyl*-^{14}C]-benzylsuccinate from [*phenyl*-^{14}C]-toluene and fumarate according to Leuthner et al. (1998). A 200-µl sample of extract was transferred into an anoxic glass vial to which 20 µl of a 100 mM fumarate solution was added (final concentration, 10 mM). The reaction was started by adding 1 µl of a 60 mM stock solution of [*phenyl*-^{14}C]-toluene (Biotrend, Köln, Germany) in isopropanol (final concentration, 272 µM; specific activity, 220 MBq·mmol^{-1}). Aliquots of 30 µl were taken after 5, 15, 28, and 60 min. The reaction was stopped by adding 10 µl of a 5 M $NaHSO_4$ solution to the samples. Precipitated proteins were removed by centrifugation and the formed amount of non-volatile radioactivity was analysed by liquid scintillation counting.

Reaction products of benzylsuccinate synthase were separated by thin-layer chromatography as described by Biegert et al. (1996).

Chemical analyses. Toluene dissolved in heptamethylnonane was measured by means of an Auto System gas chromatograph (Perkin Elmer, Überlingen, Germany) equipped with a PVMS 54 column (length, 50 m; inner diameter, 0.32 mm) and a flame ionization detector. The flow rate of H_2 was 1.7 ml·min^{-1}. The temperature program was run from 60 °C (2 min isotherm) to 120 °C at 5 °C·min^{-1}, and then from 120 °C (0.1 min isotherm) to 220 °C at 10 °C·min^{-1} (5 min isotherm at 220 °C). The temperatures at the injection port and the detector were 250 and 280 °C, respectively. Defined, freshly prepared solutions of toluene in heptamethylnonane were used for calibration.

The saturated and aromatic hydrocarbon fraction of crude oil was analyzed as described by Behar et al. (1989). Hydrocarbons were determined by using the same gas chromatograph as used for toluene analysis. Separation was achieved on a fused silica capillary column (Optima-5, length, 50 m; inner diameter, 0.32 mm; Macherey-Nagel, Düren, Germany). The

carrier gas was H_2 at a flow rate of 2.0 ml·min^{-1}. The temperature program for analysis of the alkane fraction was run from 50 °C (2 min isotherm) to 290 °C at 5 °C·min^{-1} (10 min isotherm at 290 °C). The temperature program for analysis of the aromatic hydrocarbon fraction was run from 30 °C (10 min isotherm) to 290 °C at 5 °C·min^{-1} (13 min isotherm at 290 °C). For analysis of both fractions, the temperatures at the injection port and the detector were 270 and 320 °C, respectively.

Toluene and benzylsuccinate in the aqueous phase in cultures of strain ToP1 were determined by a high-performance liquid chromatography system (Sykam, Gilching/Munich, Germany) equipped with a Spherisorb OD S2 reversed-phase column (250 by 5 mm). For toluene analysis, the eluent was an acetonitrile-water mixture (60:40, v/v) at a flow rate of 1 ml·min^{-1} and a column temperature of 25 °C. For benzylsuccinate analysis, the eluent was an acetonitrile-water mixture (25:75, v/v) at a flow rate of 0.7 ml·min^{-1} and a column temperature of 20 °C. UV absorption spectra of peaks were determined between 195 and 360 nm by using a fast scanning detector (S 3206, Linear Instruments Co., NV, USA). Absorptions at the maxima of the two compounds were used for their quantification. Defined, freshly prepared solutions of toluene and benzylsuccinate in acetonitrile-water mixture (volume ratio as in eluent) were used as standards.

Protein concentrations were determined according to Bradford (1976) with bovine serum albumin as standard.

Results

Enrichment and isolation of toluene-utilizing phototrophic bacteria. Media for enrichment of phototrophic bacteria were inoculated with activated sludge and overlaid with toluene (2% in a carrier phase of heptamethylnonane and sealed anoxically). Activated sludge has formerly been shown to harbour high population densities of various phototrophic bacteria (Siefert et al. 1978). When inoculated media were illuminated directly by a tungsten light bulb, growth of cyanobacteria and algae was favoured which led to oxygen production. Therefore, bottles were illuminated through a filter that excluded wavelengths shorter than 750 nm. All these bottles including toluene-free controls turned intensely red within one week, due to growth of purple bacteria on degradation products of the sludge biomass. When inocula from these bottles were transferred to new media, toluene-dependent phototrophic growth in comparison to controls became obvious. Subsequent transfers yielded red to brownish enrichment cultures dominated by two cell types, as revealed by light microscopy (Fig. 1A). For isolation, samples were serially diluted in agar and overlaid with toluene in a carrier phase. Two main types of colonies that could be distinguished by their colors, red and yellow, developed at approximately equal numbers within 3-4 weeks. Several colonies were

microscopically examined. Yellow colonies consisted of oval cells with a slight tendency toward unequal ("budding") devision. Red colonies contained rod-shaped cells with budding division. When transferred to liquid medium, only cells from the yellow colonies were able to grow on toluene. Cells isolated from red colonies could be subcultivated in medium with benzoate. One isolate from a yellow colony, strain ToP1, and one isolate from a red colony, Strain BP1, were maintained for further studies. The strains were further cultivated without a light filter.

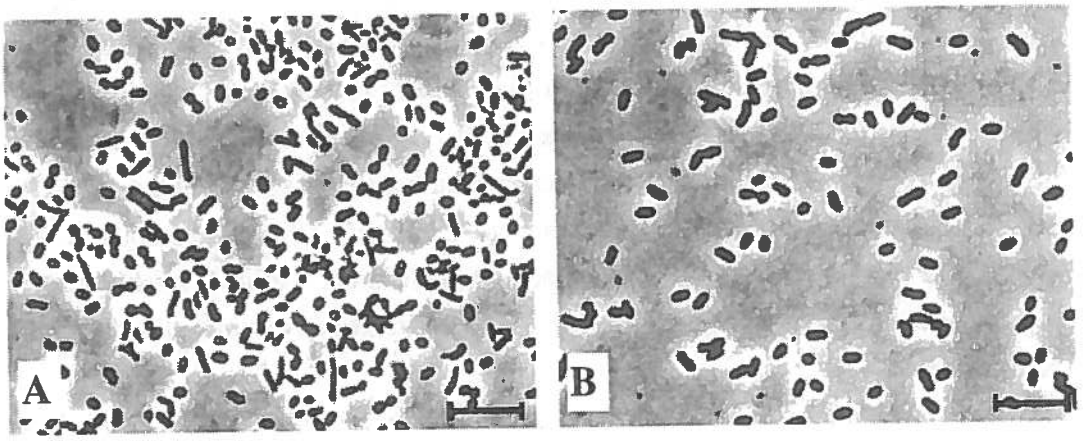

Fig. 1 Phase contrast photomicrograph of the enrichment culture (A) and of strain ToP1 (B). Bar represents 10 µm.

Morphological and physiological characteristics. The cell shape of strain ToP1 is shown in Fig. 1B. Cells were partly motile and 1.3-2 µm by 0.7 µm in size. The in vivo absorption spectrum (Fig. 2) exhibited a small peak at 833 nm and a large peak at 1017 nm, which is indicative of bacteriochlorophyll *b* (Trüper and Pfennig, 1981). Hence, by its absorption characteristics strain ToP1 resembles *Blc. sulfoviridis* (Keppen and Gorlenko 1975) and *Blc.viridis* (Drews and Giesbrecht 1966). The temperature range for growth was 12-36 °C, with an optimum at 34 °C. The pH range was between 6.5 and 8.3, with an optimum around 7.4. The doubling time on toluene was 30-35 h, as determined from the exponential growth phase of cells growing at a distance of 10-15 cm from a 25-W tungsten light bulb. In addition to toluene, strain ToP1 utilized a variety of aliphatic organic acids, yielding optical cell densities (660 nm) of 0.6 and higher within 5 days or less (Table 1). Among further aromatic compounds tested, only benzoate and cinnamate allowed growth as fast as on toluene. Benzylsuccinate was a poor substrate; an optical density (660 nm) not higher than 0.2 was reached after 6 weeks. Strain ToP1 also grew on crude oil as the only source of organic substrates. Chemical analysis revealed only consumption of toluene. Furthermore, photoautotrophic growth on H_2 and CO_2 was observed. In contrast to the type strain of *Blc.*

sulfoviridis (Trüper and Imhoff 1989), strain ToP1 did not require sulfide as a sulfur source for cell synthesis during growth on organic compounds or H_2 and CO_2. On these substrates, strain ToP1 could grow in sulfide-free medium in the presense of ascorbate as reductant. Strain ToP1 did not grow on agar plates with acetate incubated in the dark under air.

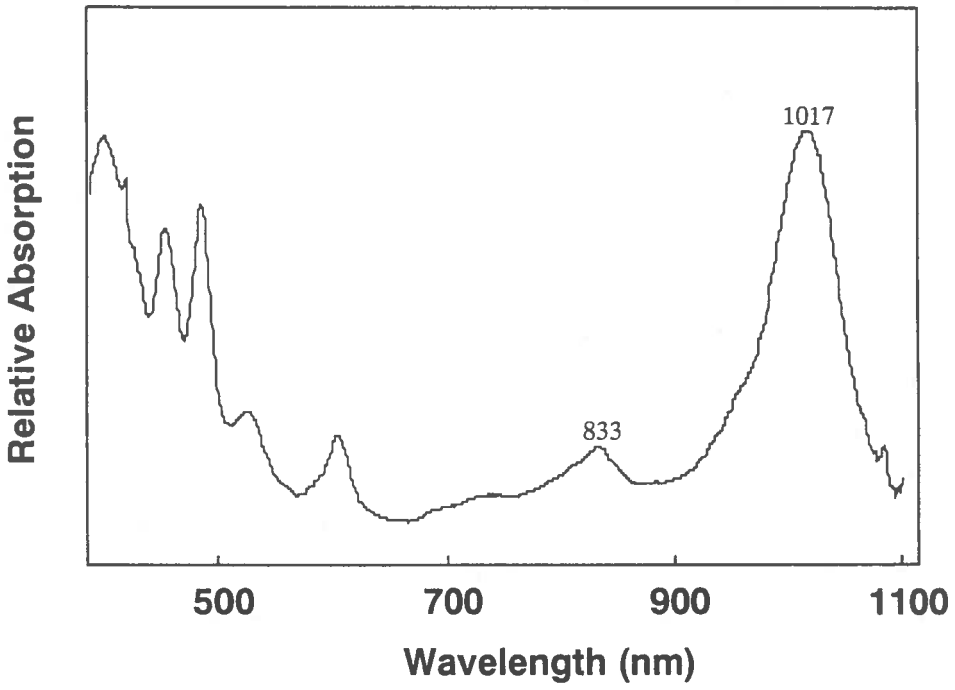

Fig. 2 *In vivo* absorption spectrum of strain ToP1.

The other type of phototroph from the enrichment culture, represented by strain BP1, was only briefly characterized. Cells were 3 μm in lengths and 0.6 μm in diameter. Strain BP1 utilized acetate, butyrate, caprylate, succinate, ascorbate, and 4-hydroxybenzoate in addition to benzoate. Benzylsuccinate, like toluene, was not a growth substrate for strain BP1. When strain ToP1 was mixed with strain BP1 and cultivated over 15 generations with toluene as the only growth substrate, cells of the latter were always detectable by microscopy at high numbers. In fact, colonies in agar dilutions with acetate revealed that cells of strains ToP1 and BP1 in the toluene-grown co-culture grew at a ratio of 3:1.

The capacity for photoheterotrophic utilization of toluene was also tested with *Rhodocyclus purpureus, Rhodomicrobium vannielii,* and *Rhodopseudomonas palustris,* which are known to be benzoate-utilizing phototrophs (Imhoff and Trüper 1992). However, none of these strains grew on toluene.

Furthermore, toluene utilization was tested with the type strains of *Blc. sulfoviridis* and *Blc. viridis*. They resemble strain ToP1 by the presence of bacteriochlorophyll *b* and on the

basis of 16S rRNA sequences (see below). However, these strains neither utilized toluene nor benzoate. Further nutritional characteristics of *Blc. sulfoviridis* in comparison to those of strain ToP1 are included in Table 1.

Genetic relationship and DNA base composition. From strain ToP1, a nearly complete 16S rRNA gene sequence of 1440 nucleotides was obtained. Phylogenetic analysis of sequences from the data bank revealed *Blc. sulfoviridis* (formlery *Rhodopseudomonas sulfoviridis*) of the α-subclass of Proteobacteria as the closest relative of strain ToP1, with a similarity value of 99.9%. The second closest relative of strain ToP1 was *Blc. viridis* (formerly *Rhodopseudomonas viridis*), the similarity value being 98.7% (Fig. 3). Because of the high sequence similarity values between these strains, a more refined analysis of their relationship was obtained from DNA-DNA hybridization (Table 2). Strain ToP1 shared 78.4% DNA-DNA similarity with the type strain of *Blc. sulfoviridis*. Furthermore, the G+C contents of total DNA of the three strains were compared (Table 2.).

From strain BP1, a 16S rRNA gene partial sequence of 432 bp (positions 13 to 499, *E. coli* numbering; Brosius et al. 1981) was obtained. Sequence comparison with the available data bank (Maidak et al. 1997) revealed 100% similarity with the type strain of *Rps. palustris.*

Quantification of light-dependent cell growth on toluene. Anaerobic growth of strain ToP1 on toluene was only observed if cultures were incubated in the light. Phototrophic substrate consumption and formation of cell mass was quantified in growth experiments with two different toluene concentrations in the carrier phase. For comparison, growth was also quantified with acetate. Results are summarized in Table 3. In the presence of cells, consumption of toluene was complete or nearly complete. No abiotic loss of toluene was detectable in a cell-free control during the incubation time of 4 weeks. The average cell yield in the two experiments was 182 g cell dry mass formed per mol toluene consumed.

Introduction

Aromatic hydrocarbons are major constituents of crude oil and gasoline that are of concern as environmental contaminants (US Environmental Protection Agency 1986). Since many aquatic environments contain anoxic zones, degradation of aromatic hydrocarbons in the absence of molecular oxygen has received much attentions during the past decade.

Anaerobic degradation of aromatic hydrocarbons has been mainly studied in freshwater environments, and in enrichment cultures or pure cultures derived from freshwater sites. Most of these studies focused on nitrate as electron acceptor. Strains of denitrifying bacteria utilizing toluene (Dolfing et al. 1990; Altenschmidt and Fuchs 1991; Evans et al. 1991; Schocher et al. 1991; Fries et al. 1994; Rabus and Widdel 1995; Hess et al. 1997), *m*-xylene (Dolfing et al. 1990; Fries et al. 1994; Rabus and Widdel 1995; Hess et al. 1997), ethylbenzene (Rabus and Widdel 1995; Ball et al. 1996), *n*-propylbenzene (Rabus and Widdel 1995), or *p*-cymene (Harms et al. 1999) so far isolated exclusively belong to the *Azoarcus-Thauera* branch of the β subclass of *Proteobacteria.* Bacteria with the same affiliation were also detected by oligonucleotide probing in hydrocarbon-contaminated aquifers (Hess et al. 1997; Zhou et al. 1997; Zarda et al. 1998) and in an anaerobic enrichment culture growing on oil and nitrate (Rabus et al. 1999). In addition ferric iron-reducing (Lovley et al. 1989) and sulfate-reducing bacteria (Beller et al. 1996), both belonging to the δ subclass of *Proteobacteria*, have been isolated from freshwater environments. Anaerobic hydrocarbon-degrading microorganisms isolated so far from marine environments are all sulfate-reducing bacteria (Rabus et al. 1993; Harms et al. 1999). Sulfate is the most significant electron acceptor in the anaerobic mineralization of biomass in marine sediments (Jørgensen 1982). Denitrifying bacteria with the capacity for anaerobic hydrocarbon degradation have neither been enriched nor isolated until now from marine environments. Even though much less abundant than sulfate, the role of nitrate in anaerobic mineralization processes particularly in coastal environments has been demonstrated (Jørgensen 1983). The goal of the present study was the search for denitrifying bacteria that degrade aromatic hydrocarbons anaerobically under the saline conditions of marine environments. If such bacteria existed, their relationships to their freshwater counterparts with the same degradative capacity would be of particular interest.

Materials and Methods

Sources of organisms and cultivation procedures. Enrichment cultures with crude oil and toluene, respectively, as well as enumeration of viable denitrifiers by the most-probable-number (MPN) technique has been performed from two different marine sediments originated from the North Sea and the Black Sea. North Sea sediment was obtained from a small North Sea harbor (Horumersiel) near Wilhelmshaven (Germany). Sediment samples from the western Black Sea were achieved during the Black Sea cruise from the Max-Planck Institute

Abstract

The occurrence of nitrate-reducing bacteria with the capacity to utilize aromatic hydrocarbons was investigated in two marine environments using serial dilutions and enrichment cultures with toluene. So far, anaerobic oxidation of hydrocarbons with nitrate has been studied in freshwater environments. Dilution series and enrichment cultures were examined by 16S rRNA-targeted whole-cell hybridization with group-specific fluorescent oligonucleotide probes. Toluene-utilizing bacterial populations originating from North Sea sediment were always dominated by members of the γ subclass of *Proteobacteria*. In contrast, populations from Black Sea sediment were dominated by members of the α subclass of *Proteobacteria*. In addition, denitrifying bacteria were enriched with crude oil as a complex mixture of hydrocarbons. Oil analysis after growth revealed that toluene was most readily consumed. Oligonucleotide probing of the enrichment culture from North Sea sediment again revealed dominance of γ *Proteobacteria*. In the enrichment culture from Black Sea sediment, β and γ *Proteobacteria* were much more abundant than α *Proteobacteria*, in contrast to toluene-grown cultures. Two new strains, Col2 and TH1 were isolated and characterized as representatives of γ and α*Proteobacteria* that are able to oxidize toluene with nitrate. So far, all denitrifying bacteria utilizing toluene or other alkylbenzenes affiliated with the β subclass of *Proteobacteria*. In accordance with their marine origin, strains Col2 and TH1 had a pronounced requirement for NaCl, the optimal concentrations being 26 and 20 g/l, respectively. No growth occurred in media without NaCl.

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Anaerobic, nitrate-dependent utilization of hydrocarbons by marine members of the alpha and gamma subclass of *Proteobacteria*

Karsten Zengler[1*], Enric Llobet-Brossa[1], Thuy Hang Dinh[1], Gerda Harms[1], Thierry Nadalig[1], Hans Hermann Richnow[2], and Friedrich Widdel[1]

Applied and Environmental Microbiology, submitted

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[1] Max-Planck-Institute for Marine Microbiology, Celsiusstr. 1, D-28359 Bremen, Germany

[2] Institute for Biogeochemisty and Marine Chemistry, University of Hamburg, Bundestr. 55, D-20146 Hamburg, Germany

* Corresponding author

Rabus R, Heider J 1998. Initial reactions of anaerobic metabolism of alkylbenzenes in denitrifying and sulfate-reducing bacteria. Arch. Microbiol. 170:377-384

Schocher RJ, Seyfried B, Vazquez F, Zeyer J 1991. Anaerobic degradation of toluene by pure cultures of denitrifying bactera. Arch. Microbiol. 157:7-12

Siefert E, Irgens RL, Pfennig N 1978. Phototrophic purple and green bacteria in a sewage treatment plant. Appl. Environ. Microbiol. 35:38-44

Springer N, Ludwig W, Amann R, Schmidt HJ, Gorth HD, Schleifer K-H 1993. Occurence of fragmented 16S rRNA in an obligate bacterial endosymbiont of *Paramecium caudatum*. Proc. Natl. Acad. Sci. USA 90:9892-9895

Strunk O, Ludwig W 1998. ARB: a software environment for sequence data, http://www.mikro.biologie.tu-muenchen.de Department of Microbiology, Technische Universität München, Munich, Germany

Trüper HG, Imhoff JF 1989. *Rhodopseudomonas*. In: Staley JT, Bryant MP, Pfennig N, Holt JG (eds) Bergey´s Manual of Systematic Bacteriology, vol 3, Williams & Wilkins, Baltimore, pp 1672-1677

Trüper HG, Pfennig N 1981. Characterization and identification of the anoxygenic phototrophic bacteria. In: Starr MP, Stolp H, Trüper HG, Balows A, Schlegel HG (eds) The Prokaryotes: a handbook on habitats, isolation, and identification of bacteria, Springer-Verlag, Berlin Heidelberg New York, pp 299-312

Widdel F, Bak F 1992. Gram-negative mesophilic sulfate-reducing bacteria. In: Balows A, Trüper HG, Dworkin M, Harder W, Schleifer K-H (eds) The Prokaryotes, 2nd ed, vol 4. Springer-Verlag, Berlin Heidelberg New York, pp 3352-3392

Imhoff JF, Trüper HG 1992. The genus Rhodospirillum and related genera. In: Balows A, Trüper HG, Dworkin M, Harder W, Schleifer K-H (eds) The Prokaryotes, 2nd ed, vol 3. Springer-Verlag, Berlin Heidelberg New York, pp 2141-2155

Jahnke K-D 1992. Basic computer program from evaluation of spectroscopic DNA renaturation data from GILFORD System 2600 spectrometer on a PC/XT/AT type personal computer. J. Microbiol. Meth. 15:61-73

Jüttner F, Henatsch JJ 1986. Anoxic hypolimnion is a significant source of biogenic toluene. Nature (London) 323:797-798

Keppen OJ, Gorlenko VM 1975. A new species of purple budding bacteria containing bacteriochlorophyll *b*. Microbiology 44:224-229

Kuhn EP, Zeyer J, Eicher P, Schwarzenbach RP 1988. Anaerobic degradation of alkylated benzenes in denitrifying laboratory aquifer colomns. Appl. Environ. Microbiol. 54:490-496

Langenhoff AAM, Nijenhuis I, Tan NCG, Briglia M, Zehnder AJB, Schraa G 1997. Characterisation of a manganese-reducing, toluene-degrading enrichment culture. FEMS Microbiol. Ecol. 24:113-125

Leuthner B, Leutwein C, Schulz H, Hörth P, Haehnel W, Schiltz E, Schäffer H, Heider J 1998. Biochemical and genetic characterization of benzylsuccinate synthase from *Thauera aromatica*: a new glycyl radical enzyme catalysing the frist step in anaerobic toluene metabolism. Mol. Microbiol. 28:615-628

Lovley DR, Baedecker MJ, Lonergan DJ, Cozzarelli IM, Phillips EJP, Siegel DI 1989. Oxidation of aromatic contaminants coupled to microbial iron reduction. Nature (London) 339:297-299

Ludwig W, Strunk O, Klugbauer N, Weizenegger M, Neumaier J, Bahleitner M, Schleifer K-H 1998. Bacterial phylogeny based on comparative sequence analysis. Electrophoresis 19:554-568

Maidak BL, Olsen GJ, Larsen N, Overbeek R, McCaughey MJ, Woese CR 1997. The RDP (Ribosomal Database Project). Nucleic Acids Res. 25:109-110

Mesbah M, Premachandran U, Whitman WB 1989. Precise measurement of G+C content of deoxyribonuleic acid by high-performance liquid chromatography. Int. J. Syst. Bacteriol. 39:159-167

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

Rabus R, Widdel F 1995. Anaerobic degradation of ethylbenzene and other aromatic hydrocarbons by new denitrifying bacteria. Arch. Microbiol. 163:96-103

Rabus R, Widdel F 1996. Utilization of alkylbenzenes during anaerobic growth of pure cultures of denitrifying bacteria on crude oil. Appl. Environ. Microbiol. 62:1238-1241

Elder DJE, Kelly DJ 1994. The bacterial degradation of benzoic acid and benzenoid compounds under anaerobic conditions. Unifying trends and new perspectives. FEMS Microbiol. Rev. 13:441-468

Escara JF, Hutton JR 1980. Thermal stability and renaturation of DNA in dimethyl sulfoxide solutions: acceleration of the renaturation rate. Biopolymers 19:1315-1327

Evans PJ, Ling W, Goldschmidt B, Ritter ER, Young LY 1992. Metabolites formed during anaerobic transformation of toluene and *o*-xylene and their proposed relationship to the initial steps of toluene mineralization. Appl. Environ. Microbiol. 58:496-501

Evans PJ, Mang DT, Kim KS, Young LY 1991. Anaerobic degradation of toluene by a denitrifying bacterium. Appl. Environ. Microbiol. 57:1139-1145

Fischer-Romero C, Tindall BJ, Jüttner F 1996. *Tolumonas auensis* gen. nov., sp. nov., a toluene-producing bacterium from anoxic sediments of a freswater lake. Int. J. Syst. Bacteriol. 46:183-188

Fries MR, Zhou J, Chee-Sanford J, Tiedje JM 1994. Isolation, charaterization, and distribution of denitrifying toluene degraders from avariety of habitats. Appl. Environ. Microbiol. 60:2802-2810

Fuchs G, Mohamed MES, Altenschmidt U, Koch J, Lack A, Brackmann R, Lochmeyer C, Oswald B 1994. Biochemistry of anaerobic biodegradation of aromatic compounds. In: Ratledge R (ed) Biochemistry of Microbial Degradation, Kluwer Academic Publishers, Dordrecht, pp 513-553

Grbic-Galic D, Vogel TM 1987. Transformation of toluene and benzene by mixed methanogenic cultures. Appl. Environ. Microbiol. 53:254-260

Harder W, van Dijken JP 1975. Theoretical consideration on the relation between energy production and growth of methane-utilizing bacteria. In: Gottschalk G, Schlegel HG, Pfennig N (eds) Microbial production and utilization of gases, Akademie der Wissenschaften zu Göttingen, Göttingen, pp 403-418

Heider J, Fuchs G 1997. Anaerobic metabolism of aromatic compounds. Eur. J. Biochem. 243:577-596

Heider J, Spormann AM, Beller HR, Widdel F 1998 Anaerobic bacterial metabolism of hydrocarbons. FEMS Microbiol. Rev., in press

Hiraishi A 1997. Transfer of the bacteriochlorophyll *b*-containing phototrophic bacteria *Rhodopseudomonas viridis* and *Rhodopseudomonas sulfoviridis* to the genus *Blastochloris* gen. nov. Int. J. Syst. Bacteriol. 47:217-219

Holliger C, Zehnder AJB 1996. Anaerobic biodegradation of hydrocarbons. Curr. Opin. Biotechnol. 7:326-330

Huss VAR, Festl H, Schleifer K-H 1983. Studies on the spectrophotometric determination of DNA hybridazation from renaturation rates. System. Appl. Microbiol. 4:184-192

References

Aeckersberg F, Bak F, Widdel F 1991. Anaerobic oxidation of saturated hydrocarbons to CO_2 by a new type of sulfate-reducing bacterium. Arch. Microbiol. 156:5-14

Altenschmidt U, Fuchs G 1991. Anaerobic degradation of toluene in denitrifying *Pseudomonas* sp.: indication for toluene methylhydroxylation and benzoyl-CoA as central aromatic intermediate. Arch. Microbiol. 156:152-158

Amesz J, Duysens LN, Brandt DC 1961. Methods for measuring and correcting the absorption spectrum of scattering suspensions. J. Theoret. Biol. 1:59-74

Ball HA, Johnson HA, Reinhard M, Spormann AM 1996. Initial reactions in anaerobic ethylbenzene oxidation by a denitrifying bacterium, strain EB1. J. Bacteriol. 178:5755-5761

Behar F, Leblond C, Saint Paul C 1989. Analyse quantitative des effluents de pyrolyse en milieu ouvert et fermé. Revue de l'Institut Français du Pétrole 44:387-411

Beller HR, Spormann AM 1997. Benzylsuccinate formation as a means of anaerobic toluene activation by sulfate-reducing strain PRTOL1. Appl. Environ. Microbiol. 63:3729-3731

Beller HR, Spormann AM, Sharma PK, Cole JR, Reinhard M 1996. Isolation and characterization of a novel toluene-degrading, sulfate-reducing bacterium. Appl. Environ. Microbiol. 62:1188-1196

Biegert T, Fuchs G, Heider J 1996. Evidence that anaerobic oxidation of toluene in the denitrifying bacterium *Thauera aromatica* is initiated by formation of benzylsuccinate from toluene and fumarate. Eur. J. Biochem. 238:661-668

Bradford M 1976. A rapid and sensitive method for the quantification of microgramm quantities of protein utilizing the principle of protein-dye binding. Anal. Biochem. 72:248-254

Brosius J, Dull TL, Sleeter DD, Noller HF 1981. Gene organization and primary structure of a ribosomal RNA operon from *Escherichia coli.* J. Molec. Biol. 148:107-127

Coschigano PW, Wehrmann TS, Young LY 1998. Identification and analysis of genes involved in anaerobic toluene metabolism by strain T1: Putative role of a glycine free radical. Appl. Environ. Microbiol. 64:1650-1656

de Man JC 1975. The probability of most probable numbers. Eur. J. Appl. Microbiol. 1:67-78

Dolfing J, Zeyer J, Binder-Eicher P, Schwarzenbach RP 1990. Isolation and characterization of a bacterium that mineralizes toluene in the abscene of molecular oxygen. Arch. Microbiol. 154:336-341

Drews G, Giesbrecht P 1966. *Rhodopseudomonas viridis*, nov. spec., ein neu isoliertes, obligat phototrophes Bakterium. Arch. Mikrobiol. 53:255-262

Acknowledgments. We thank Thierry Nadalig, Arcachon, France, for analysis of crude oil. This work was supported by the Max-Planck-Gesellschaft, the Deutsche Forschungsgemeinschaft and Fonds der Chemischen Industrie.

According to the equation, the cell yield would be 216 g cell dry mass per mol toluene consumed. The experimentally determined average value (see Results) was 15.8% less than the theoretical value. It can be excluded that this deviation was due to CO_2 limitation. Even the highest amount of toluene consumed in 450 ml medium, 1.4 mmol (Table 3), would need no more than 2.06 mmol CO_2; the medium in the experiment contained as much as 13.5 mmol bicarbonate (30 mmol/l) and there was additional CO_2 in the gas phase. Furthermore, toluene consumption in this experiment was shown to be complete. One explanation for the deviation is that the used formula for cell mass is only an approximation. Also in case of acetate, the experimental value, (43 g/mol; Table 3) differs from the theoretically expected one (51 g/mol).

Experiments with cell-free extracts confirmed that the phototrophic isolate activates toluene by addition to fumarate, a mechanism previously elucidated in denitrifying (Evans et al. 1992; Biegert et al. 1996; Coschigano et al. 1998; Leuthner et al. 1998) and sulfate-reducing (Beller and Spormann 1997; Rabus and Heider 1998) bacteria. The observed rate of benzylsuccinate formation in cell extract was rather low. From the cell yield (Y = 182 g/mol toluene) and the doubling time (30 h, corresponding to a growth rate of 0.023 h^{-1}), a minimum activity of $0.126 \cdot 10^{-3}$ $mol \cdot g^{-1} \cdot h^{-1}$ or 2 $nmol \cdot min^{-1} \cdot (mg\ dry\ mass)^{-1}$ would be expected. Assuming a protein content in cell dry mass of 50%, the minimum activity would be 4 $nmol \cdot min^{-1} \cdot (mg\ protein)^{-1}$. The measured enzyme activity is nearly 1% of the expected value. Also previous studies exhibited a significant loss of benzylsuccinate synthase activity upon extract preparation (Beller and Spormann 1997; Leuthner et al. 1998).

In the original enrichment culture as well as in defined binary cultures, *Rps. palustris*-like phototrophs grew at significant cell numbers concomitantly with toluene-utilizing *Blc. sulfoviridis*. Counting series also indicated that these two types of phototrophs grew simultaneously with toluene. However, the *Rps. palustris*-like strain BP1 is unable to grow on toluene. Since these cultures contained only toluene as added organic substrate, strain BP1 must have grown by scavenging of metabolites excreted by *Blc. sulfoviridis*, strain ToP1. Benzylsuccinate can be excluded among the scavenged metabolites because this compound is not a growth substrate of strain BP1.

Phototrophs with the capacity for toluene utilization were detected in three different habitats. Their number was up to nearly 2% of the cultivable phototrophic population. The distribution of toluene-utilizing phototrophic bacteria in various aquatic habitats is conceivable from an ecological point of view. Besides being a constituent of crude oil and refined products, toluene is a natural product formed by microorganisms, as demonstrated by studies of anoxic hypolimnia (Jüttner and Henatsch 1986; Fischer-Romero et al. 1996). Anoxic aquatic habitats with access of light have probably been wide-spread during bacterial evolution.

Table 4 Enumeration of phototrophic bacteria from freshwater habitats via most probable number (MPN) dilution series.

Habitat	Number of bacteria per ml sample[a] determined with		
	Acetate	Benzoate	Toluene
Activated sludge	$1.5 \cdot 10^5$	$1.5 \cdot 10^4$	$1.5 \cdot 10^3$
Weser River	$2.4 \cdot 10^3$	$9.3 \cdot 10^2$	$4.0 \cdot 10^1$
Eutrophic pond	$4.6 \cdot 10^4$	$1.4 \cdot 10^4$	$3.0 \cdot 10^1$

[a] The contents of dry mass in activated sludge, Weser river sediment, and eutrophic pond sediment were 8.2, 7.3, and 3.3 mg/ml, respectively.

Discussion

Anaerobic utilization of toluene has hitherto been demonstrated in chemotrophic bacteria. These belong to the β-subclass and δ-subclass of Proteobacteria. The presently isolated bacterium, strain ToP1, is the first phototrophic organism and first member of the α-subclass that has been shown to grow with toluene in the absence of oxygen. With respect to cell shape, spectral properties, DNA-DNA similarity, G+C content of DNA, and relatedness of 16S rRNA genes, strain ToP1 is very similar to *Blc. sulfoviridis*. Nutritionally strain ToP1 and *Blc. sulfoviridis* share several substrates, but also exhibit differences, for instance in their capacities to utilize aromatic compounds. The type strain of *Blc. sulfoviridis* did not grow on any of the aromatic compounds tested (Table 1). Nevertheless, due to the relatively small number of distinctive nutritional characteristics, strain ToP1 should be regarded as a member of the species, *Blc. sulfoviridis*. Therefore the species description (Trüper and Imhoff 1989; Hiraishi 1997, former name *Rhodopseudomonas sulfoviridis*) should be modified by including toluene and benzoate as potential substrates of strains.

Since phototrophic bacteria conserve energy from light, they are expected to convert their organic substrates completely into cell material. If substrates are more reduced than cell material, CO_2 has to be assimilated simultaneously. If an approximate formula of bacterial cell mass, $C_4H_8O_2N$ (Harder and van Dijken 1975) is assumed, the theoretical assimilation equation is as follows:

$$17\ C_7H_8 + 25\ HCO_3^- + 36\ NH_4^+ \rightarrow 36\ C_4H_8O_2N + 3\ H_2O + 11\ H^+$$

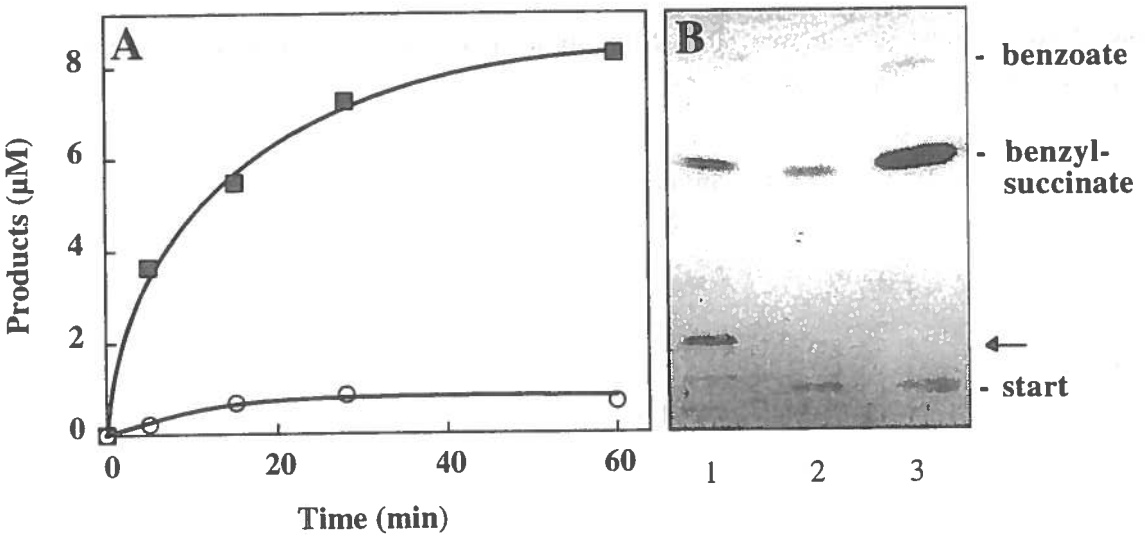

Fig. 4 Anaerobic conversion of [*phenyl*-^{14}C]-toluene to nonvolatile products by cell-free extracts of strain ToP1 grown phototrophically on toluene. The protein concentration was 21.9 mg·ml^{-1}. **A** Time course of product formation from labelled toluene in assays without fumarate (○) and with addition of fumarate (■). **B** Thin-layer chromatography analysis of reaction products. An assay carried out with cell-free extract from a toluene-grown sulfate-reducing bacterium (Rabus and Heider 1998) was used as standard (lane 1). Analyzed samples from a time course experiment (**A**) without fumarate (lane 2) and with fumarate (lane 3) were withdrawn after 28 min. The start positions and the migration positions of benzylsuccinate and benzoate are indicated. The additional band (arrow) in lane 1 is probably the glycerol ester of benzylsuccinic acid, due to the presence of glycerol formerly added to buffer for cell disruption.

Natural abundance of phototrophic bacteria with the capacity for toluene utilization. The abundance of phototrophic bacteria capable of toluene utilization in freshwater habitats was estimated via liquid dilution series with toluene as sole substrate (in a carrier phase of heptamethylnonane). To compare numbers of phototrophs that utilize the aromatic hydrocarbon with numbers of those that grow on other aromatic compounds, parallel dilution series were carried out with benzoate as substrate. Among the aromatic compounds degraded by anaerobic bacteria, benzoate is probably the most commonly utilized substrate (Elder and Kelly 1994; Fuchs et al. 1994; Holliger and Zehnder 1996; Heider and Fuchs 1997). Furthermore, phototrophic bacteria were counted with acetate. Acetate is a substrate for many purple bacteria (Imhoff and Trüper 1992) and was used to detect as many as possible of the phototrophic bacteria in a sample. Results of the counting series are shown in Table 4. The highest dilutions with bacterial growth in the light exhibited the same red to brownish color and contained cells with the same shapes as observed in the initial enrichment culture. Dilutions with benzoate yielded intensely red cultures with cell forms resembling those of *Rps. palustris*.

Table 2 Relatedness of genomic DNA from strain ToP1, *Blastochloris* (*Blc.*) *sulfoviridis* and *Blc. viridis*.

	Similarity (%)			G+C content of DNA (mol%)
	Strain ToP1	*Blc. sulfovirids*	*Blc. viridis*	
Strain ToP1	100.0			67.1[a]
Blc. sulfovirids	78.4	100.0		67.9[b]
Blc. viridis	41.4	31.0	100.0	66.5[b]

[a] This study
[b] Data from Hiraishi (1997)

Table 3 Quantification of photoheterotrophic growth of strain ToP1 on toluene and acetate. Experiments were carried out in 500-ml bottles with 450 ml medium under a head space of N_2/CO_2 (90:10, v/v). Toluene was dissolved in 15 ml heptamethylnonane as carrier phase. Cultures were illuminated with a 25-W tungsten light bulb at a distance of 20 cm.

Substrate	Amount of substrate (mmol)		Cell dry mass formed[b] (mg)	Cell yield (mg/mmol)
	added	consumed[a]		
Toluene	0.7	0.69	124	177.1
Toluene	1.4	1.4	261	186.4
Acetate	4.5	4.5	193	42.8

[a] Values after substraction of remaining amount of substrate
[b] Values after substraction of cell mass control without substrate

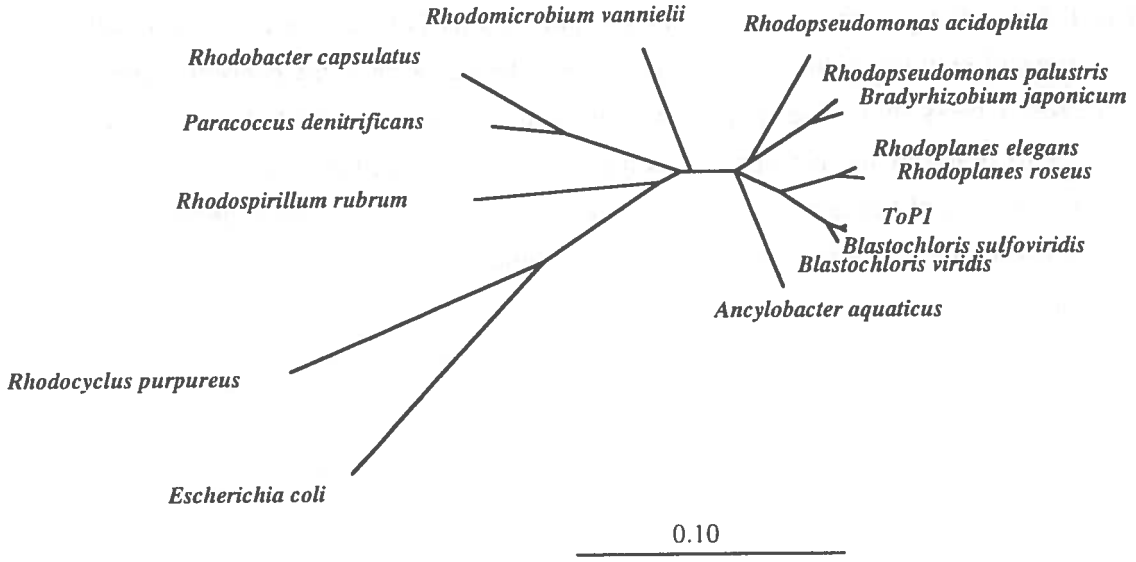

Fig. 3 Reconstructed phylogenetic tree based on 16S rRNA relationships of strains ToP1 and a selection of species from the α-subclass of Proteobacteria. The tree is based on the results of distance matrix analysis including complete or almost complete 16S rRNA gene sequences from representative bacteria of this subclass (Maidak et al., 1997). The topology of the tree was evaluated and corrected according to the results of distance-matrix, maximum-parsimony and maximum-likelihood analyses of various data sets. Phylogenetic positions of strain ToP1 did not differ in any of the treeing approaches. Topologies that could not be resolved unambiguously are shown as a multifurcation branching patterns, as recommended by Ludwig et al. (1998). The bar indicates 10% estimated sequence divergence. Accession numbers of sequences from shown strains were as follows: *Escherichia coli*, U00006; *Rhodocyclus purpureus*, M34132; *Rhodospirillum rubrum*, D30778; *Paracoccus denitrificans*, X69159; *Rhodobacter capsulatus*, D16428; *Rhodomicrobium vannielii*, M34127; *Rhodopseudomonas acidophila*, M34128; *Rhodopseudomonas palustris*, L11664; *Bradyrhizobium japonicum*, X87272; *Rhodoplanes elegans*, D25311; *Rhodoplanes roseus*, D25313; *Blastochloris sulfoviridis*, D86514; *Blastochloris viridis*, D25314; *Ancylobacter aquaticus*, M27803.

Study of anaerobic toluene activation. In the supernatant of toluene-grown cultures, up to 10 μM benzylsuccinate was detected by HPLC analysis. In denitrifying and sulfate-reducing bacteria, benzylsuccinate formation by addition of fumarate to the methyl group was previously shown as the initial reaction of anaerobic toluene catabolism (Evans et al. 1992; Biegert et al. 1996; Beller and Spormann 1997; Coschigano et al. 1998; Leuthner et al. 1998; Rabus and Heider 1998). It appeared therefore likely that the phototrophic bacterium, strain ToP1, initiates toluene utilization in the same manner as chemotrophic anaerobes. This assumption was confirmed by enzyme assays. Cell-free extracts of strain ToP1 catalyzed the formation of a non-volatile compound from ^{14}C-toluene and fumarate. Formation of this compound was 19-fold slower without added fumarate. The compound was identified as benzylsuccinate by co-chromatography with standard compounds on thin-layer chromatography plates (Fig. 4). The rate of benzylsuccinate formation in the cell-free extracts was 33.8 pmol·min^{-1}·(mg protein)$^{-1}$.

Table 1 Anaerobic growth tests of the phototrophic strains ToP1 and *Blastochloris sulfoviridis* (*Blc. sulfoviridis*) in the light. Each compound was tested twice at the concentrations given in parentheses. Unless otherwise noted, concentrations are given in mM. Concentrations in % (v/v) refer to dilutions of hydrophobic compounds in heptamethylnonane as an inert carrier phase. The volume of heptamethylnonane was 0.5 ml; the volume of the aqueous medium was 15 ml (in 20-ml tube). Symbols: ++ good growth within about 2 weeks or less; + growth time longer than 4 weeks; - no growth

Compound tested[a, b]	Strain ToP1	*Blc. sulfoviridis*, type strain
$H_2 + CO_2$	++	-
Ethanol (1, 5)	-	++
Glycerol (5, 10)	-	++[c]
Propionate (5, 10)	++	-[c]
Malate (0.5, 2)	-	++[c]
Fumarate (1, 5)	-	++
Glucose (1, 5)	-	++
Toluene (1%)	++	-
Benzoate (0.5, 2)	++	-
Cinnamate (0.5, 2)	++	nd
Benzylsuccinate (0.5, 2)	+	-
Crude oil[d]	++	-

[a] Further substrates utilized by both strains:
Sulfide (1, 2), thiosulfate (10, 20), acetate (10, 20), pyruvate (1, 5), lactate (5, 10), caproate (1, 5), caprylate (1, 2), succinate (1, 5), fructose (0.5, 2)

[b] Further compounds tested but not utilized by strain ToP1:
Formate (10, 20), alanine (1, 5), arginine (1, 5), aspartate (1, 5), glutamate (1, 5), ethylbenzene (2, 5%), *n*-propylbenzene (2, 5%), *o*-xylene (2, 5%), *m*-xylene (2, 5%), phenol (0.5, 2), benzyl alcohol (0.5, 2), 4-hydroxybenzoate (0.5, 2), phenylacetate (0.5, 2), phenylalanine (0.5, 2), cyclohexane carboxylate (0.5, 2)

[c] Data obtained from Imhoff and Trüper (1992)

[d] Added amounts: 0.45 and 0.9 ml per 15 ml culture volume

for Marine Microbiology Bremen, Germany onboard the Russian R/V PETR KOTTSOV in September 1997. The sampling stations were situated along a transect (except one station near the oil platform, Gloria), which extended over a length of ~ 225 km off the Romanian coast from 62 m to 2046 m water depth following the transition from fully oxic to sulfidic conditions.

Techniques for preparation of media and for cultivation of marine nitrate-reducing bacteria under anoxic conditions were as described for sulfate-reducing bacteria (Widdel and Bak 1992). Cultures were grown in defined, bicarbonate-buffered, sulfide-reduced (0.5 mM) mineral medium, essentially having the same composition of sodium, magnesium, potassium, calcium and chloride ions as natural seawater (Widdel and Bak 1992) but only 2 mM sulfate ions. Concentration of nitrate in the media were 5 mM. After nitrate was consumed a second portion of 5 mM nitrate was resupplied. Butyl-rubber sealed bottles (100, 250, and 500 ml) and tubes (20 ml) under a head space of N_2-CO_2 (90:10 [vol/vol]) were used for routine cultivation. Filter-sterilized (via solvent-resistant cellulose filters, pore size 0.2 µm) hydrocarbons, aromatic alcohols, aldehydes, and ketones were diluted (0.5 - 5 % [vol/vol]) in a carrier phase (0.5 ml per tube) of deaerated 2,2,4,4,6,8,8-heptamethylnonane to avoid toxic effects of the pure substances (Rabus et al. 1993). Crude oil (5 to 7.5 ml per 250 ml bottle) was added without dilution in a carrier phase. Bottles with the overlaid, insoluble hydrocarbon phases were incubated nearly horizontally to facilitate diffusion of substances into the aqueous medium. The orifices sealed with black rubber stoppers were kept somewhat below the surface level of the medium to avoid adsorption of hydrophobic compounds from the overlying phase by the rubber (Aeckersberg et al. 1991; Rabus et al. 1993). Heptamethylnonane and crude oil were gently deaerated and stored in a special flask under N_2 as described (Rabus and Widdel 1996). Water-soluble substrates were added from concentrated, separately sterilized stock solutions in water to yield the indicated concentrations.

Quantitative growth experiments with toluene dissolved in a carrier phase (concentration indicated in experiments) were carried out in flat glass bottles (250 ml) containing defined medium (190 ml) and heptamethylnonane (5 ml) under a head space of N_2-CO_2 (90:10 [vol/vol]). Bottles were incubated horizontally while contact of the carrier phase with the stoppers was avoided.

All used chemicals were of analytical grade.

Isolation, purity control, and maintenance. Toluene-utilizing strains were isolated via repeated dilution in agar tubes (Widdel and Bak 1992) each overlaid with 0.5 ml heptamethylnonane containing 1% (vol/vol) toluene. The salinity of the medium for preparation of the dilution series was increased by a factor of 1.5, such that mixing with the aqueous agar (6 ml medium added to 3 ml molten aqueous agar) yielded the same salt concentration as in the original liquid medium (Widdel and Bak 1992). Purity of isolates was routinely checked by examination by phase-contrast microscopy. In addition, cultures were supplied with yeast extract (0.5 g/l) and glucose or fructose (both 5 mM) and examined microscopically.

Sediment samples for enumeration of viable cells were homogenized by shaking under nitrogen. The most-probable-number (MPN) technique was used in three replicate series with 10-fold dilutions in liquid medium. Applied substrates were 10 mM acetate, 1 mM benzoate and toluene (1% [vol/vol], in heptamethylnonane). MPN tubes were incubated for at least 20 weeks. Numbers of bacteria per ml sediment of the respective sample were calculated by standard procedures (de Man 1975).

Sequence analyses of 16S rRNA genes. In vitro amplification of the 16S rRNA gene and direct sequencing was performed as previously described (Rainey et al. 1996). Sequencing reactions were performed by using the ABI PRISM™ Dye Terminator Cycle Sequencing Ready Reaction Kit (Applied Biosystems, Foster City, CA, USA). Sequences were determined using a 373A DNA sequencer (Applied Biosystems). The new sequence was added to an alignment of about 16,000 homologous primary structures of 16S rRNA genes (Maidak et al. 1999) by using the aligning tool of the ARB program package (Strunk and Ludwig 1998). Distance matrix, maximum parsimony and maximum likelihood methods were applied as implemented in the ARB software package. Phylogenetic trees were constructed using subsets of data that included sequences from representative members of the α and γ subclass of *Proteobacteria* as well as from outgroup species (Maidak et al. 1999). Topologies in the tree were evaluated by using the different approaches to elaborate a consensus tree (Ludwig et al. 1998). Sequence data of strains Col2 and TH1 are deposited under EMBL accession number AJ133761 and AJ133762, respectively.

Determination of G+C content of DNA. DNA was purified by using hydroxyapatite according to the method of Cashion et al. (Cashion et al. 1977). The G+C content of the DNA was determined by high-performance liquid chromatography (HPLC), (Mesbah et al. 1989). Analyses were performed at the Deutsche Sammlung von Mikroorganismen und Zellkulturen (Braunschweig, Germany).

Cell fixation and whole cell hybridization. Samples from enrichment cultures and MPN tubes were fixed in 4% formaldehyde-phosphate-buffered saline (PBS) for 2 hours on ice . Samples were washed in PBS and stored in a mixture of PBS-ethanol (1:1 [vol/vol]) at -20 °C, like described (Manz et al. 1998). Samples from enrichment cultures with crude oil and toluene, respectively, and MPN tubes were then hybridized with group-specific fluoresently labeled, rRNA-targeted oligonucleotides using 5'-labelled oligodeoxynucleotides with indocarbocyanine dye Cy3 (Llobet-Brossa et al. 1998; Manz et al. 1998). Total cell counts were determined by DAPI (4',6-diamidino-2-phenylindole) staining (Llobet-Brossa et al. 1998). Slides were examined with an Axiophot II microscope (Zeiss, Jena, Germany). For each sample between 700 and 1,000 DAPI-stained cells and the respective hybridized cells in 10 to 20 independent fields were counted. Counting results were corrected with signals observed with the probe NON338 (Snaidr et al. 1997).

Chemical analyses. Nitrate and nitrite were measured by high-performance liquid chromatography as described (Rabus and Widdel 1995). Before measurement, liquid samples

were diluted 1 : 5 with NaOH (10 mM), incubated for 1 hour at 4 °C, centrifuged (4 min at 13,000 rpm) and filtered (0.2 µm). N_2O was determined using a gas chromatograph coupled to conductivity detector (Rabus and Widdel 1995). Ammonium was measured by the indophenol formation reaction according to Marr (Marr et al. 1988).

Toluene dissolved in heptamethylnonane was measured by means of an Auto System gas chromatograph (Perkin Elmer, Überlingen, Germany) equipped with a PVMS 54 column (length, 50 m; inner diameter, 0.32 mm) and a flame ionization detector. The flow rate of H_2 was 1.7 ml·min^{-1}. The temperature program was run from 60 °C (2 min isotherm) to 120 °C at 5 °C·min^{-1}, and then from 120 °C (0.1 min isotherm) to 220 °C at 10 °C·min^{-1} (5 min isotherm at 220 °C). The temperatures at the injection port and the detector were 250 and 280 °C, respectively. Defined, freshly prepared solutions of toluene in heptamethylnonane were used for calibration.

Crude oil was analyzed by gas chromatography (GC-6000 Vega 2, Carlo Erba Instruments, Milano, Italy). Separation was achieved on a fused silica capillary column (DB-5, length, 30 m; inner diameter, 0.25 mm; J&W Scientific, Folsom, USA). The carrier gas was H_2 at a flow rate of 2.5 ml·min^{-1}. The temperature program run from 35 °C (3 min isotherm) to 310 °C at 4 °C·min^{-1} (15 min isotherm at 310 °C). The temperature at the injection port and the detector was 310 °C.

Toluene in the aqueous phase in cultures of strain Col2 were determined by a high-performance liquid chromatography system (Sykam, Gilching/Munich, Germany) equipped with a Spherisorb OD S2 reverse-phase column (250 by 5 mm). The eluent was an acetonitrile-water mixture (60:40, [vol/vol]) at a flow rate of 1 ml·min^{-1} and a column temperature of 25 °C. UV absorption spectra of peaks were determined between 195 and 360 nm by using a fast scanning detector (S 3206, Linear Instruments Co., NV, USA). Absorption at the maxima was used for its quantification. Defined, freshly prepared solutions of toluene in acetonitrile-water mixture (volume ratio as in eluent) were used as standards.

Results and Discussion

Counting series and batch enrichment cultures with toluene and nitrate. To search for marine denitrifying bacteria which degrade monoaromatic hydrocarbons anaerobically, toluene was chosen as the most intensely studied representative of this class of compounds. Anoxic serial dilutions with toluene (1% [vol/vol], in carrier phase of heptamethylnonane) and nitrate were carried out with sediment from the North Sea coast and from the western Black Sea. For comparison, parallel dilution series were performed with benzoate and acetate. Benzoate is expected to allow growth of most denitrifiers able to utilize alkylbenzenes as well as polar aromatic compounds (Heider and Fuchs 1997). Acetate, the most common product from fermentation breakdown of biomass (Schink 1992), was expected to allow growth of many of the cultivable denitrifying bacteria. Numbers of denitrifying bacteria

determined in North Sea sediment with toluene, benzoate, and acetate were $1.1 \cdot 10^4$, $1.5 \cdot 10^5$, and $9.3 \cdot 10^5$, respectively. The respective numbers determined in Black Sea sediment were $6.0 \cdot 10^1$, $1.8 \cdot 10^3$, and $2.2 \cdot 10^5$, respectively. Highest dilutions with toluene as substrate were fully grown after 16 weeks, dominated by coccoid bacteria in case of North Sea sediment and by rod-shaped bacteria in case of sediment originated from the Black Sea.

In addition, selection of marine denitrifying bacteria with toluene as the only organic substrate was studied in batch enrichment cultures. Enrichment of bacteria was originally attempted with two different concentrations of nitrate, 5 and 10 mM. Enrichment was only successful with 5 mM of nitrate. Immediately after consumption, a second portion of 5 mM nitrate was resupplied. If a total concentration of 10 mM nitrate was added at once, denitrification came to a standstill, probably due to the high accumulation of nitrite. Consumption of nitrate in comparison to controls without toluene increased after 4 and 8 weeks in bottles with North Sea sediment and Black Sea sediment, respectively. Sediment-free subcultures obtained after repeated transfer (5% inoculum [vol/vol]) with two subsequently added portion of 5 mM nitrate were fully grown after 2 and 3 weeks, respectively. In each enrichment cultures, the same cell types were observed as in the counting series of the respective origin.

Batch enrichment cultures with crude oil. Former denitrifying enrichment cultures of freshwater origin with crude oil as a natural complex mixture of hydrocarbons revealed complete consumption of toluene, ethylbenzene, and *m*-xylene and partial consumption of some other alkylbenzenes and *n*-alkanes (Rabus et al. 1999). In the present study, degradative capacities of marine denitrifying bacteria with respect to crude oil components was examined in enrichment cultures inoculated with North Sea and Black Sea sediment. From both habitats, denitrifying enrichment cultures were obtained and subcultivated. Their anaerobic growth on oil and nitrate was approximately half as fast as that of the respective enrichment cultures with toluene (see above).

Oil analysis after growth of the enrichment culture from North Sea sediment revealed utilization only of toluene; consumption was complete. In the enrichment culture from Black Sea sediment, toluene was also completely consumed. In addition, partial consumption of *o*-xylene, *m*/*p*-xylene (not distinguishable in presently applied analysis), *n*-heptane, *n*-octane, *n*-nonane, and *n*-decane was observed (respective consumption: 40, 60, 50, 50, 25, 30%). Due to the use of *n*-hexane as extractant, its possible consumption could not be measured in the present study. The range of hydrocarbons consumed by the marine enrichment culture from Black Sea is similar to the range consumed by a denitrifying enrichment culture of freshwater origin; however, the latter consumed in addition ethylbenzene from crude oil (Rabus et al. 1999).

Group-specific whole-cell hybridization. To investigate whether the marine counting series and enrichment cultures with hydrocarbons were dominated by distinctive phylogenetic groups, fluoresently labelled rRNA-targeted oligonucleotide probes were applied. Results are shown in Table 1. In the batch enrichment cultures, more than 83% of cells

Table 1. Total DAPI cell counts and relative percentages of hybridized cells with specific probes.

Habitiat	% of cells hybridized with probe[a]			
	EUB338	ALFA968	BET42a	GAM42a
North Sea				
MPN tubes with toluene[b]	55.3	1.3	>1.0	31.2
Enrichment with toluene	93.3	1.5	>1.0	79.8
Enrichment with crude oil	86.7	2.0	19.3	63.7
Black Sea				
MPN tubes with toluene[b]	65.6	21.1	6.3	>1.0
Enrichment with toluene	91.3	73,7	5.3	3.3
Enrichment with crude oil	83.5	1.7	53.3	17.8

[a]Oligonucleotide probes (formamide concentrations used for experiments): domain *Bacteria*, EUB338 (35%); α subclass of *Proteobacteria*, ALFA968 (30%), (Neef 1997); β subclass of *Proteobacteria*, BET42a (35%); γ subclass of *Proteobacteria*, GAM42a (35%); sulfate-reducing bacteria of the δ subclass of *Proteobacteria*, SRB385 (30%). Probes BET42a and GAM42a were used with a competitor (Neef 1997).
[b]Hybridizations were carried out with the highest dilution of most-probable number tubes that exhibit growth.

detectable by DAPI staining yielded a hybridization signal with probe EUB338 that covers the bacterial domain. The lower fraction of cells hybridizing with this probe in the MPN tubes was probably due to the fact that these cultures have been in the stationary phase for a longer time than the examined batch enrichment cultures. Often, cells reduce their ribosome content after the onset of starvation (Fukui et al. 1996). In all toluene-grown cultures (MPN tubes and batch enrichments) from North Sea sediment, most bacterial cells yielded a hybridization signal with probe GAM42a that is specific for the γ subclass of *Proteobacteria* (Fig. 1, A and B). In the toluene-grown cultures from Black Sea sediment, most cells reacted with probe ALFA968 that is specific for the α subclass of *Proteobacteria* (Fig. 1, C and D). In both batch enrichment cultures grown on crude oil, a significant fraction of cells belonging to the β subclass was observed; in the oil-grown enrichment culture from Black Sea sediment, bacteria of the β subclass were even the dominant ones. One may conclude that alkylbenzene in marine sediments favour growth of a phylogenetically more diverse population of denitrifiers than in freshwater sediment. All denitrifying bacteria enriched and isolated so far from freshwater habitats with toluene or other alkylbenzenes were members of the β subclass of *Proteobacteria*.

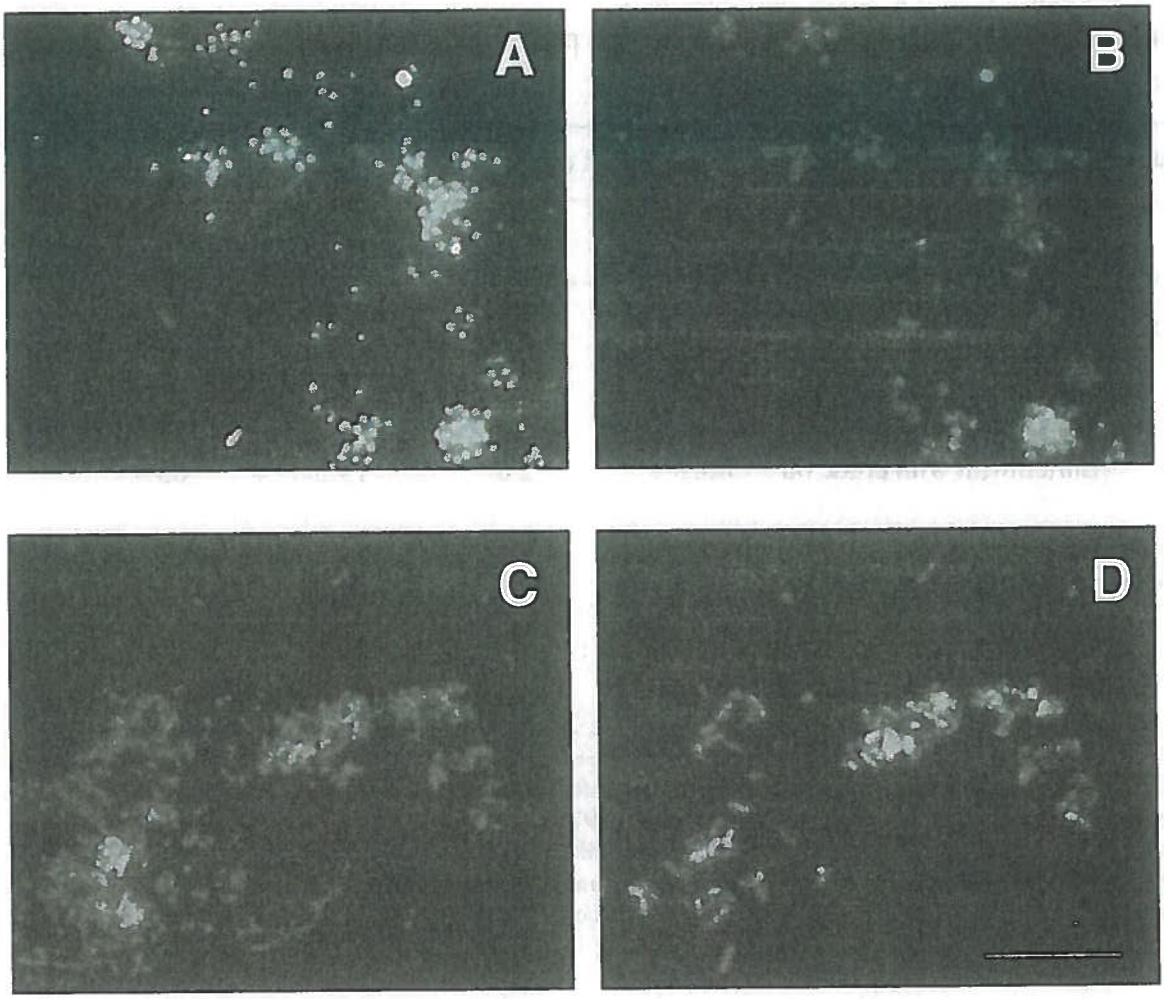

Fig. 1 Epifluorescence photomicrographs of bacterial cells enriched from (**A**, **B**) North Sea sediment and (**C**, **D**) Black Sea sediment in anoxic medium with toluene and nitrate. Photographs were taken under excitation with UV light showing fluorescence of DAPI staining (**A**, **C**), or the same microscopic fields under excitation with green light showing fluorescence of probe GAM42a (**B**) or probe ALFA968 (**D**). The bar in panel D represents 5 μm and applies to all photomicrographs.

Isolation and characterization of marine toluene-degrading denitrifying bacteria. The observed abundance of bacteria of the α and γ subclass that have not been detected so far among alkylbenzene-degrading denitrifying bacteria prompted us to attempt the isolation of two novel representatives of these phylogenetic groups. Via repeated agar dilution series, two new strains of toluene-utilizing denitrifying bacteria were isolated. Strain Col2 was from the oil-degrading enrichment culture originating from North Sea sediment, and strain TH1 was from the toluene-degrading enrichment culture originating from Black Sea sediment. Cells of strain Col2 were oval-shaped to spherical and tended to from loose aggregates (Fig. 2A). Cells of strain TH1 were rod-shaped (Fig. 2B). Strains did not exhibit motility. Characteristics including substrate tests are shown in Table 2.

Table 2. Morphological and physiological characteristics of the two new marine denitrifying strains Col2 and TH1

Characteristics	Strain Col2	Strain TH1
Width × length (µm)	1.2 - 1.5 × 1.5 - 1.7	0.7 - 1.0 × 1.7 - 2.5
pH-range of growth	6.4 - 9.8	6.4 - 8.6
pH-optimum	7.6	7.2
Temperature range of growth (°C)	5 - 40	15 - 30
Temperature optimum (°C)	37	28
G+C content of DNA (mol%)	68.4	64.9
Compounds tested[a]		
Toluene (2%)	+	+
Benzyl alcohol (0.5, 2)	+	-
Benzoate (1, 4)	+	+
Phenylacetate (0.5, 2)	+	-
Ethanol (1, 5)	+	-
Formate (1, 5)	-	+
Acetate (5, 10)	+	-
Propionate (5, 10)	+	-
n-Butyrate (5, 10)	+	-
Lactate (5, 10)	+	+
Pyruvate (1, 5)	+	+
Succinate (1, 5)	+	+
Fumarate (1, 5)	+	+
D,L-Malate (1, 5)	+	+

[a]Each compound was tested twice at the concentrations given in parentheses. Unless otherwise noted, concentrations are millimolar. Concentrations in percentages (vol/vol) refer to dilutions of hydrophobic compounds in heptamethylnonane as an inert carrier phase. Symbols: +, growth observed; -, no growth observed. Further compounds tested but utilized by neither strain: benzene (0.5, 2%), ethylbenzene (0.5, 2%), *o*-xylene, *m*-xylene, and *p*-xylene (0.5, 2% each), cyclohexane (0.5, 2%), phenol (0.5, 2), benzylsuccinate (1, 4), glucose (1, 5), fructose (1, 5), hydrogen

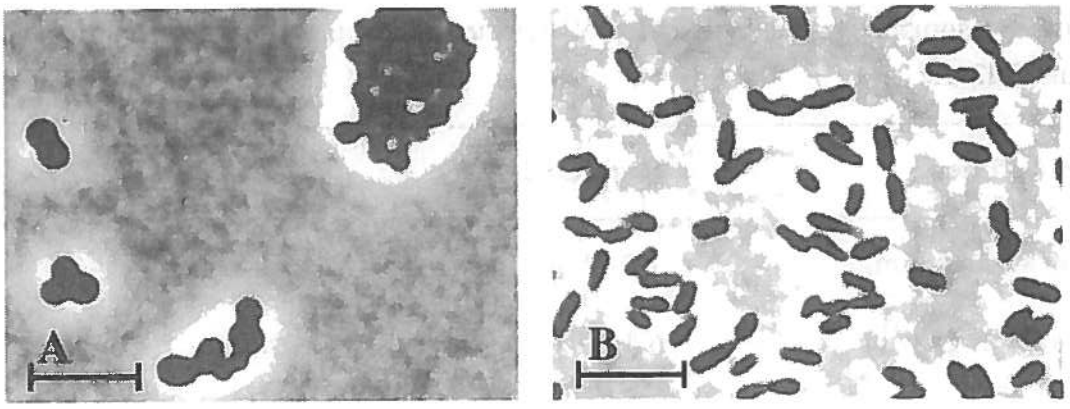

Fig. 2 Phase contrast photomicrographs of newly isolated marine denitrifying bacteria utilizing toluene. (**A**) Strain Col2 originating from North Sea sediment and (**B**) strain TH1 originating from Black Sea sediment. Bar, 5 μm.

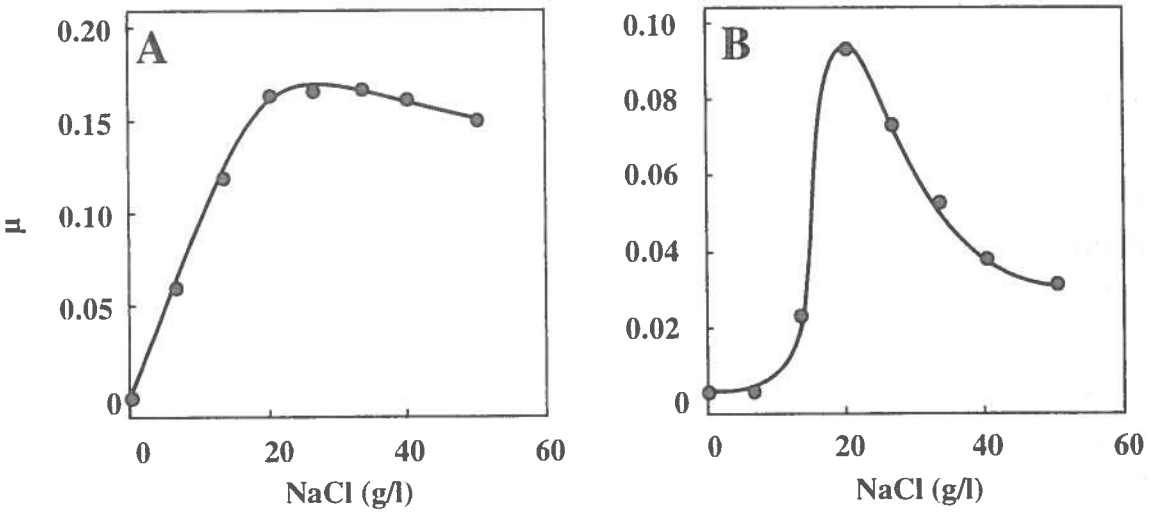

Fig. 3 Effect of NaCl concentration on growth of (**A**) strain Col2 and (**B**) TH1. Growth rates (μ) were calculated from growth curves (not shown).

Since both strains were of marine origin, the effect of the NaCl concentration on growth was tested. Both strains exhibited a pronounced requirement for NaCl and did not grow without the salt (Fig. 3). The result confirms that both isolates are typical marine denitrifying bacteria.

Analysis of the 16S rRNA genes revealed that strain Col2 and strain TH1 affiliated with the γ and α subclass, respectively, of the *Proteobacteria* (Fig. 4). Bacteria with the same phylogenetic affiliation and the same morphology as strain Col2 and strain TH1 dominated in the counting series with toluene from the respective habitats. The new isolates are the first alkylbenzene-degrading denitrifying bacteria not belonging to the *Azoarcus-Thauera* branch of the β subclass of *Proteobacteria*.

Acknowledgments. This work was supported by the Max-Planck-Gesellschaft and the Fonds der Chemischen Industrie.

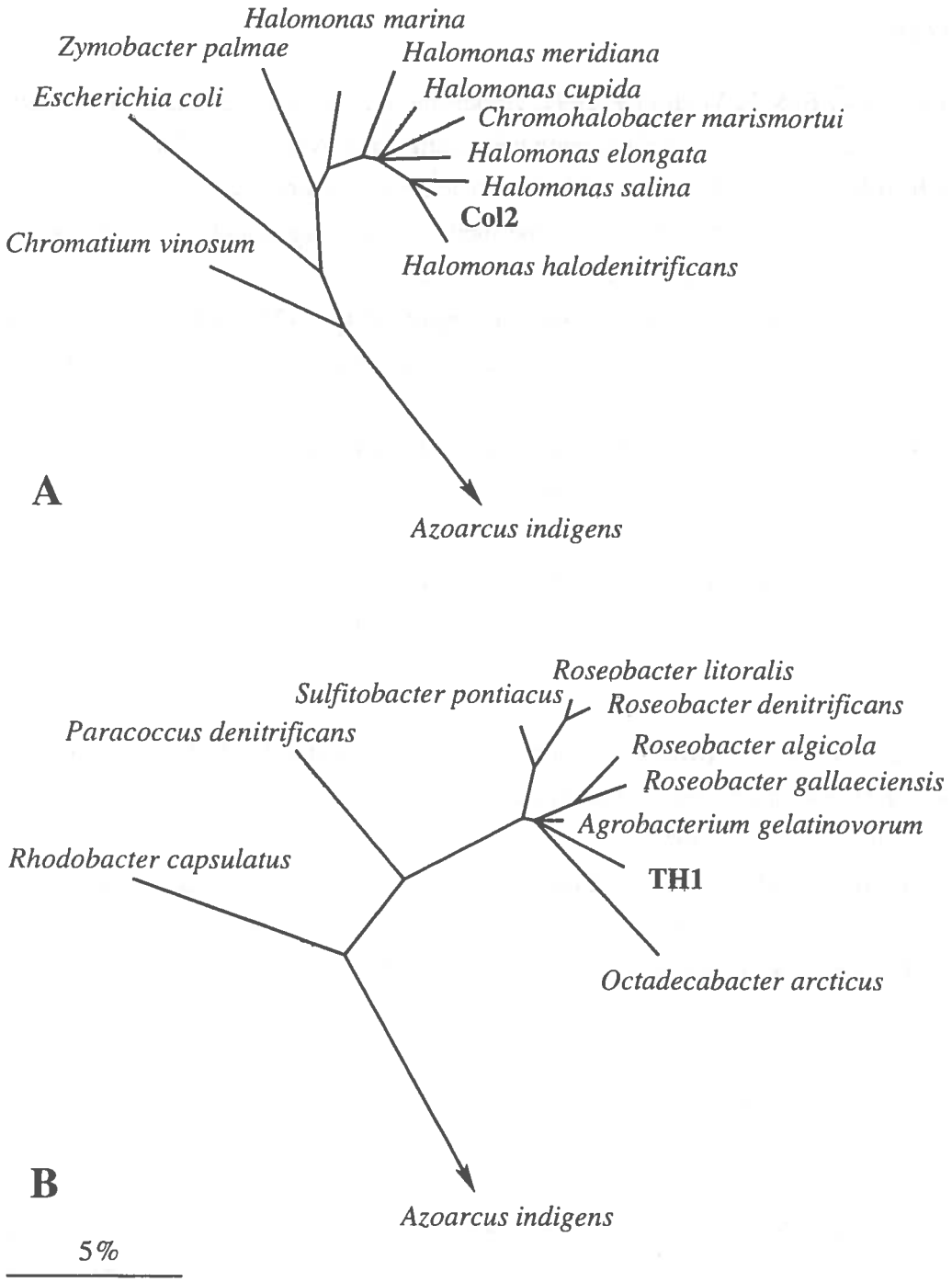

Fig. 4 Reconstructed phylogenetic tree based on 16S rRNA reflecting relationships of new marine toluene-utilizing denitrifiers. (**A**) Relationships of strain Col2 (from North Sea) within the gamma subclass of *Proteobacteria*; (**B**) relationships of strain TH1 (from Black Sea) within the alpha subclass of *Proteobacteria*. New sequences were added to an alignment of about 16,000 homologous bacterial 16S rRNA primary structures by using the aligning tool of the ARB program package (Strunk and Ludwig 1998; Maidak et al. 1999). The trees are based on maximum parsimony analysis including complete or almost complete 16S rRNA sequences from representative bacteria (Strunk and Ludwig 1998; Maidak et al. 1999). Topologies were evaluated and corrected according to distance matrix, maximum parsimony and maximum likelihood analyses of various data sets to construct the consensus trees (Ludwig et al. 1998). Multifurcations indicate topologies that could not be unambiguously resolved. Bar indicates 5% estimated sequence divergence.

References

Aeckersberg F, Bak F, Widdel F 1991. Anaerobic oxidation of saturated hydrocarbons to CO_2 by a new type of sulfate-reducing bacterium. Arch. Microbiol. 156:5-14.

Altenschmidt U, Fuchs G 1991. Anaerobic degradation of toluene in denitrifying *Pseudomonas* sp.: indication for toluene methylhydroxylation and benzoyl-CoA as central aromatic intermediate. Arch. Microbiol. 156:152-158.

Ball HA, Johnson HA, Reinhard M, Spormann AM 1996. Initial reactions in anaerobic ethylbenzene oxidation by a denitrifying bacterium, strain EB1. J. Bacteriol. 178:5755-5761.

Beller HR, Spormann AM, Sharma PK, Cole JR, Reinhard M 1996. Isolation and characterization of a novel toluene-degrading, sulfate-reducing bacterium. Appl. Environ. Microbiol. 62:1188-1196.

Cashion P, Holder-Franklin MA, McCully J, Franklin M 1977. A rapid method for the base ratio determination of bacterial DNA. Anal. Biochem. 81:461-466.

de Man JC 1975. The probability of most probable numbers. Eur. J. Appl. Microbiol. 1:67-78.

Dolfing J, Zeyer J, Binder-Eicher P, Schwarzenbach RP 1990. Isolation and characterization of a bacterium that mineralizes toluene in the abscene of molecular oxygen. Arch. Microbiol. 154:336-341.

Evans PJ, Mang DT, Kim KS, Young LY 1991. Anaerobic degradation of toluene by a denitrifying bacterium. Appl. Environ. Microbiol. 57:1139-1145.

Fries MR, Zhou J, Chee-Sanford J, Tiedje JM 1994. Isolation, charaterization, and distribution of denitrifying toluene degraders from avariety of habitats. Appl. Environ. Microbiol. 60:2802-2810.

Fukui M, Suwa Y, Urushigawa Y 1996. High survival efficiency and ribosomal RNA decaying pattern of *Desulfobacter latus*, a highly specific acetate-utilizing organism, during starvation. FEMS Microbiol. Ecol. 19:17-25.

Harms, G., R. Rabus, and F. Widdel. 1999. Anaerobic oxidation of the aromatic hydrocarbon, *p*-isopropyltoluene (*p*-cymene) by two new types of denitrifying bacteria. Arch. Microbiol., in press.

Harms G, Zengler K, Rabus R, Aeckersberg F, Minz D, Rosselló-Mora R, Widdel F 1999. Anaerobic oxidation of *o*-xylene, *m*-xylene, and homologous alkybenzenes by new types of sulfate-reducing bacteria. Appl. Environ. Microbiol. 65:999-1004.

Heider J, Fuchs G 1997. Anaerobic metabolism of aromatic compounds. Eur. J. Biochem. 243:577-596.

Hess A, Zarda B, Hahn D, Häner A, Stax D, Höhener P, Zeyer J 1997. In situ analysis of denitrifying toluene- and *m*-xylene-degrading bacteria in a diesel fuel-contaminated laboratory aquifer column. Appl. Environ. Microbiol. 63:2136-2141.

Jørgensen BB 1982. Mineralization of organic matter in the sea bed - the role of sulphate reduction. Nature (London) 296:643-645.

Jørgensen BB 1983. Processes at the sediment-water interface, p. 477-509. *In* Bolin B and Cook RB (ed.), The Major Biogeochemical Cycles and Their Interactions. J. Wiley & Sons, New York.

Llobet-Brossa E, Rosselló-Mora R, Amann R 1998. Microbial community composition of Wadden Sea sediments as revealed by fluorescence in situ hybridization. Appl. Environ. Microbiol. 64:2691-2696.

Lovley DR, Baedecker MJ, Lonergan DJ, Cozzarelli IM, Phillips EJP, Siegel DI 1989. Oxidation of aromatic contaminants coupled to microbial iron reduction. Nature (London) 339:297-299.

Ludwig W, Strunk O, Klugbauer N, Weizenegger M, Neumaier J, Bahleitner M, Schleifer K-H 1998. Bacterial phylogeny based on comparative sequence analysis. Electrophoresis 19:554-568.

Maidak BL, Cole JR, Parker CT, Garrity GM Jr, Larsen N, Li B, Lilburn TG, McCaughey MJ, Olsen GJ, Overbeek R, Pramanik S, Schmidt TM, Tiedje JM, Woese CR 1999. A new version of the RDP (Ribosomal Database Project). Nucleic Acids Res. 27:171-173.

Manz W, Eisenbrecher M, Neu TR, Szewzyk U 1998. Abundance and spatial organization of Gram-negative sulfate-reducing bacteria in activated sludge investigated by in situ probing with specific 16S rRNA targeted oligonucleotides. FEMS Microbiol. Ecol. 25:43-61.

Marr IL, Cresser MS, Ottendorfer LJ 1988. Umweltanalytik. Eine allgemein Einführung. Thieme Verlag, Stuttgart.

Mesbah M, Premachandran U, Whitman WB 1989. Precise measurement of G+C content of desoxyribonuleic acid by high-performance liquid chromatography. Int. J. Sys. Bacteriol. 39:159-167.

Neef A. 1997. Anwendung der in situ-Einzelzell-Identifizierung von Bakterien zur Populationsanalyse in Komplexen mikrobiellen Biozönosen. Dissertation. Technische Universität München, Munich, Germany.

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

Rabus R, Widdel F 1995. Anaerobic degradation of ethylbenzene and other aromatic hydrocarbons by new denitrifying bacteria. Arch. Microbiol. 163:96-103.

Rabus R, Widdel F 1996. Utilization of alkylbenzenes during anaerobic growth of pure cultures of denitrifying bacteria on crude oil. Appl. Environ. Microbiol. 62:1238-1241.

Rabus R, Wilkes H, Schramm A, Harms G, Behrends A, Amann R, Widdel F 1999. Anaerobic utilisation of alkylbenzenes and *n*-alkanes from crude oil in an enrichment culture of denitrifying bacteria affiliated with *Azoarcus/Thauera* cluster. Environ. Microbiol. 1:145-157

Rainey FA, Ward-Rainey N, Kroppenstedt RM, Stackebrandt E 1996. The genus *Nocardiopsis* represents a phylogenetically coherent taxon and a distinct actinomycete lineage: proposal of *Nocardiapsaceae* fam. nov. Int. J. Sys. Bacteriol. 46:1088-1092.

Schink B 1992. Syntrophism among prokaryotes, p. 276-299. *In* Balows AT, Trüper HG, Dworkin M, Harder W, Schleifer K-H (eds.), The Prokaryotes, 2nd ed, vol. 1. Springer-Verlag, Berlin Heidelberg New York.

Schocher RJ, Seyfried B, Vazquez F, Zeyer J 1991. Anaerobic degradation of toluene by pure cultures of denitrifying bactera. Arch. Microbiol. 157:7-12.

Snaidr J, Amann R, Huber I, Ludwig W, Schleifer K-H 1997. Phylogenetic analysis and in situ identification of bacteria in activated sludge. Appl. Environ. Microbiol. 63:2884-2896.

Strunk O, Ludwig W 1998. ARB: a software environment for sequence data, http://www.mikro.biologie.tu-muenchen.de. Department of Microbiology, Technische Universität München, Munich, Germany.

US Environmental Protection Agency 1986. Underground motor fuel storage tanks: a national survey. Publication NTIS PB 86-216512. U.S. Environmental Protection Agency, Washington, D.C.

Widdel F, Bak F. 1992. Gram-negative mesophilic sulfate-reducing bacteria, p. 3352-3392. *In* Balows AT, Trüper HG, Dworkin M, Harder W, Schleifer K-H (eds.), The Prokaryotes, 2nd ed, vol. 4. Springer-Verlag, Berlin Heidelberg New York.

Zarda B, Mattison G, Hess A, Hahn D, Höhener P, Zeyer J 1998. Analysis of bacterial and protozoan communities in an aquifer contaminated with monoaromatic hydrocarbons. FEMS Microbiol. Ecol. 27:141-152.

Zhou J, Palumbo AV, Tiedje JM 1997. Sensitive detection of a novel class of toluene-degrading denitrifiers, *Azoarcus tolulyticus*, with small-subunit rRNA primers and probes. Appl. Environ. Microbiol. 63:2384-2390.

3

Anaerobic oxidation of *o*-xylene, *m*-xylene, and homologous alkylbenzenes by new types of sulfate-reducing bacteria

Gerda Harms, Karsten Zengler, Ralf Rabus, Frank Aeckersberg, Dror Minz, Ramon Rosselló-Mora, and Friedrich Widdel*

Applied and Environmental Microbiology (1999) 65: 999-1004

Max-Planck-Institute for Marine Microbiology, Celsiusstr. 1, D-28359 Bremen, Germany

* Corresponding author

Abstract

Various alkylbenzenes were depleted during growth of an anaerobic, sulfate-reducing enrichment culture with crude oil as the only source of organic substrates. From this culture, two new types of mesophilic, rod-shaped sulfate-reducing bacteria, strains oXyS1 and mXyS1, were isolated with *o*-xylene and *m*-xylene, respectively, as organic substrates. Sequence analyses of 16S rRNA genes revealed that the isolates affiliated with known completely oxidizing sulfate-reducing bacteria of the δ-subclass of *Proteobacteria*. Strain oXyS1 showed the highest similarities with *Desulfobacterium cetonicum* and *Desulfosarcina variabilis* (similarity values, 98.4 and 98.7%, respectively). Strain mXyS1 was less closely related to known species, the closest relative being *Desulfococcus multivorans* (similarity value, 89.6%). Complete mineralization of *o*-xylene and *m*-xylene was demonstrated in quantitative growth experiments. Strain oXyS1 was able to utilize toluene, *o*-ethyltoluene, benzoate, and *o*-methylbenzoate in addition to *o*-xylene. Strain mXyS1 oxidized toluene, *m*-ethyltoluene, *m*-isoproyltoluene, benzoate, and *m*-methyl-benzoate in addition to *m*-xylene. Strain oXyS1 did not grow *m*-alkyltoluenes, whereas strain mXyS1 did not utilize *o*-alkyltoluenes. Like the enrichment culture, both isolates grew anaerobically on crude oil with concomitant reduction of sulfate to sulfide.

Introduction

Sulfate-reducing bacteria are an important group of anaerobes in the global carbon and sulfur cycle. Oxidation of organic compounds coupled to the reduction of sulfate to sulfide may account for more than 50% of carbon mineralization in marine sediments (Jørgensen 1982). In oil field waters, however, the activity of sulfate-reducing bacteria may be detrimental. Their product, hydrogen sulfide, is toxic and corrosive, increases the sulfur content of oil and gas, and leads to the precipitation of ferrous sulfide, which plugs oil-bearing strata and stabilizes undesirable oil-water emulsions (Cord-Ruwisch et al. 1987, Herbert 1987, Odom 1993). Recently it was demonstrated that in a mesophilic (around 30°C) enrichment culture, sulfate-reducing bacteria can utilize various alkylbenzenes directly from crude oil (Rabus et al. 1996, Rueter et al. 1994). Besides alkanes, alkylbenzenes and other aromatic hydrocarbons are major constituents of crude oil (Tissot and Welte 1984). A moderately thermophilic (≤65°C) sulfate-reducing isolate grew by consumption of *n*-alkanes from oil (Rueter et al. 1994). Hence, oil hydrocarbons were supposed to serve as growth substrates for sulfate-reducing bacteria under in situ conditions in oil reservoirs, at least at temperatures not higher than the optima of the respective cultures. More recent measurements with enrichment cultures under sulfate-reducing conditions at 50 to 70°C with added aromatic hydrocarbons revealed partial consumption, especially of xylenes and ethylbenzene (Chen and Taylor 1997). This suggested that anaerobic degradation of aromatic hydrocarbons coupled to sulfate reduction in oil reservoirs is in principle possible also under thermophilic conditions. Also, extremely thermophilic (85°C) sulfate-reducing *Archaea* have been detected in oil reservoirs (L`Haridon et al. 1995, Stetter et al. 1993); however, utilization of hydrocarbons by these *Archaea* has not been demonstrated.

16S rRNA-targeted oligonucleotide probing of the mesophilic enrichment culture by using alkylbenzenes from crude oil revealed that the major part of the bacterial population belonged to a cluster (suggested family, *Desulfobacteriaceae* [Widdel and Bak 1992]) that comprises known species of completely oxidizing sulfate-reducing bacteria within the δ-subclass of *Proteobacteria* (Rabus et al. 1996). Besides toluene, the enrichment culture also consumed *o*-xylene and *m*-xylene (Rabus et al. 1996, Rueter et al. 1994). This paper reports on the isolation of two new types of sulfate-reducing bacteria from the enrichment culture that are able to grow on *o*-xylene or *m*-xylene. This degradative capacity has not been observed so far in pure cultures of sulfate-reducing bacteria. Among aromatic hydrocarbons, only toluene was shown before to serve as growth substrate for isolated strains of sulfate-reducing bacteria (Beller et al. 1996, Rabus et al. 1993). Anaerobic growth of pure cultures on *m*-xylene has been shown with denitrifying bacteria (Dolfing et al. 1990, Fries et al. 1994, Hess et al. 1997, Rabus and Widdel 1995a, Seyfried et al. 1994). However, to our knowledge, no strain of any type of anaerobic bacterium that grows on *o*-xylene or *p*-xylene has been isolated to date. In some instances, pure cultures converted these xylenes cometabolically to aromatic dead-end metabolites (Beller et al. 1996, Biegert and Fuchs 1995, Evans et al. 1991b, Rabus and Widdel 1995b). In contrast, enriched bacterial populations (microcosms and consortia) have been

repeatedly shown to utilize the isomers of xylene (Chen and Taylor 1997, Edwards et al. 1992, Edwards and Grbic-Galic 1994, Evans et al. 1991a, Häner et al. 1995, Zeyer et al. 1986).

Materials and Methods

Sources of bacteria. The new types of sulfate-reducing bacteria were isolated from a previously described mesophilic enrichment culture growing anaerobically with crude oil and sulfate in seawater medium (Rabus et al. 1996, Rueter et al. 1994). The enrichment culture originated from the water phase of a North Sea oil tank in Wilhelmshaven, Germany. *Desulfobacterium cetonicum* (DSM 7267) was from the Deutsche Sammlung von Mikroorganismen und Zellkulturen, Braunschweig, Germany.

Media, cultivation techniques, and quantitative growth experiments. Techniques for preparation of media and for cultivation of sulfate-reducing bacteria under anoxic conditions were as described previously (Widdel and Bak 1992). Cultures were grown in defined bicarbonate-buffered, sulfide-reduced mineral medium, essentially having the same sodium, magnesium, potassium, calcium, chloride, and sulfate ion compositions as natural seawater (Widdel and Bak 1992). Butyl-rubber sealed tubes (20 ml) containing 15 ml of medium under a headspace of N_2-CO_2 (90:10 [vol/vol]) were used for routine cultivation. Filter-sterilized (via solvent-resistant cellulose filters, pore size 0.2 µm) hydrocarbons and aromatic alcohols, aldehydes, and ketones were diluted (0.5 to 5% [vol/vol]) in a carrier phase (0.5 ml per tube) of deaerated 2,2,4,4,6,8,8-heptamethylnonane to avoid the toxic effects of the pure substances (Rabus et al. 1993). Anoxic, sterile crude oil (0.5 ml per cultivation tube) was added directly to the cultures without dilution in a carrier phase. The tubes with the overlaid insoluble hydrocarbon phases were incubated nearly horizontally to facilitate diffusion of substances into the aqueous medium. The orifices sealed with black rubber stoppers were kept somewhat below the surface of the medium to avoid adsorption of hydrophobic compounds from the overlying hydrocarbon phase by the rubber (Aeckersberg et al. 1991, Rabus et al. 1993). Heptamethylnonane and crude oil were deaerated, sterilized, and stored in a special flask under an atmosphere of N_2 as described previously (Aeckersberg et al. 1991, Rabus and Widdel 1996). Water-soluble substrates were added from autoclaved or filter-sterilized aqueous stock solutions to yield the indicated concentrations.

Quantitative growth experiments with xylenes dissolved in a carrier phase (the concentrations are indicated in the descriptions of the individual experiments) were carried out by using flat glass bottles (250 and 500 ml) containing defined mineral medium (190 and 450 ml, respectively) and heptamethylnonane (5 and 15 ml, respectively) under a headspace of N_2-CO_2 (90:10 [vol/vol]). The bottles were incubated horizontally on a rotary shaker (65 rpm), while contact of the carrier phase with the stoppers was avoided, as described for cultivation in tubes.

All the chemicals used were analytical grade.

Isolation, purity control and maintenance. Strains oXyS1 and mXyS1 were isolated via repeated dilution in agar tubes (Widdel and Bak 1992). The agar was overlaid with 0.5 ml of heptamethylnonane containing 2% (vol/vol) *o*-xylene or *m*-xylene, respectively. The salinity (NaCl, $MgCl_2$, $MgSO_4$ and $CaCl_2$) of the medium for preparation of the dilution series was increased by a factor of 1.5, so that mixing with the aqueous agar (6 ml of medium added to 3 ml of molten aqueous agar) yielded the same salt concentration as was present in the original liquid medium (Widdel and Bak 1992).

The purity of isolates was routinely checked by phase-contrast microscopy. In addition, cultures were supplied with yeast extract (0.5 g/l) and glucose or fructose (both 5 mM) and examined microscopically.

For maintenance, strains oXyS1 and mXyS1 were grown on *o*-xylene and *m*-xylene, respectively, stored at 4°C, and transferred every 6 to 8 weeks.

Sequence analyses of 16S rRNA genes. Genomic DNA of strain oXyS1 was extracted and a 16S rRNA gene sequence was amplified as described by Rainey et al. (Rainey et al. 1992). The PCR products were sequenced directly. Analysis was carried out by Fred A. Rainey at the Deutsche Sammlung von Mikroorganismen und Zellkulturen. Genomic DNA of strain mXyS1 was extracted as described by Tsai and Olson (Tsai and Olson 1991). The 16S rRNA gene sequence was amplified with bacterial primers GM3F and GM4R (Muyzer et al. 1995). The amplified fragment was cloned into the pGM-T vector (Promega, Madison, Wis.). Plasmids were purified with the Wizard plasmid purification kit (Promega). Sequencing reactions were performed with the *Taq* Dye-Deoxy™ terminator cycle-sequencing kit (Applied Biosystems [ABI], Foster City, Calif.). Sequences were determined with an ABI 373S DNA sequencer according to manufacturer's instructions.

New sequences were added to an alignment of about 5,300 homologous primary structures of bacterial 16S rRNA genes by using the alignment tool of the ARB program package (Strunk and Ludwig). Similarity and distance matrices were calculated with the ARB-PHYL program of the same package. Phylogenetic trees were constructed by using subsets of data that included representative sequences of members of the δ-subclass of the *Proteobacteria* (Maidak et al. 1997). We used distance matrix, maximum-likelihood, and maximum-parsimony methods as implemented in the programs PHYLIP (Felsenstein 1992), ARB, and fastDNAml (Maidak et al. 1997).

Chemical analyses. For routine detection of sulfide formed in enrichment cultures, a simple test with Cu^{2+} ions yielding CuS was applied (Cord-Ruwisch 1985). Sulfide in quantitative growth experiments was determined colorimetrically by the methylene blue formation reaction as described previously (Aeckersberg et al. 1991, Cline 1969).

Xylenes dissolved in heptamethylnonane were measured by means of an Auto System gas chromatograph (Perkin Elmer, Norwalk, Conn.) equipped with a PVMS 54 column (length 50 m; inner diameter, 0.32 mm) and a flame ionization detector. H_2 was used as the carrier gas at a flow rate of 1.7 $ml \cdot min^{-1}$. The temperature program was run from 80°C (2-min isotherm) to

120°C at 20°C·min^{-1} and then from 120°C (0.1-min isotherm) to 300°C at 40°C·min^{-1}. The temperatures at the injection port and the detector were 250 and 350°C, respectively. Defined, freshly prepared solutions of xylenes in heptamethylnonane were used for calibration.

Xylenes in the aqueous phase were determined by a high-performance liquid chromatography system (Sykam, Gilching/Munich, Germany) equipped with a Spherisorb OD S2 reverse-phase column (250 by 5 mm); the eluent was an acetonitrile-water mixture (80:20 [vol/vol]) in distilled water. The flow rate was 1 ml·min^{-1} and the temperature of the column was 25°C (Rabus and Widdel 1995a). Detection was performed by UV absorption at 265 nm. Defined, freshly prepared solutions of xylenes in acetonitrile-water (80:20 [vol/vol]) were used as standards.

The G+C content of the DNA was determined by high-performance liquid chromatography as described previously (Mesbah et al. 1989). The analysis was carried out by Fred A. Rainey.

Enzymatic tests. Anoxic preparation of cell-free extracts and enzyme assays were carried out as described previously (Aeckersberg et al. 1991, Rabus et al. 1993). Protein was quantified by the method of Bradford (Bradford 1976).

Nucleotide sequence accession numbers. The new sequences determined in this study are deposited under EMBL accession no. Y17286 (strain oXyS1) and AJ006853 (strain mXyS1).

Results and Discussion

Isolation. An anaerobic enrichment culture with crude oil as the only source of organic substrates and sulfate as the electron acceptor consumed toluene, *o*-xylene, *m*-xylene, *o*-ethyltoluene, *m*-ethyltoluene, *m*-propyltoluene and *m*-isopropyltoluene, with concomitant production of sulfide (Rabus et al. 1996, Rueter et al. 1994). In counting series using agar dilutions with benzoate or overlaid with crude oil or toluene (in carrier phases), large numbers of colonies of sulfate-reducing bacteria were formerly obtained that were also able to grow with these substrates when transferred back to liquid medium (Rabus et al. 1996). Counting of bacteria with defined alkylbenzenes other than toluene was not carried out during the initial study. In the present study, we therefore attempted to isolate sulfate-reducing bacteria from the enrichment culture growing with oil directly by using *o*-xylene and *m*-xylene as defined hydrocarbons for dilution series. These two compounds were chosen because they were consumed the most rapidly, next to toluene. Furthermore, strains of sulfate-reducing bacteria utilizing *o*-xylene and *m*-xylene would present novel metabolic types; their study would add to our knowledge of the nutritional and phylogenetic diversity of sulfate-reducing bacteria that degrade hydrocarbons. Dilution series from the enrichment culture were carried out by using agar overlaid with *o*-xylene and *m*-xylene in a carrier phase. The largest colonies developed next to the overlying hydrocarbon phase. Pure cultures were isolated by a second agar dilution

series. One strain isolated with *o*-xylene and one strain isolated with *m*-xylene were chosen for further investigations. These strains were designated as oXyS1 and mXyS1, respectively.

Morphological and other characteristics. The cells of both new isolates were rod shaped. The cells of strain oXyS1 were more elongated than those of strain mXyS1 (Fig. 1). The dimensions were 0.8 to 1.0 by 2.5 to 4.0 µm for strain oXyS1 and 0.6 to 1.0 by 1 to 2 µm for strain mXyS1.

Growth of strain oXyS1 was observed within a temperature range of 15 to 35°C, with an optimum around 32°C, and within a pH range of 6.2 to 7.9, with an optimum around 7.5. Growth of strain mXyS1 occurred within a temperature range of 16 to 35°C, with an optimum around 30°C, and within a pH range of 5.8 to 8, with an optimum around 7.2.

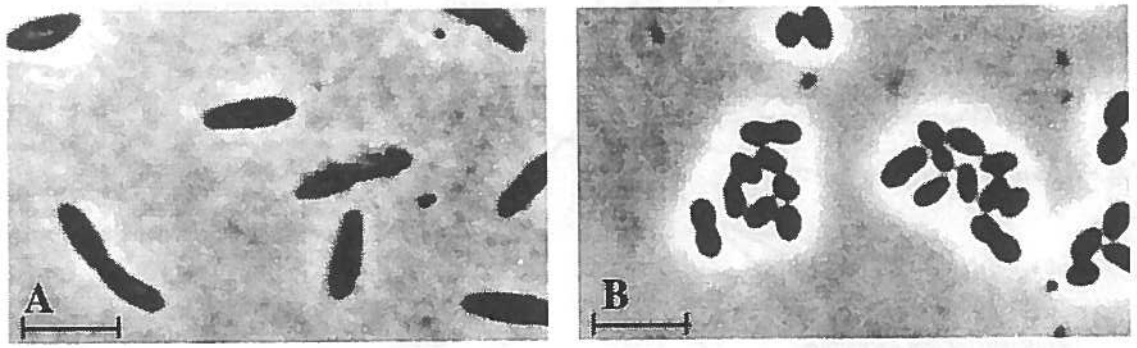

Fig. 1 Phase contrast photomicrographs of newly isolated sulfate-reducing bacteria utilizing xylenes. (A): Strain oXyS1 isolated with *o*-xylene; (B): strain mXyS1 isolated with *m*-xylene. Bar, 5 µm.

Relationships based on 16S rRNA gene sequences and G+C content of DNA. Analyses of sequences derived from the 16S rRNA genes revealed that both strains affiliated with the δ-subclass of the *Proteobacteria* and branch within a group for which the family designation *Desulfobacteriaceae* has been proposed (Widdel and Bak 1992). This group of mesophilic sulfate-reducing bacteria contains, among a few incompletely oxidizing species, the above described completely oxidizing species. Many of these are, like the presently obtained isolates, nutritionally rather versatile, and several have been reported to grow with aromatic compounds, most commonly with benzoate (Widdel and Bak 1992). The closest relatives of strain oXyS1 among the described species were *Desulfobacterium cetonicum* and *Desulfosarcina variabilis*, for which the similarity values being as high as 98.4 and 98.7%, respectively. As shown in Fig. 2, the branching of strain oXyS1 and its closest relatives is drawn as a multifurcation because the topology could not be resolved unambiguously by the different treeing approaches. Due to the high similarity values among the sequences from these three organisms, they may be even regarded as members of one species (Goodfellow et al. 1997, Stackebrandt and Goebel 1994). For definite classification in the future, however,

additional characteristics, especially DNA-DNA hybridization data, should be taken into consideration. Strain mXyS1 did not show a specific relationship to a known species. The closest relative was *Desulfococcus multivorans*, for which the similarity value was 89.6% (Fig. 2). According to this relatively deep branching, strain mXyS1 may be regarded as the first represenative of a so far unknown, distinct line of descent within the δ-subclass of the *Proteobacteria.*

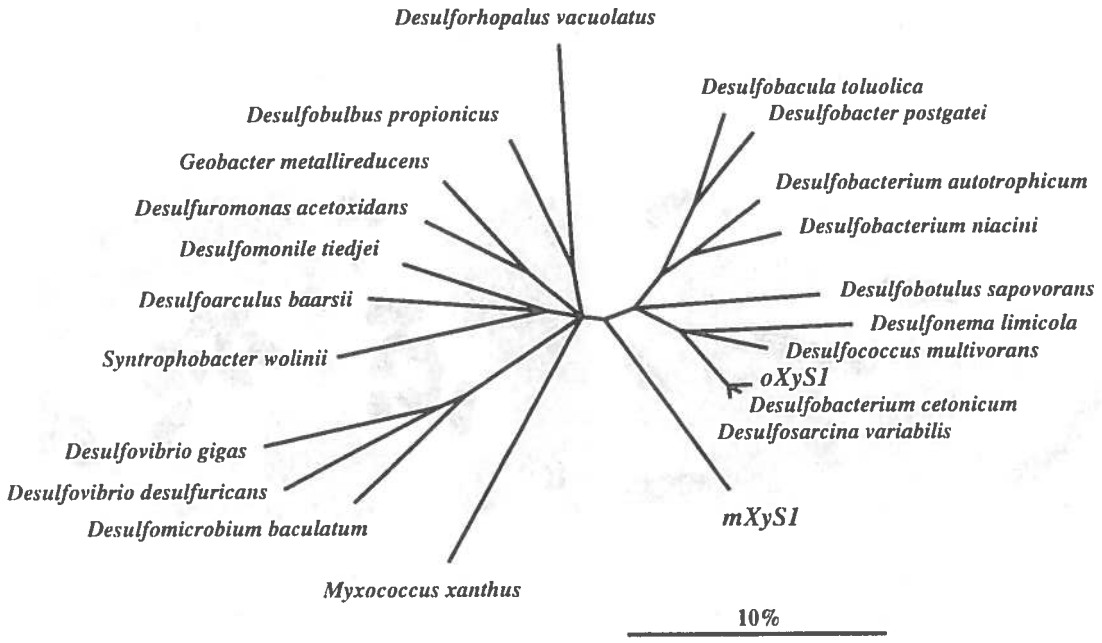

Fig. 2 16S rRNA-based tree reflecting the relationships of strains oXyS1 and mXyS1 to a selected number of members of the δ-subclass of the Proteobacteria. The tree is based on maximum-parsimony analysis; this includes only sequence positions that have identical residues in at least 50% of all available complete or almost complete 16S rRNA sequences from representative bacteria of this subgroup (23). For most species shown in the tree, the sequences are those of the type strains. The sequence of *Desulfomicrobium baculatum* was from strain 9974 (DSM 1743), which is not the type strain. *Desulfoarculus baarsii*, *Desulfobotulus sapovorans,* and *Desulfomicrobium baculatum* are the newly suggested names of the former *Desulfovibrio baarsii*, *Desulfovibrio sapovorans,* and *Desulfovibrio baculatus*, respectively (for a discussion, see reference 42). The topology of the tree was evaluated and corrected on the basis of the results of distance matrix, maximum-parsimony, and maximum likelihood analyses of various data sets. Phylogenetic positions of strains oXyS1 and mXyS1 did not differ in all treeing approaches. Multifurcations indicate topologies that could not be resolved unambiguously. The bar indicates estimated 10% sequence divergence.

The G+C content of the DNA of strain oXyS1 and mXyS1 determined via HPLC analysis was 51 and 49 mol%, respectively. Reported G+C values of *Desulfobacterium cetonicum* and *Desulfosarcina variabilis* were 59 and 51 mol % (Galushko and Rozanova 1995, Widdel and Bak 1992). Even though these two values were previously determined by thermal denaturation, i.e.. a method different from the high-pressure liquid chromatography analysis

used in the present study, the latter value is in good agreement with the close relationship between strain oXyS1 and *Desulfosarcina variabilis* revealed by sequence analysis of 16S rRNA genes.

Study of substrate utilization. Strains oXyS1 and mXyS1 were able to grow on a variety of aromatic and aliphatic compounds (Table 1). Growth on toluene was observed for both strains, whereas utilization of other alkylbenzenes was strain specific. Strain oXyS1 used only one higher homologue of *o*-xylene, *o*-ethyltoluene, but no *meta*-substituted alkyltoluenes. In contrast, strain mXyS1 used two homologues of *m*-xylene, *m*-ethyltoluene and *m*-isopropyltoluene, but no *ortho*-substituted alkylbenzenes. *p*-Alkylbenzenes did not allow the growth of either strain. Both strains were able to grow on crude oil. One may therefore speculate that sulfate-reducing bacteria of the types represented by strains oXyS1 and mXyS1 consume toluene as well as the respective xylenes and higher homologues also from the crude oil in the enrichment culture.

Desulfobacterium cetonicum, a very close relative of strain oXyS1, was originally isolated from an oil field by using butyrate as a substrate (Galushko and Rozanova 1995). The origin of *Desulfobacterium cetonicum* as well as its capacity to utilize benzoate suggested that this species can also oxidize aromatic hydrocarbons. Aromatic hydrocarbons have not been tested so far with this species. Actually, growth tests during the present study revealed utilization of toluene but not of *o*-xylene. Growth tests on aromatic hydrocarbons were also carried out with the other close relative of strain oXyS1, *Desulfosarcina variabilis*, which has been originally isolated on benzoate (Pfennig et al. 1981, Widdel and Bak 1992). However, *Desulfosarcina variabilis* neither utilized *o*-xylene, *m*-xylene nor toluene. Two sulfate-reducing bacteria formerly isolated on toluene did not grow on *o*-xylene or *m*-xylene (Beller et al. 1996, Rabus et al. 1993).

Quantitative growth experiments. Growth of the new isolates was slower than that of the previously described toluene-degrading sulfate-reducing bacteria (Beller et al. 1996, Rabus et al. 1993). The growth curve of strain oXyS1 on *o*-xylene is shown in Fig. 3. The exponential growth phase was rather short, as could be seen in a semilogarithmic plot of the sulfide concentration or the optical density versus time (results not shown). Exponential growth soon turned into pronounced linear growth, i.e., the growth rate decreased steadily. The relatively short exponential phase allowed only an estimate of the doubling time, which was around 75 h for strain oXyS1 and around 55 h for strain mXyS1. The sulfate-reducing strains PROTL1 and Tol2 exhibited the shortest doubling times on toluene of 36 h (Beller et al. 1996) and 27 h (Rabus et al. 1993), respectively.

The balance of substrate consumption and sulfide formation by strains oXyS1 and mXyS1 was quantitatively determined in separate experiments with two different initial concentrations of *o*-xylene and *m*-xylene, respectively, in the presence of sulfate (28 mM). To avoid changes in the volumes of the carrier phase and the medium, samples were not taken

during growth but only at the end of the experiments. The results obtained with strain mXyS1 are shown in Table 2. In the presence of cells, more than 90% of the originally added *m*-xylene.

Table 1 Anaerobic growth tests of sulfate-reducing strains oXyS1 and mXyS1 on aromatic and non aromatic compounds.

Compound tested[a]	Presence in strains[b]	
	oXyS1	mXyS1
Aromatic hydrocarbons		
Toluene (2%)	+	+
o-Xylene (2%)	+	-
m-Xylene (2%)	-	+
o-Ethyltoluene (2%)	+	-
m-Ethyltoluene (2%)	-	+
m-Isopropyltoluene (2%)	-	+
Aromatic compounds with functional groups		
o-Methylbenzyl alcohol (0.5, 2)	+	-
m-Methylbenzyl alcohol (0:5, 2)	-	-
Benzoate (1, 4)	+	+
o-Methylbenzoate (1, 2)	+	-
m-Methylbenzoate (1, 2)	-	+
Benzylsuccinate (1, 4)	+	-
Other compounds		
Ethanol (1, 5)	+	-
Formate (10, 20)	+	+
Acetate (5, 10)	+	+
Propionate (5, 10)	-	+
n-Butyrate (5, 10)	+	+
Lactate (5, 10)	+	-
Pyruvate (1, 5)	+	+
Succinate (1, 5)	+	-
D,L-Malate (1, 5)	+	-

Each compound was tested twice at the concentrations given in parentheses. Unless otherwise noted, concentrations are given in millimolar. Concentrations in percentages (vol/vol) refer to dilutions of hydrophobic compounds in heptamethylnonane as an inert carrier phase.

[a] Further compounds tested but utilized by neither strain:
Benzene (2 %), ethylbenzene (2 %), *p*-xylene (2 %), *p*-ethyltoluene (2 %), *o*-isopropyltoluene (2 %), *p*-isopropyltoluene (2 %), *n*-hexane (2, 5 %), *n*-decane (5, 10 %), cyclohexane (2 %), methylcyclohexane (2 %), phenol (0.5, 2), *o*-cresol (0.5, 2), *m*-cresol (0.5, 2), *p*-cresol (0.5, 2), acetophenone (1 %), *p*-methylbenzoate (0.5, 2), methanol (5, 10), glucose (1, 5), fructose (1, 5).

[b] Symbols: +, growth observed; -, no growth observed.

had disappeared, whereas abiotic loss of *m*-xylene in cell-free controls was not observed. The amount of electrons that can be theoretically derived from the amount of disappeared *m*-xylene is 13 to 17 % higher than the amount of electrons required for sulfide production from sulfate. This deviation can be explained by partial utilization of the substrate for cell synthesis. Various sulfate-reducing bacteria were shown to assimilate approximately 10 to 15 % of the organic substrate (Rabus et al. 1993, Schnell et al. 1989, Widdel and Pfennig 1981). Furthermore,

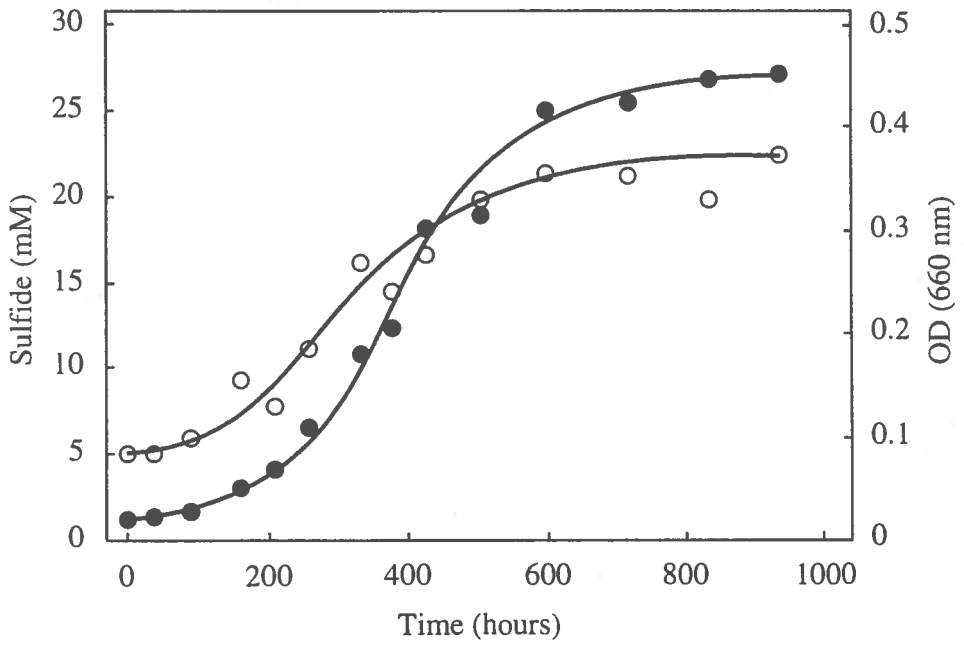

Fig. 3 Sulfide production (●) and increase in cell turbidity (○) in a culture of strain oXyS1 growing in defined medium on *o*-xylene. The growth experiment was carried out in a flat bottle (500 ml) with 450 ml of medium and 0.3 ml of *o*-xylene dissolved in 15 ml of heptamethylnonane. The bottle was incubated horizontally and shaken slightly. OD, optical density.

some undetected organic metabolites may have been excreted. In conclusion, the result is in good agreement with the occurrence of complete oxidation of *m*-xylene to CO_2. Essentially the same result was obtained with strain oXyS1 incubated on *o*-xylene. Again, more than 90% of the added growth substrate was consumed in inoculated bottles whereas disappearance of *o*-xylene in sterile bottles was not measurable (further data not shown). The ability of both strains for complete oxidation is also obvious from their growth on acetate which according to our present biochemical knowledge necessarily leads to CO_2 as the end product. The equation to undergo complete oxidation of xylenes is as follows:

$$(CH_3)_2C_6H_4 + 5.25\ SO_4^{2-} + 2.5\ H^+ + 3\ H_2O \rightarrow 8\ HCO_3^- + 5.25\ H_2S$$

To our knowledge, this is the first demonstration of the mineralization of *o*-xylene and *m*-xylene in pure cultures of sulfate-reducing bacteria.

Table 2 Quantification of *m*-xylene consumption and sulfide formation by strain mXyS1.

Experiment[a]	*m*-Xylene added (mmol)	*m*-Xylene disappeared[b] (mmol)	Sulfide produced[c] (mmol)	Electrons from *m*-xylene disappeared[d] (mmol)	Electrons consumed by SO_4^{2-} reduction[e] (mmol)
Strain mXyS1 with small amount of *m*-xylene	0.242	0.231	1.07	9.7	8.56
Strain mXyS1 with large amount of *m*-xylene	1.21	1.11	5.0	46.6	40.0
Strain mXyS1 without *m*-xylene (control)	0.0	0.0	0.067	0.0	0.54
Sterile medium without cells (control)	1.21	0.0	0.0	0.0	0.0

[a] Experiments were carried out under anoxic conditions with flat bottles (250 ml) with a culture volume of 190 ml. The total amount of sulfate added to each bottle was 5.23 mmol (27.5 mM). The medium was overlaid with 5 ml of heptamethylnonane as the carrier phase for *m*-xylene. The volume of *m*-xylene to be added was calculated from the density (0.866 g · cm^{-3} at 20 °C) and molecular mass (106.2 g · mol^{-1}).

[b] Difference between *m*-xylene added and *m*-xylene recovered in the carrier and aqueous phase at the end of the experiment.

[c] The small amount of sulfide produced in the control with cells without substrate was subtracted from the amount of sulfide produced in experiments with cells and *m*-xylene.

[d] Stoichiometrically, 42 mmol of electrons is derived from 1 mmol of *m*-xylene oxidized to CO_2.

[e] Stoichiometrically, 8 mmol of electrons is required for complete reduction of 1 mmol of SO_4^{2-} to 1 mmol of H_2S.

Key enzymes of acetyl-coenzyme A oxidation. Enzyme tests for detection of key enzymes for acetyl-coenzyme A oxidation were carried out with cell-free extract of strain oXyS1. Carbon monoxide dehydrogenase and formate dehydrogenase were measured at specific activities (with respect to protein) of 0.12 and 0.23 µmol $min^{-1} \cdot mg^{-1}$, respectively. No activity of 2-oxoglutarate dehydrogenase was detectable. The results suggest that terminal oxidation occurs via the carbon monoxide dehydrogenase pathway (reverse Wood pathway, or

C_1 pathway), as occurs in most species of completely oxidizing sulfate-reducing bacteria (Widdel and Hansen 1992).

Acknowledgements. We are indebted to Fred A. Rainey, Deutsche Sammlung von Mikroorganismen und Zellkulturen, Braunschweig, Germany, for performing sequence analysis and for providing unpublished sequence data. We thank Jens Harder, Bremen, for instrumental help.

G. Harms and K. Zengler contributed equally to this study.

This work was supported by the Max-Planck-Gesellschaft and the Fonds der Chemischen Industrie.

References

Aeckersberg F, Bak F, Widdel F 1991. Anaerobic oxidation of saturated hydrocarbons to CO_2 by a new type of sulfate-reducing bacterium. Arch. Microbiol. 156:5-14.

Beller HR, Spormann AM, Sharma PK, Cole JR, Reinhard M 1996. Isolation and characterization of a novel toluene-degrading, sulfate-reducing bacterium. Appl. Environ. Microbiol. 62:1188-1196.

Biegert T, Fuchs G 1995. Anaerobic oxidation of toluene (analogues) to benzoate (analogues) by whole cells and by cell extracts of a denitrifying *Thauera* sp. Arch. Microbiol. 163:407-417.

Bradford MM 1976. A rapid and sensitive method for the quantification of microgram quantities of protein using the principle of protein-dye binding. Anal. Biochem. 72:248-254.

Chen C-I, Taylor RT 1997. Thermophilic biodegradation of BTEX by two consortia of anaerobic bacteria. Appl. Microbiol. Biotechnol. 48:121-128.

Cline JD 1969. Spectrophotometric determination of hydrogen sulfide in natural waters. Limnol. Oceanogr. 14:454-458.

Cord-Ruwisch R 1985. A quick method for the determination of dissolved and precipitated sulfides in cultures of sulfate-reducing bacteria. J. Microbiol. Methods 4:33-36.

Cord-Ruwisch R, Kleinitz W, Widdel F 1987. Sulfate-reducing bacteria and their activities in oil production. J. Petrol. Technol. January:97-106.

Dolfing J, Zeyer J, Binder-Eicher P, Schwarzenbach RP 1990. Isolation and characterization of a bacterium that mineralizes toluene in the absence of molecular oxygen. Arch. Microbiol. 154:336-341.

Edwards EA, Wills LE, Reinhard M, Grbic-Galic D 1992. Anaerobic degradation of toluene and xylene by aquifer microorganisms under sulfate-reducing conditions. Appl. Environ. Microbiol. 58:794-800.

Edwards EA, Grbic-Galic D 1994. Anaerobic degradation of toluene and *o*-xylene by a methanogenic consortium. Appl. Environ. Microbiol. 60:313-322.

Evans PJ, Mang DT, Young LY 1991a. Degradation of toluene and *m*-xylene and transformation of *o*-xylene by denitrifying enrichment cultures. Appl. Environ. Microbiol. 57:450-454.

Evans PJ, Mang DT, Kim KS, Young LY 1991b. Anaerobic degradation of toluene by a denitrifying bacterium. Appl. Environ. Microbiol. 57:1139-1145.

Felsenstein J 1992. Numerical methods for inferring phylogenetic trees. Q. Rev. Biol. 57:379-404.

Fries MR, Zhou J, Chee-Sanford J, Tiedje JM 1994. Isolation, characterization and distribution of denitrifying toluene degraders from a variety of habitats. Appl. Environ. Microbiol. 60:2802-2810.

Galushko AS, Rozanova EP 1995. *Desulfobacterium cetonicum* sp. nov.: a sulfate-reducing bacterium which oxidizes fatty acids and ketones. Microbiology 60:742-746.

Goodfellow M, Manfio GP, Chun J 1997. Towards a practical species concept for cultivable bacteria, p. 25-59. *In* M. F. Claridge, H. A. Dawah, and M. R. Wilson (ed.), The units of biodiversity. Chapman & Hall, London, United Kingdom.

Häner A, Höhener P, Zeyer J 1995. Degradation of *p*-xylene by a denitrifying enrichment culture. Appl. Environ. Microbiol. 61:3185-3188.

Herbert BN 1987. Reservoir souring, p. 63-71. *In* E. C. Hill, J. L. Shennan, and R. J. Walkinson (ed.), Microbial problems in the offshore oil industry. John Wiley & Sons Ltd., London, United Kingdom.

Hess A, Zarda B, Hahn D, Häner A, Stax D, Höhener P, Zeyer J 1997. In situ analysis of denitrifying toluene- and *m*-xylene-degrading bacteria in a diesel fuel-contaminated laboratory aquifer column. Appl. Environ. Microbiol. 63:2136-2141.

Jørgensen BB 1982. Mineralization of organic matter in the sea bed - the role of sulphate reduction. Nature (London) 296:643-645.

L'Haridon S, Reysenbach A-L, Glénat P, Prieur D, Jeanthon C 1995. Hot subterranean biosphere in a continental oil reservoir. Nature (London) 377:223-224.

Maidak BL, Olsen GJ, Larsen N, Overbeek R, McCaughey MJ, Woese CR 1997. The RDP (Ribosomal Database Project). Nucleic Acids Res. 25:109-110.

Mesbah M, Premachandran U, Whitman WB 1989. Precise measurement of the G+C content of deoxyribonucleic acid by high-performance liquid chromatography. Int. J. Syst. Bacteriol. 39:159-167.

Muyzer G, Teske A, Wirsen CO, Jannasch HW 1995. Phylogenetic relationships of *Thiomicrospira* species and their identification in deep-sea hydrothermal vent samples by denaturing gradient gel electrophoresis of 16S rDNA fragments. Arch. Microbiol. 164:165-172.

Odom JM 1993. Industrial and environmental activities of sulfate-reducing bacteria, p. 189-210. *In* J. M. Odom and R. Singleton, Jr. (ed.), The sulfate-reducing bacteria: contemporary perspectives. Springer-Verlag, New York, N.Y.

Pfennig N, Widdel F, Trüper HG 1981. The dissimilatory sulfate-reducing bacteria, p. 926-940. *In* M. P. Starr, H. Stolp, H. G. Trüper, A. Balows, and H. G. Schlegel (ed.), The Prokaryotes, 1st ed, vol 1. Springer-Verlag, New York, N.Y.

Rabus R, Widdel F 1995a. Anaerobic degradation of ethylbenzene and other aromatic hydrocarbons by new denitrifying bacteria. Arch. Microbiol. 163:96-103.

Rabus R, Widdel F 1995b. Conversion studies with substrate analogues of toluene in a sulfate-reducing bacterium, strain Tol2. Arch. Microbiol. 164:448-451.

Rabus R, Widdel F 1996. Utilization of alkylbenzenes during anaerobic growth of pure cultures of denitrifying bacteria on crude oil. Appl. Environ. Microbiol. 62:1238-1241.

Rabus R, Fukui M, Wilkes H, Widdel F 1996. Degradative capacities and 16S rRNA-targeted whole-cell hybridization of sulfate-reducing bacteria in an anaerobic enrichment culture utilizing alkylbenzenes from crude oil. Appl. Environ. Microbiol. 62:3605-3613.

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

Rainey FA, Dorsch M, Morgan HW, Stackebrandt E 1992. 16S rDNA analysis of *Spirochaeta thermophila*: position and implications for the systematics of the order *Spirochaetales*. Syst. Appl. Microbiol. 16:224-226.

Rueter P, Rabus R, Wilkes H, Aeckersberg F, Rainey FA, Jannasch HW, Widdel F 1994. Anaerobic oxidation of hydrocarbons in crude oil by new types of sulfate-reducing bacteria. Nature (London) 372:455-458.

Schnell S, Bak F, Pfennig N 1989. Anaerobic degradation of aniline and dihydroxybenzenes by newly isolated sulfate-reducing bacteria and description of *Desulfobacterium anilini*. Arch. Microbiol. 152:556-563.

Seyfried B, Glod G, Schocher R, Tschech A, Zeyer J 1994. Initial reactions in the anaerobic oxidation of toluene and *m*-xylene by denitrifying bacteria. Appl. Environ. Microbiol. 60:4047-4052.

Stackebrandt E, Goebel BM 1994. Taxonomic note: a place for DNA:DNA reassociation and 16S rRNA sequence analysis in the present species definition in bacteriology. Int. J. Syst. Bacteriol. 44:846-849.

Stetter KO, Huber R, Blöchl E, Kurr M, Eden RD, Fielder M, Cash H, Vance I 1993. Hyperthermophilic archaea are thriving in deep North Sea and Alaskan oil reservoirs. Nature (London) 365:743-745.

Strunk O, Ludwig W ARB: a software environment for sequence data. http://www.mikro.biologie.tu-muenchen.de. Department of Microbiology, Technische Universität München, Munich, Germany.

Tissot BP, Welte DH 1984. Petroleum formation and occurrence, 2nd ed. Springer-Verlag KG, Berlin, Germany.

Tsai YL, Olson BH 1991. Rapid method for direct extraction of DNA from soil and sediments. Appl. Environ. Microbiol. 57:1070-1074.

Widdel F, Bak F 1992. Gram-negative mesophilic sulfate-reducing bacteria, p. 3352-3378. *In* A. Balows, H. G. Trüper, M. Dworkin, W. Harder, and K.-H. Schleifer (ed.), The Prokaryotes, 2nd ed, vol. 4 Springer-Verlag, New York, N.Y.

Widdel F, Hansen TA 1992. The dissimilatory sulfate- and sulfur-reducing bacteria, p. 583-624. *In* A. Balows, H. G. Trüper, M. Dworkin, W. Harder, and K.-H. Schleifer (ed.), The Prokaryotes, 2nd ed., vol. 1 Springer-Verlag, New York, N.Y.

Widdel F, Pfennig N 1981. Studies on dissimilatory sulfate-reducing bacteria that decompose fatty acids. I. Isolation of new sulfate-reducing bacteria enriched with acetate from saline environments. Description of *Desulfobacter postgatei* gen. nov. sp. nov. Arch. Microbiol. 129:395-400.

Zeyer J, Kuhn EP, Schwarzenbach RP 1986. Rapid microbial mineralization of toluene and 1,3-dimethylbenzene in the absence of molecular oxygen. Appl. Environ. Microbiol. 52:944-947.

4

Methane formation from long-chain alkanes by anaerobic microorganisms

Karsten Zengler[1], Hans Hermann Richnow[2], Ramon Rosselló-Mora[1], Walter Michaelis[2], and Friedrich Widdel[1*]

Nature (London), in press

[1] Max-Planck-Institute for Marine Microbiology, Celsiusstr. 1, D-28359 Bremen, Germany

[2] Institute for Biogeochemisty and Marine Chemistry, University of Hamburg, Bundestr. 55, D-20146 Hamburg, Germany

* Corresponding author

Abstract

Biological formation of methane is the terminal process of biomass degradation in aquatic habitats where oxygen, nitrate, ferric iron, and sulphate have been depleted as electron acceptors. The pathway leading from dead biomass through the metabolism of anaerobic bacteria and archaea to methane is well-understood in case of easily degradable biomolecules such as carbohydrates, proteins or lipids (Ferry 1993; Schink 1997). Little is known about the organic compounds that lead to methane in old anoxic sediments where easily degradable biomolecules are no longer available. One class of naturally formed, long-lived compounds in such sediments are saturated hydrocarbons (alkanes) (Cheesbrough and Kolattukudy 1984; Blumer et al. 1971; Tissot and Welte 1984). Alkanes are usually considered as inert in the absence of oxygen, nitrate or sulphate (Heider et al. 1999), and analysis of alkane patterns is often used for biogeochemical characterisation of sediments (Eglinton 1968; Simoneit 1986). On the other hand, there are hints that alkanes can be consumed in anoxic sediments also below the zone of sulphate reduction (Giger et al. 1980; Wersin et al. 1991), but the underlying process has not been elucidated. In the present study we used enrichment cultures to demonstrate that biological conversion of long-chain saturated hydrocarbons to the simplest hydrocarbon, methane, is possible under strictly anoxic conditions.

To study the possibility of methane formation (methanogenesis) from long-chain saturated hydrocarbons, hexadecane (n-$C_{16}H_{34}$) as one representative of this class of compounds was incubated under strictly anoxic conditions in sulphate-free medium inoculated with anoxic ditch sediment. After four months, gas formation in the presence of hexadecane increased steadily in comparison to an inoculated control without hexadecane. Via repeated transfer of aliquots to new media, a sediment-free enrichment culture was obtained that formed gas in the presence of hexadecane as substrate (Fig. 1). The formed gas was mainly methane. Methane production in newly inoculated cultures was always favoured if low concentrations of sulphate (≤2 mM) were added. This stimulatory effect was probably due to the resulting background growth of sulphate-reducing bacteria which favoured growth conditions for other anaerobic microorganisms involved in the process of methanogenesis. Microscopic examination of the sediment-free enrichment culture revealed oval, long hair-like, and rod-shaped microorganisms mainly occurring in loose aggregates, as well as vibrioid forms homogeneously distributed in the medium. Methane formation and growth of microorganisms also occurred with pentadecane, but not thus far with decane or hexane.

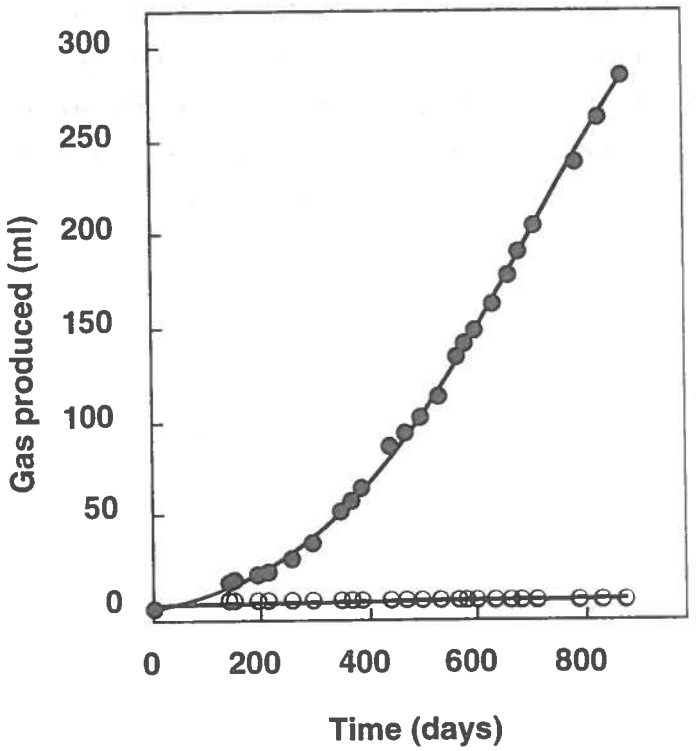

Fig. 1 Formation of gas in an anaerobic enrichment culture growing in 100 ml mineral medium in the presence of hexadecane (●) as the only organic substrate. An inoculated parallel control without hexadecane (○) did not show gas formation.

During a study of methanogenesis in oil wells it has been formerly supposed that aerobic hydrocarbon-degrading bacteria excrete polar oxidation products (e.g. fatty acids) that may be

subsequently consumed by anaerobic methanogenic communities (Rozanova 1997). However, for the present enrichment culture we could refute the suspicion that aerobic bacteria initiated alkane degradation by using traces of oxygen eventually diffusing through the stoppers. No growth occurred when aliquots were incubated with hexadecane under atmospheric or lower oxygen partial pressure, indicating the absence of aerobic or microaerobic alkane-utilising microorganisms. Furthermore, methane-forming enrichment cultures with hexadecane grew just as well if the butyl rubber-sealed bottles were incubated inside an anoxic jar containing pure N_2 and an oxygen trap.

Fatty acid analysis revealed only acetate at low concentration (7.5 µM) in the enrichment culture. Further accumulation during growth was not observed. With repeated release of overpressure during growth, methane and carbon dioxide became the dominant gases in the culture head speace (molar ratio approx. 15:2). Ethane or other gaseous hydrocarbons were never detected. Hydrogen was only detectable at trace concentrations (partial pressure, 1.7 Pa). The finding of acetate and H_2 at low concentration is in agreement with their reported role as key intermediates during methanogenesis from natural organic compounds (Ferry 1993; Schink 1997). Decomposition of such compounds by anaerobic bacteria leads to acetate and H_2 which are consumed by methanogenic archaea and hence maintained at very low concentration. Conversion of hexadecane to CH_4 and CO_2 is expected to involve at least three groups of microorganisms, analogous to methane formation from other molecules with more than two C-atoms (e.g., propionate, higher fatty acids) (Ferry 1993; Schink 1997). These microorganisms are assumed to be acetogenic (syntrophic) bacteria decomposing hexadecane to acetate and H_2 (equation 1), a group of archaea cleaving acetate into CH_4 and CO_2 (equation 2), and another group of archaea converting CO_2 and H_2 to methane (equation 3).

$4\ C_{16}H_{34} + 64\ H_2O$	$\rightarrow$	$32\ CH_3COO^- + 32\ H^+ + 68\ H_2$	$\Delta G =$ -907 kJ	(1)
$32\ CH_3COO^- + 32\ H^+$	$\rightarrow$	$32\ CH_4 + 32\ CO_2$	$\Delta G =$ -385 kJ	(2)
$68\ H_2 + 17\ CO_2$	$\rightarrow$	$17\ CH_4 + 34\ H_2O$	$\Delta G =$ -282 kJ	(3)
Net reaction:				
$4\ C_{16}H_{34} + 30\ H_2O$	$\rightarrow$	$49\ CH_4 + 15\ CO_2$	$\Delta G =$ -1574 kJ	(4)

Free energy changes are indicated for given stoichiometries in mol; they were calculated for mentioned concentrations determined in the enrichment culture (and pH 7.0). All partial reactions (equations 1-3) are thermodynamically feasible (exergonic). The acetogenic reaction (equation 1) would not be possible (endergonic) at standard activities of acetate and H_2. The free energy change per mol CH_4 in the net reaction is -32.1 kJ.

For an experimental proof of the postulated net reaction (equation 4), the degradation balance was determined by quantification of remaining hexadecane and formed methane in an enrichment culture. From 1.7 mmol hexadecane initially added, 0.59 mmol were consumed

during formation of 4.6 mmol CH_4 (sum from analysed gas samples taken anoxically during 810 days of incubation). Theoretically (equation 4), the consumed amount of hexadecane would yield 7.2 mmol CH_4. The deviation can be explained by partial utilisation of organic carbon and reducing equivalents from hexadecane for cell synthesis of the involved microorganisms, and a certain loss of methane during the repeated sampling procedures.

Conversion of consumed hexadecane to CH_4 and CO_2 was finally verified in a subsequent growth experiments with ^{13}C-labelled substrate. Pure ^{13}C-hexadecane in the enrichment culture retarded growth significantly. Therefore, a 1:9 (vol:vol) mixture of ^{13}C-labelled and unlabelled hexadecane was applied. The ^{13}C-content in methane and CO_2 after 158 days of incubation was 10.1 (±0.8) and 1.85 (±0.001) atom%, respectively. In a control experiment with unlabelled hexadecane, the ^{13}C-content in CH_4 and CO_2 was 1.07 and 1.09 atom%, respectively.

Both quantitative experiments confirm that hexadecane was converted to methane. The high ^{13}C-content of methane in the labelling experiment is in accordance with methane formation mainly by acetate cleavage according to equation (2). Methane produced alone by this reaction should have the same ^{13}C-content as the applied hexadecane which was 11.0 atom% (calculated from mixing ratio). However, this highly ^{13}C-enriched methane mixes with methane with lower ^{13}C-content originating from CO_2 of the bicarbonate buffer (equation 3). This buffer was initially unlabelled and became only gradually enriched by $^{13}CO_2$ from acetate cleavage (equation 2). Exact calculation of the part of methane formed by CO_2 reduction during this experiment is not possible, due to a certain sulphate concentration and background growth of sulphate-reducing bacteria (*Desulfovibrio*; see next paragraph). These bacteria most likely consumed a part of the hydrogen formed by acetogenic bacteria (equation 1). Hence, probably significantly less than one third of total methane was formed by CO_2 reduction during the incubation period.

To prove the presence of microorganismic groups that may catalyse the postulated reactions (equations 1-3), we screened 30 bacterial and 30 archaeal 16S rRNA gene sequences retrieved from the enrichment culture. Four distinct patterns were observed among the bacterial clones, and three patterns among the archaeal clones. Representative clones of each pattern were chosen for sequencing followed by phylogenetic reconstruction (Ludwig et al. 1998). All bacterial clones affiliated with the delta subclass of *Proteobacteria* (Fig. 2a). Three of them (representing 26 screened clones) were closely related to cultured syntrophic bacteria of the genus *Syntrophus*. One bacterial clone affiliated with *Desulfovibrio*, a genus that comprises incompletely oxidising, H_2-utilising sulphate-reducing bacteria (Widdel and Bak 1992). All archaeal clone types affiliated with methanogenic *Archaea* (Fig. 2b). One clone type was most closely related with the genus *Methanosaeta*, which comprises acetoclastic methanogens. Two clone types were related to the genera *Methanospirillum* and *Methanoculleus*, which comprise methanogens utilising H_2 and CO_2. These results are in agreement with the postulated presence of microorganisms catalysing reactions (1) - (3). *Desulfovibrio* is probably present due to the low concentration of sulphate added to stimulate growth of the microbial community.

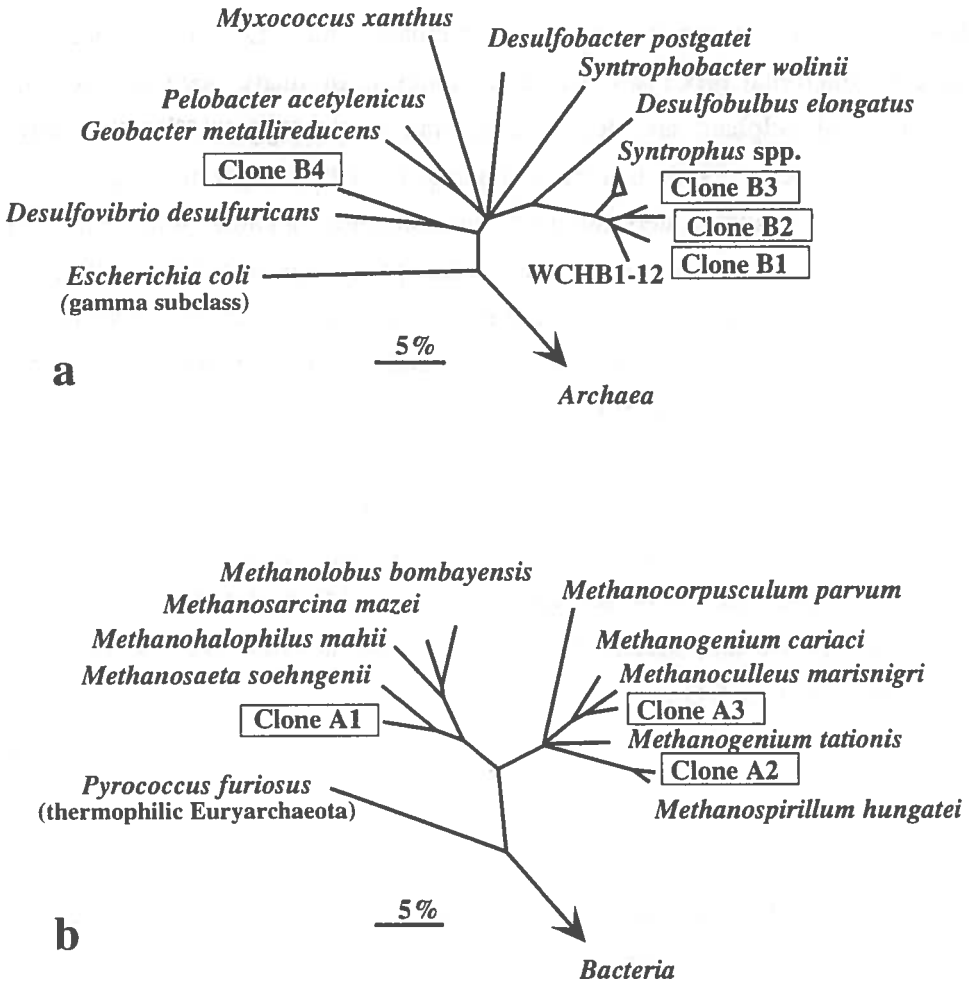

Fig.2 Reconstructed phylogenetic tree based on 16S rRNA sequences that were retrieved from the methanogenic enrichment culture on hexadecane. **a**, Relationships of bacterial clones (B1 through B4) within the delta subclass of *Proteobacteria*. **b**, Relationships of archaeal clones (A1 through A3) within methanogenic *Archaea*. Bars indicate 5% estimated sequence divergence. Group designated as *Syntrophus* spp. comprises sequences of species *S. gentianae, S. buswellii* and *S. acidotrophicus;* the latter together with clone WCHB1-12 from a solvent-contaminated aquifer (Dojka et al. 1998) were the closest relatives of the presently obtained clones (sequence similarities around 95%).

In conclusion, results show that anaerobic bacterial communities can convert long-chain saturated hydrocarbons to methane, the simplest hydrocarbon. Therefore, the net process may be regarded as a "microbial alkane cracking". To our knowledge, such a biological process has not been demonstrated and studied so far. The only hydrocarbons shown to be degraded under conditions of methanogenesis were certain monoaromatic compounds (Heider et al. 1999) and alkenes (Schink 1985; Harder and Foss, in press). Alkanes contain only apolar sigma bonds and are, therefore, chemically even less reactive than aromatic hydrocarbons or alkenes. Few bacteria have been isolated that oxidise alkanes anaerobically, however, they required sulphate or nitrate as electron acceptor. Their biochemistry of anaerobic alkane activation is only speculative so far (Heider et al. 1999).

Demonstration of alkane conversion to methane and CO_2 has implications for the understanding of microbial processes during diagenesis. In many deep sediments, oxygen, nitrate, iron(III) and sulphate are depleted, leaving methanogenesis as the only terminal degradation process (Ferry 1993; Lovley and Chapelle 1995). Due to diagenetic processes including saturation of unsaturated aliphatic biomolecules, alkanes represent a significant fraction among the organic substances in aged sediments (Tissot and Welte 1984; Beier et al. 1991). It is therefore possible that alkanes are part of the hitherto largely unknown substrates that maintain slowly growing microbial communities and lead to methane formation in deep, old sediments (Lovley and Chapelle 1995; Parkes et al. 1994). Bacterial communities as observed in the present enrichment culture may also contribute to the disappearance of alkanes and methane formation in coalbed reservoirs where these processes have been partly attributed to bacterial activity (Scott et al. 1994). Bacterial methanogenesis from alkanes appears in principle possible also in organic-rich shales that show formation of highly ^{13}C-depleted ("biological") methane Martini et al. 1996; Rowe and Muehlenbachs 1999), and in crude oil reservoirs depleted of electron acceptors other than CO_2.

Methods

Growth of enrichment cultures. Enrichment cultures were grown in anoxic mineral medium buffered (pH 7) with bicarbonate (30 mM)/CO_2 and reduced with sulphide (1 mM) (Widdel and Bak 1992); sulphate was omitted in the initial culture; for subcultures, limited concentrations of sulphate were added (1-2 mM). Butyl rubber-sealed growth bottles contained an anoxic head space (one third or less of bottle volume) initially gassed with N_2/CO_2 (90/10, vol/vol). Cultures were incubated at 28 °C. Overpressure due to microbial gas formation was discontinuously released by insertion of a hypodermic needle connected to a volumetric device. The volume of the intial enrichment culture was 400 ml (including 150 ml ditch mud). Hexadecane was distributed by brief shaking causing hydrocarbon droplets to associate with sediment particles. Hexadecane in sediment-free subcultures (100 ml) was provided on the surface of Teflon filter foil to allow a large contact area between the alkane and medium. The foil (pore size, 0.45 µm) was autoclaved in a bottle under N_2 and then allowed to soak anoxically with a defined amount of hexadecane (regularly 0.5 ml; for labelling experiment 0.05 ml). The hexadecane-containing filter foil was fixed on the bottom by means of a glass rod and overlaid with anoxic mineral medium (100 ml). Decane and hexane for growth tests were diluted (ratio 1:3 and 1:50, respectively) in 2,2,4,4,6,8,8-heptamethylnonane as an inert carrier phase to avoid toxic effects of the shorter hydrocarbons. Anoxic jars for incubation of sealed culture bottles contained alkaline pyrogallol as oxygen trap. Aerobic growth tests were carried out in sulphide-free medium (20 ml; with sulphate) with hexadecane and incubated in bottles (500 ml) under 1.0% and 20% O_2 (added to an N_2 atmosphere containing in addition 10% CO_2).

Chemical analyses. Methane, carbon dioxide and hexadecane were determined by standard gas chromatograophic methods. Trace concentrations of hydrogen were analyzed on a gas chromatograph equipped with a mercury oxide reduction detector (Seiler et al. 1980). Fatty acids were converted to 2-nitrophenylhydrazides and analysed by high-performance liquid chromatography (Albert and Martens 1997). The ratio of carbon isotopes was determined by mass spectroscopy after gas chromatographic separation (IRM-GC-MS) (Hayes et al. 1990) using a DELTA XL instrument (Finnigan MAT, Bremen).

Chemical synthesis. Labelled hexadecane was synthesised from uniformly ^{13}C-labelled palmitic acid (Campro Scientific, Emmerich, Germany) via conversion to the methyl ester (with dimethyl sulphate), reduction to hexadecanol (with $NaAlH_4$), conversion to the *p*-tosylate ester, and reduction to the hydrocarbon (with $NaAlH_4$). After chromatographic separation, purity was confirmed by gas chromatography/mass spectrometry and NMR.

Nucleic acid-based analyses. Sequences of 16S rRNA genes from the enrichment culture were amplified by PCR by using general primers for the domains *Bacteria* and *Archaea*. After cloning (pGEM-T Vector System II, Promega, Madison, WI, USA), inserts were screened by their restriction pattern (ARDRA) obtained with *Sau*3A. Almost full sequences from each restriction group were added to an alignment of about 13,000 homologous 16S rRNA primary structures by using the aligning tool of the ARB program package (Strunk and Ludwig 1998). Consensus trees were constructed after evaluation and correction according to distance matrix, maximum parsimony, and maximum likelihood analyses of data sets (Ludwig et al. 1998). Ambiguous topologies are shown as multifurcations.

Calculation of free energy. Free energy changes were calculated from ΔG_f° values (Dean 1992). The used ΔG_f° value of hexadecane in the pure liquid state (+46.7 kJ/mol) as present in the culture was derived from the value for a (theoretical) gaseous standard state (+83.7 kJ/mol) (Dean 1992) via the real vapour pressure (0.308 Pa) at 298 K. The latter was obtained by extrapolation from literature values (Lide 1998) of vapour pressures (p) at other temperatures (T), using a plot of lg(p) versus 1/T.

Acknowledgements. We thank Christian Garms, University of Hamburg, for synthesis of labelled hexadecane. We are grateful to J. Oesselmann and A. Hilkert at Finnigan-MAT, Bremen, for providing the IRM-GC-MS, and to N. Finke, Max Planck Institute, Bremen, for fatty acid analysis. This study was supported by the Max-Planck-Gesellschaft and the Fonds der Chemischen Industrie.

Correspondence and request for materials should be addressed to F.W. (e-mail: fwiddel@mpi-bremen.de). The nucleotide sequences of clones have been deposited at EMBL Genbank under accesion numbers AJ133791-AJ133793 (clones A1 through A3) and AJ133794-AJ133797 (clones B1 through B4).

References

Albert DB, Martens CS 1997. Determination of low-molecular-weight organic acid concentrations in seawter and pore-water samples via HPLC. Mar. Chem. 56:27-37.

Beier JA, Wakeham SG, Pilskaln CH, Honjo S 1991. Enrichment in saturated compounds of Black Sea interfacial sediment. Nature (London) 351:642-644.

Blumer M, Guillard R, Chase T 1971. Hydrocarbons of marine phytoplankton. Mar. Biol. 8:183-189.

Cheesbrough TM, Kolattukudy PE 1984. Alkane biosynthesis by decarbonylation of aldehyds catalyzed by a particulate preparation from *Pisum sativum*. Proc. Natl. Acad. Sci. U.S.A. 81:6613-6617.

Dean JA. 1992. Lange´s handbook of chemistry. McGraw-Hill, New York.

Dojka MA, Hugenholtz P, Haack SK, Pace NR 1998. Microbial diversity in a hydrocarbon- and chlorinated-solvent-contaminated aquifer undergoing intrinsic bioremediation. Appl. Environ. Microbiol. 64:3869-3877.

Eglinton G. 1968. Hydrocarbons and fatty acids in living organisms and recent and ancient sediments, p. 1-24. *In* Schenck PA and Hayenaar I (ed.), Advances in Organic Geochemistry, vol. 31. Pergamon, New York.

Ferry JG. 1993. Methanogenesis. Chapman & Hall, New York.

Giger W, Schaffner C, Wakeham SG 1980. Aliphatic and olefinic hydrocarbons in recent sediments of Greifensee, Switzerland. Geochim. Cosmochim. Acta. 44:119-129.

Harder J, Foss S. Anaerobic formation of the aromatic hydrocarbon *p*-cymene from monoterpenes by methanogenic enrichment cultures. Geomicrobiol. J.:in press.

Hayes JM, Freeman KH, Popp BN, Hoham CH 1990. Compound-specific isotope analyses: a novel tool for reconstruction of ancient biogeochemical processes. Org. Geochem. 16:1115-1128.

Heider J, Spormann AM, Beller HR, Widdel F 1999. Anaerobic bacterial metabolism of hydrocarbons. FEMS Microbiol. Rev. 22:459-473.

Lide DR. 1998. Handbook of Chemistry and Physics. CRC, Boca Raton.

Lovley DR, Chapelle FH 1995. Deep subsurface microbial processes. Rev. Geophys. 33:365-381.

Ludwig W, Strunk O, Klugbauer N, Weizenegger M, Neumaier J, Bahleitner M, Schleifer K-H 1998. Bacterial phylogeny based on comparative sequence analysis. Electrophoresis 19:554-568.

Martini AM, Budai JM, Walter LM, Schoell M 1996. Microbial generation of economic acumulations of methane within a shallow organic-rich shale. Nature (London) 383:155-158.

Parkes RJ, Cragg BA, Bale SJ, Getliff JM, Goodman K, Rochelle PA, Fry JC, Weightman AJ, Harvey SM 1994. Deep bacterial biosphere in Pacific ocean sediments. Nature (London) 371:410-413.

Rowe D, Muehlenbachs A 1999. Low-temperature thermal generation of hydrocarbon gases in shallow shales. Nature (London) 398:61-63.

Rozanova EP, Savvichev AS, Miller YM, Ivanov MV 1997. Microbial processes in a West Siberian oil field flooded with waters containing a complex of organic compounds. Microbiology 66:718-725.

Schink B 1985. Degradation of unsaturated hydrocarbons by methanogenic enrichment cultures. FEMS Microbiol. Ecol. 31:69-77.

Schink B 1997. Energetics of syntrophic cooperation in methanogenic degradation. Microbiol. Mol. Biol. Rev. 61:262-280.

Scott AR, Kaiser WR, Ayers WD, Jr. 1994. Thermogenic and secondary biogenic gases, San Juan Basin, Colorado and New Mexico - Implications for coalbed gas producibility. Am. Assoc. Pet. Geol., Bull. 78:1186-1209.

Seiler W, Giehl H, Roggendorf P 1980. Detection of carbon monoxide and hydrogen by conversion of mercury oxide to mercury vapor. Atmos. Technol. 12:40-45.

Simoneit BRT 1986. Biomarker geochemistry of black shales from Creataceous oceans — an overview. Mar. Geol. 70:9-41.

Strunk O, Ludwig W 1998. ARB: a software environment for sequence data, http://www.mikro.biologie.tu-muenchen.de. Department of Microbiology, Technische Universität München, Munich, Germany.

Tissot B, Welte D. 1984. Petroleum Formation and Occurence, 2nd edn. Springer-Verlag, New York.

Wersin P, Höhener P, Giovanoli R, Stumm W 1991. Early diagentic influences on iron transformations in a fresh-water lake sediment. Chemical Geology. 90:233-252.

Widdel F, Bak F. 1992. Gram-negative mesophilic sulfate-reducing bacteria, p. 3352-3392. *In* Balows A; Trüper H.G.; Dworkin, M.; Harder, W.; Schleifer, K.-H. (ed.), The Prokaryotes, 2nd ed, vol. 4. Springer-Verlag, New York.

5

Anaerobic degradation of oil hydrocarbons by sulfate-reducing and nitrate-reducing bacteria

Manabu Fukui[1], Chris Boreham[2], Gerda Harms[1], Ralf Rabus[1], Andreas Schramm[1], Friedrich Widdel[1]*, Heinz Wilkes[3], and Karsten Zengler[1]

Proceedings of the Eighth International Symposium on Microbial Ecolog (ISME), Halifax, Canada, 1998, in press

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[1] Max-Planck-Institute for Marine Microbiology, Celsiusstr. 1, D-28359 Bremen, Germany

[2] Australian Geological Survey Organization, Canberra A.C.T., Australia 2601

[3] Forschungszentrum Jülich GmbH, ICG-4, D-52425 Jülich, Germany

* Corresponding author

Abstract

Crude oil is a complex mixture mainly constituted of various saturated and aromatic hydrocarbons. Whereas degradation of hydrocarbons by oxygen-respiring microorganisms is known since nearly one century, utilization of hydrocarbons under anoxic conditions has been investigated only during the past ten years. The present paper summarizes investigations into the anaerobic degradability of crude oil as a complex mixture of hydrocarbons. Anaerobic growth on crude oil was observed in enrichment cultures and pure cultures of sulfate-reducing bacteria. Several alkylbenzenes and *n*-alkanes were specifically consumed with concomitant reduction of sulfate to sulfide; consumed hydrocarbons together accounted for up to approximately 10% of the crude oil. Incompletely consumed alkylbenzenes exhibited an enrichment in ^{13}C versus ^{12}C. Sulfate-reduction with oil hydrocarbons may offer an explanation for ancient microbial processes in oil reservoirs where reduced sulfur species are present, and for the undesirable sulfide formation in oil production plants. Furthermore, an anaerobic consumption of alkylbenzenes and *n*-alkanes from crude oil in enrichment cultures and pure cultures of denitrifying bacteria could be demonstrated.

Introduction

Most crude oils are constituted by more than 75% of aliphatic and aromatic hydrocarbons (Schmidt and Romey 1981; Tissot and Welte 1984). Oil hydrocarbons belong to the large global fraction of organic carbon that has been preserved from an ancient biosphere due to burial followed by diagenetic and catagenetic transformation processes. The withdrawal of photosynthetically fixed carbon from reoxidation and recycling into the inorganic pool by bacteria and other organisms gave rise to our oxic atmosphere. The total mass of O_2 on our planet amounts to 1,200 Tt (1,185 Tt in the atmosphere, and around 12 Tt dissolved in water reservoirs; values calculated from data summarized by Greenwood and Earnshaw 1986. One Tt = 10^{12} t). Assuming that for each molecule of O_2 generated by water cleavage one atom of carbon (C) is fixed from CO_2, according to the net equation of photosynthesis, 450 Tt organic C must have been deposited (C-loss due to diagenetic decarboxylation processes not considered). The real amount of biologically fixed organic C may be even higher since some O_2 has been consumed by reaction with originally reduced inorganic compounds such as ferrous minerals. Living organisms and dead biomass (in soil and water) on our planet contain 0.8 and 2.8 Tt C, respectively, which is 0.18% and 0.62%, respectively, of the total organic C (summarized from Bolin 1983). The estimated amounts in accessible fuel reservoirs are 0.23 Tt C in oil harbored in conventional reservoirs as well as in shales and sands, 0.044 Tt C in gas, 0.22 Tt C in lignite and 0.95 Tt C in coal (calculated from data summarized by Schmidt and Romey 1981; Tissot and Welte 1984). The sum is 1.4 Tt accessible fuel-C, which is 0.32% of the total organic C. Hence, one has to postulate that around 445 Tt, i.e. around 99% of photosynthetically produced and presently preserved organic C is distributed in sediments of various ages where its inaccessibility to biological reoxidation processes guarantees the maintenance of our oxic biosphere. The hydrocarbon content of this organic material is unknown. But even if one assumes that this enormous reservoir of organic carbon consists on the average of no more than 1% of hydrocarbons, as the less maturated kerogens (the diagenetically transformed biomass that gave rise to oil), it would add around 4 Tt hydrocarbon-C to the 1.4 Tt organic C in accessible fuel reservoirs. Hence, studying the biological degradation or degradability of hydrocarbons means directing our research interest not only to an environmentally and technologically important, but also to a globally rather dominant group of organic compounds.

Brief historical overview

Biological hydrocarbon oxidation was first demonstrated around the turn of the century in fungal and bacterial cultures (for summary see Bühler and Schindler 1984). The biochemical mechanisms of hydrocarbon oxidation have been elucidated (Britton 1984; Bühler and Schindler 1984). The only aerobic activation mechanism of saturated hydrocarbons (open-chain and cyclic alkanes) that has been substantiated thus far is the monooxygenase reaction. Aromatic

hydrocarbons are initially attacked by monooxygenases or dioxygenases, depending on the type of alkyl side chain or the type of microorganism (Gibson and Subramanian 1984).

During the 1940s and again twenty years later, there were reports of an anaerobic oxidation of alkanes by sulfate-reducing bacteria of the genus *Desulfovibrio* (Davis and Yarbrough 1966; Novelli and ZoBell 1944; Rosenfeld 1947). However, cultures have not been preserved, or attempts to reproduce hydrocarbon oxidation by *Desulfovibrio* strains failed (Aeckersberg et al. 1991; Aeckersberg 1994). Interest in possible hydrocarbon oxidation by sulfate-reducing bacteria arose from their frequent presence in oil production plants where these bacteria produce sulfide with its many undesirable effects (for overview see Cord-Ruwisch et al. 1987; Odom 1993; Rabus et al. 1996). Even though sulfate-reducing bacteria were recognized as the source of sulfide produced in oil fields in the 1920s (Bastin 1926), their electron donor and carbon substrates in these habitats remained a matter of discussion for several decades. In his review written in 1958, ZoBell supposed that crude itself, provided it is dispersed in mineral solution, supports growth of certain sulfate-reducing bacteria; but at that time he expressed certain doubts about an anaerobic utilization of hydrocarbons as reported before. Furthermore, geochemical studies invoked an interest in possible hydrocarbon oxidation by sulfate-reducing bacteria. Investigations into the genesis of sulfur deposits led to the assumption that hydrocarbons from oil formerly served as electron donors for sulfate-reducing bacteria and yielded sulfide that was subsequently oxidized with oxygen to sulfur (Ruckmick et al. 1979).

The first hydrocarbons for which an anaerobic degradation could be unequivocally shown were alkylbenzenes; degradation of these, especially of toluene, was shown in enriched bacterial populations (Grbic-Galic and Vogel 1987; Häner et al. 1995; Kuhn et al. 1988; Rueter et al. 1994) and in pure cultures of iron-reducing (Lovley and Lonergan 1990), denitrifying (Altenschmidt and Fuchs 1991; Ball et al. 1996; Dolfing et al. 1990; Evans et al. 1991; Fries et al. 1994; Rabus and Widdel 1995; Schocher et al. 1991) and sulfate-reducing (Beller et al. 1996; Rabus et al. 1993) bacteria. In the meantime, several details of the biochemistry and underlying genes of anaerobic toluene activation have become known (Beller and Spormann 1997; Biegert et al. 1996; Coschigano et al. 1998; Leuthner et al. 1998; Rabus and Heider 1998). Toluene is activated by condensation with fumarate yielding benzylsuccinate. This is further oxidized by reactions somewhat resembling β-oxidation of fatty acids and yielding benzoyl-CoA and succinyl-CoA. Furthermore, anaerobic degradation of the unsubstituted aromatic hydrocarbons, benzene and naphthalene could be measured in enriched bacterial communities (Edwards and Grbic-Galic 1992; Lovley et al. 1995; Zhang and Young 1997).

Consumption of an *n*-alkane as the only organic growth substrate under anoxic conditions was demonstrated in quantitative experiments with newly isolated, mesophilic types of sulfate-reducing bacteria under strict exclusion of air (Aeckersberg et al. 1991; Rueter et al. 1994). Furthermore, three strains of denitrifying bacteria have been isolated and shown to grow anaerobically on defined *n*-alkanes (Ehrenreich 1996; A. Behrends, J. Harder, F. Rainey, F.

Widdel, unpublished). The mechanism of anaerobic alkane oxidation is still insufficiently understood. Fatty acid analyses after anaerobic growth on *n*-alkanes suggested that in one type of sulfate-reducing bacterium the carbon chain of the alkane is altered at the end by one carbon atom during activation; one possibility would be the terminal addition of a C_1-unit. This mechanism may not occur in other species (Aeckersberg et al. 1998).

Growth of sulfate-reducing bacteria on crude oil

In addition to individual hydrocarbons, crude oil as a natural, complex mixture of hydrocarbons was also tested as growth substrate for sulfate-reducing bacteria. A mesophilic enrichment culture from an oil tank was shown to utilize alkylbenzenes from crude oil added as the only source of organic compounds to defined anoxic mineral medium (Rueter et al. 1994). Whole-cell hybridization with fluorescently labelled 16S rRNA-targeted oligonucleotide probes revealed that more than 95% of the enriched population were members of the suggested family of the *Desulfobacteriaceae* (Rabus et al. 1996). Members of this family of sulfate-reducing bacteria are distinctive from *Desulfovibrio* and *Desulfomicrobium* species, for which the family *Desulfovibrionaceae* has been suggested. This observation is in agreement with the finding that many sulfate-reducing bacteria that degrade aromatic compounds are members of the *Desulfobacteriaceae*. Subsequent attempts to isolate the microorganisms responsible for depletion of alkylbenzenes from crude oil in the enrichment culture yielded two types of novel sulfate-reducing bacteria. One strain oxidized *o*-xylene, *o*-ethyltoluene and toluene, the other strain oxidized *m*-xylene, *m*-ethyltoluene, *m*-isopropyltoluene and toluene (Harms et al. 1999). The anaerobic consumption of alkylbenzenes by the enrichment culture caused an isotopic discrimination, as obvious from analysis of the remaining part of the respective hydrocarbons (Table 1).

Furthermore, utilization of *n*-alkanes from crude oil by sulfate-reducing bacteria has been demonstrated. A moderately thermophilic sulfate-reducing bacterium (optimum around 60 °C) isolated on *n*-decane consumed *n*-alkanes from crude oil especially in the range from C_8 to C_{11} (Rueter et al. 1994). Furthermore, an enrichment culture exhibited sulfate-dependent consumption of *n*-alkanes from oil (Caldwell et al. 1998).

It can be calculated from the volume of oil added to the cultures of sulfate-reducing bacteria and from the amount of sulfide produced (Rueter et al. 1994) that around 10% (vol/vol) of the crude oil is anaerobically consumed by alkylbenzene- and alkane-utilizing sulfate-reducing bacteria.

The enriched and isolated cultures of sulfate-reducing bacteria growing with crude oil may serve as model systems that help to understand microbiological processes in oil wells, tanks and pipelines. Even if sulfate-reducing bacteria under the presumably non-optimal growth conditions in oil reservoirs and production plants exhibit slower and poorer growth than in the

enrichment culture, a utilization of alkylbenzenes and alkanes from crude oil as in the model culture should in principle be possible also under *in situ* conditions.

Table 1 Fractionation of carbon isotopes of alkylbenzenes during their utilization from crude oil in a sulfate-reducing enrichment culture after 54 days of incubation. 1,2,4-Trimethylbenzene is included as a representative of alkylbenzenes that did not show noticeable consumption in this enrichment culture.

Compound	Degradation (%)	^{13}C enrichment in remaining compound[a] (‰)
Toluene	100	ND[b]
o-Xylene	69	4.0
m/p-Xylene[c]	63	2.4
o-Ethyltoluene	77	6.0
m/p-Ethyltoluene[c]	58	2.6
1,2,4-Trimethylbenzene	0	0.3

[a] Reproducibility of the analysis was 0.3‰.
[b] ND, not determined because of complete consumption during the experiment.
[c] The *m*- and *p*-isomers could not be separated by the applied gas-chromotographic method. Additional analysis by another separation method revealed that the enrichment culture oxidized the *m*-isomers.

The assumption of an anaerobic utilization of oil hydrocarbons by sulfate-reducing bacteria has to be reconciled with the observation that alkylbenzenes are still present in oils. There might be several explanations: (a) Many unexploited oil reservoirs exhibit very low concentrations of sulfate (Belyaev et al. 1991; Nielsen et al. 1991; Nilsen et al. 1996). Sulfate in these reservoirs has probably become the limiting factor for anaerobic oxidation processes, due to depletion by bacterial sulfate reduction during early diagenesis and eventually during oil maturation. (b) Oil in reservoirs is trapped in the pores of rocks (Tissot and Welte 1984) which may impede diffusion of substrates into aqueous niches where bacteria could develop. Only during the process of oil extraction by water injection may conditions for bacterial growth become favorable by mixing processes, formation of emulsions and new contact areas between oil and water. (c) Sulfate-reducing bacteria that were formerly buried with the ancient sediments may have died off in the reservoirs, due to unfavorable conditions (extreme temperature and salinity) at the deep sites of catagenesis, and due to sulfate depletion. During secondary oil recovery, new, viable bacteria are introduced with the injected water. Upon cooling and dilution, the temperature and salinity, respectively, may become favorable for growth of a wide variety of sulfate-reducing bacteria. Also, high concentrations of sulfate are introduced if seawater is injected.

In addition to alkylbenzenes and *n*-alkanes in crude oil, there are other sources of reducing equivalents for sulfate-reduction, and of organic carbon for cell synthesis. Further potential substrates for sulfate-reducing bacteria in oil fields are (a) anaerobic biodegradation products from substances such as xanthan that are added during secondary oil recovery (Cord-Ruwisch et al. 1987), (b) fatty acids from ancient maturation processes (Barth 1991; Means and Hubbard 1987), and (c) polar organic products formed by aerobic bacteria during growth on hydrocarbons (Nazina et al. 1985).

Also extremely thermophilic sulfate-reducing archaea have been detected in oil reservoirs and oil fields (Beeder et al. 1994; L'Haridon 1995; Stetter et al. 1993; Stetter, this volume). Their organic electron donors under *in situ* conditions are unknown. So far, archaea have not been shown to utilize hydrocarbons.

At temperatures higher than approximately 150 °C, sulfide may be formed by a chemical process (Orr 1974). Initially present sulfide reacts with sulfate to yield elemental sulfur. The latter attacks hydrocarbons and is reduced to sulfide. Sulfide then reacts with further sulfate. The net reaction is the reduction of sulfate to sulfide with hydrocarbons providing the reducing equivalents.

Growth of denitrifying bacteria on crude oil

Research on anaerobic degradation of oil hydrocarbons especially by denitrifying bacteria has been stimulated by the fact that dispersal of oil and fuel in the environment may lead to the contamination of deep aquifers. Since groundwater aquifers are frequently anoxic, knowledge of bacterial capacities for degradation of oil hydrocarbons in the absence of molecular oxygen is important to predict the fate of hydrocarbons and the effectiveness of bioremediation efforts under such conditions. Addition of nitrate to oil-contaminated sites, e.g. groundwater aquifers (Gersberg et al., 1993; Hunkeler et al., 1995), has been regarded as a potential means of enhancing bioremediation efforts on site. Furthermore, the study of anaerobic hydrocarbon oxidation by nitrate reducers may help to understand future side effects of the suggested control of sulfide production during oil production by the addition of nitrate (Jenneman, this volume).

Strains of denitrifying bacteria which had been originally enriched and isolated on toluene, ethylbenzene, propylbenzene, and *m*-xylene as defined substrates were shown to grow also on crude oil by utilization of alkylbenzenes (Rabus and Widdel 1996). In a subsequent study with denitrifying bacteria directly enriched from freshwater mud samples in the presence of crude oil as a source of organic substrates, we investigated which oil constituents are preferentially degraded by denitrifying bacteria (Rabus et al. 1999). The enrichment culture exhibited biphasic growth. Analyses of oil samples taken during incubation revealed that alkylbenzenes were only utilized during the first growth phase and *n*-alkanes only during the second growth phase. Toluene, ethylbenzene and *m*-xylene disappeared completely during the first growth phase within 10 days, while *o*-xylene, *o*-ethyltoluene and 1,2,4-trimethylbenzene

were partially consumed. During the second growth phase, partial consumption of *n*-alkanes in the range of C_5-C_{12}, in particular of hexane, was observed. It could be calculated that 3.1% (vol/vol) of the oil was anaerobically oxidized by denitrification. Application of a newly designed, fluorescently labeled 16S rRNA-targeted oligonucleotide probe specific for the *Azoarcus/Thauera* group within the β-subclass of *Proteobacteria* allowed an affiliation of the vast majority of the enriched denitrifiers with this phylogenetic group. This result is in agreement with the observation that all denitrifiers known so far to degrade alkylbenzenes belong to the *Azoarcus/Thauera* group. Recently, other oligonucleotide probes constructed according to sequences from isolated alkylbenzene-degrading denitrifiers related to *Azoarcus* species allowed the *in situ* detection of such bacteria in subsurface soil exposed to fuel hydrocarbons (Hess et al. 1997).

References

Aeckersberg F 1994. Anaerober Abbau von Alkanen und 1-Alkenen durch sulfatreduzierende Bakterien. Dissertation, University of Bremen. Verlag Mainz, Aachen.

Aeckersberg F, Bak F, Widdel F 1991. Anaerobic oxidation of saturated hydrocarbons to CO_2 by a new type of sulfate-reducing bacterium. Arch. Microbiol. 156:5-14.

Aeckersberg F, Rainey FA, Widdel F 1998. Growth, natural relationships, cellular fatty acids and metabolic adaptation of sulfate-reducing bacteria that utilize long-chain alkanes under anoxic conditions. Arch. Microbiol. 170:361-369.

Altenschmidt U, Fuchs G 1991. Anaerobic degradation of toluene in denitrifying *Pseudomonas* sp.: indication for toluene methylhydroxylation and benzoyl-CoA as central aromatic intermediate. Arch. Microbiol. 156:152-158.

Bastin ES, Greer FE, Merritt CA, Moulton G 1926. The presence of sulphate reducing bacteria in oil field waters. Science 63:21-24.

Ball HA, Johnson HA, Reinhard M, Spormann AM 1996. Initial reactions in anaerobic ethylbenzene oxidation by a denitrifying bacterium, strain EB1. J. Bacteriol. 178:5755-5761.

Barth T 1991. Organic acids and inorganic ions in waters from petroleum reservoirs, Norwegian continental shelf: a multivariate statistical analysis and comparison with American reservoir formation waters. Appl. Geochem. 6:1-15.

Beeder J, Nilsen RK, Rosnes JT, Torsvik T, Lien T 1994. *Archaeoglobus fulgidus* isolated from hot North Sea oil field waters. Appl. Environ. Microbiol. 60:1227-1231.

Beller HR, Spormann AM 1997. Anaerobic activation of toluene and *o*-xylene by addition to fumarate in denitrifying strain T. J. Bacteriol. 179:670-676.

Beller HR, Spormann AM, Sharma PK, Cole JR, Reinhard M 1996. Isolation and characterization of a novel toluene-degrading, sulfate-reducing bacterium. Appl. Environ. Microbiol. 62:1188-1196.

Biegert T, Fuchs G, Heider J 1996. Evidence that anaerobic oxidation of toluene in the denitrifying bacterium *Thauera aromatica* is initiated by formation of benzylsuccinate from toluene and fumarate. Eur. J. Biochem. 238:661-668.

Belyaev SS, Rozanova EP, Borzenkov IA, Charakhch'yan IA, Miller YuM, Sokolov MYu, Ivanov MV 1991. Characteristics of microbiological processes in a water-flooded oilfield in the middle Ob' region. Microbiol. 59:754-759.

Bolin B 1983. The carbon cycle. In: Bolin B, Cook RB (eds.) The major biogeochemical cycles and their interactions, SCOPE 21. John Wiley & Sons, Chichester, pp 41-45.

Britton LN 1984. Microbial degradation of aliphatic hydrocarbons. In: Gibson TD (ed) Microbial degradation of organic compounds. Marcel Dekker, New York Basel, pp 89-129

Bühler M, Schindler J 1984. Aliphatic hydrocarbons. In: Kieslich K (ed) Biotechnology, vol 6a. VCH, Weinheim, pp 329-385.

Caldwell ME, Garrett RM, Prince RC, Suflita JM 1998. Anaerobic biodegradation of long-chain *n*-alkanes under sulfate-reducing conditions. Environ. Sci. Technol. 32:2191-2195.

Coschigano PW, Wehrman TS, Young LY 1998. Identification and analysis of genes involved in anaerobic toluene metabolism by strain T1: putative role of a glycine free radical. Appl. Environ. Microbiol. 64:1650-1656.

Cord-Ruwisch R, Kleinitz W, Widdel F 1987. Sulfate-reducing bacteria and their activities in oil production. J. Petrol. Technol., January:97-106.

Davis JB, Yarbrough HF 1966. Anaerobic oxidation of hydrocarbons by *Desulfovibrio* desulfuricans. Chem. Geol. 1:137-144.

Dolfing J, Zeyer J, Binder-Eicher P, Schwarzenbach RP 1990. Isolation and characterization of a bacterium that mineralizes toluene in the absence of molecular oxygen. Arch. Microbiol. 154:336-341.

Edwards EA, Grbic-Galic D 1992. Complete mineralization of benzene by aquifer microorganisms under strictly anaerobic conditions. Appl. Environ. Microbiol. 58:2663-2666.

Ehrenreich P 1996. Anaerobes Wachstum neuartiger sulfatreduzierender und nitratreduzierender Bakterien auf *n*-Alkanen und Erdöl. Dissertation, University of Bremen. Shaker Verlag, Aachen.

Evans PJ, Mang DT, Kim KS, Young LY 1991. Anaerobic degradation of toluene by a denitrifying bacterium. Appl. Environ. Microbiol. 57:1139-1145.

Fries MR, Zhou J, Chee-Sanford J, Tiedje JM 1994. Isolation, characterization and distribution of denitrifying toluene degraders from a variety of habitats. Appl. Environ. Microbiol. 60:2802-2810.

Gersberg RM, Dawsey WJ, Bradley MD 1993. Nitrate enhancement of in situ bioremediation of monoaromatic compounds in groundwater. Remediation, Spring:233-245.

Gibson DT, Subramanian V 1984. Microbial degradation of aromatic hydrocarbons. In: Gibson DT (ed) Microbial degradation of organic compounds. Marcel Dekker, New York, pp 181-294.

Greenwood NN, Earnshaw A 1986. Chemistry of the elements. Pergamon Press, Oxford.

Grbic-Galic D, Vogel TM .1987. Transformation of toluene and benzene by mixed methanogenic cultures. Appl. Environ. Microbiol. 53:254-260.

Häner A, Höhener P, Zeyer J 1995. Degradation of *p*-xylene by a denitrifying enrichment culture. Appl. Environ. Microbiol. 61:3185-3188.

Harms G, Zengler K, Rabus R, Aeckersberg F, Minz D, Rosselló-Mora R, Widdel F 1999. Anaerobic oxidation of *o*-xylene, *m*-xylene, and homologous alkylbenzenes by new types of sulfate-reducing bacteria. Appl Environ Microbiol, in press.

Hess A, Zarda B, Hahn D, Häner A, Stax D, Höhener P, Zeyer J 1997. In situ analysis of denitrifying toluene- and *m*-xylene degrading bacteria in a diesel fuel contaminated laboratory aquifer column. Appl. Environ. Microbiol. 63:2136-2141.

Hunkeler D, Höhener P, Häner A, Bregnard T, Zeyer J 1995. Quantification of hydrocarbon mineralization in a diesel fuel contaminated aquifer treated by *in situ* biorestoration. In: Kovar K, and Krasny J (eds) Groundwater quality: remediation and protection (Proceedings of the Prague Conference, May 1995) IAHS Publication No. 225, Prague, pp 421-430.

Kuhn EP, Zeyer J, Eicher P, Schwarzenbach RP 1988. Anaerobic degradation of alkylated benzenes in denitrifying laboratory aquifer columns. Appl. Environ. Microbiol. 54:490-496.

Leuthner B, Leutwein C, Schulz H, Hörth P, Haehnel W, Schiltz E, Schägger H, Heider J 1998. Biochemical and genetic characterization of benzylsuccinate synthase from *Thauera aromatica*: a new glycyl radical enzyme catalysing the first step in anaerobic toluene metabolism. Mol. Microbiol. 28:615-628.

L'Haridon S, Reysenbach A-L, Glénat P, Prieur D, Jeanthon C 1995. Hot subterranean biosphere in a continental oil reservoir. Nature (London) 367:223-224.

Lovley DR, Coates JD, Woodward JC, Phillips EJP (1995) Benzene oxidation coupled to sulfate reduction. Appl. Environ. Microbiol. 61:953-958.

Lovley DR, Lonergan DJ 1990. Anaerobic oxidation of toluene, phenol, and *p*-cresol by the dissimilatory iron-reducing organism, GS-15. Appl. Environ. Microbiol. 56:1858-1864.

Means JL, Hubbard N 1987. Short-chain aliphatic acid anions in deep subsurface brines: a review of their origin, occurrence, properties, and importance and new data on their distribution and geochemical implications in the Palo Duro Basin, Texas. Org. Geochem. 11:177-191.

Nazina TN, Rozanova EP, Kuznetsov SI 1985. Microbial oil transformation processes accompanied by methane and hydrogen sulfide formation. Geomicrobiol J 4:103-130.

Nielsen H, Pilot J, Grinenko LN, Grinenko VA, Lein AYu, Smith JW, Pankina RG 1991. Lithospheric source of sulphur. In: Krouse HR, and Grinenko VA (eds) Stable isotopes: natural and anthropogenic sulphur in the environment, SCOPE 43. John Wiley & Sons, Chichester, pp 65-132.

Nilsen RK, Beeder J, Thorstenson T, Torsvik T 1996. Distribution of thermophilic marine sulfate reducers in North Sea oil field waters and oil reservoirs. Appl. Environ. Microbiol. 62:1793-1798.

Novelli GD, ZoBell CE 1944. Assimilation of petroleum hydrocarbons by sulfate-reducing bacteria. J. Bacteriol. 47:447-448.

Odom JM 1993. Industrial and environmental activities of sulfate-reducing bacteria. In: Odom JM, and Rivers Singleton, Jr. (eds) The sulfate-reducing bacteria: contemporary perspectives. Springer-Verlag, New York, pp 189-210.

Orr WL 1974. Changes in sulfur content and isotopic ratios of sulfur during petroleum maturation - study of Big Horn Basin paleozoic oils. Am. Assoc. Pet. Geol., Bull. 58:2295-2318.

Rabus R, Fukui M, Wilkes H, Widdel F 1996. Degradative capacities and 16S rRNA-targeted whole-cell hybridization of sulfate-reducing bacteria in an anaerobic enrichment culture utilizing alkylbenzenes from crude oil. Appl. Environ. Microbiol. 62:3605-3613.

Rabus R, Heider J 1998. Initial reactions of anaerobic metabolism of alkylbenzenes in denitrifying and sulfate-reducing bacteria. Arch. Microbiol. 170:377-384.

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

Rabus R, Widdel F 1995. Anaerobic degradation of ethylbenzene and other aromatic hydrocarbons by new denitrifying bacteria. Arch. Microbiol. 163:96-103.

Rabus R, Widdel F 1996. Utilization of alkylbenzenes during anaerobic growth of pure cultures of denitrifying bacteria on crude oil. Appl. Environ. Microbiol. 62:1238-1241.

Rabus R, Wilkes H, Schramm A, Harms G, Behrends A, Amann R, Widdel F 1999. Anaerobic utilization of alkylbenzenes and *n*-alkanes from crude oil in an enrichment culture of denitrifying bacteria affiliated with the *Azoarcus/Thauera* cluster. Environ. Microbiol., in press.

Rosenfeld WD 1947. Anaerobic oxidation of hydrocarbons by sulfate-reducing bacteria. J. Bacteriol. 54:664-665.

Ruckmick JC, Wimberly BH, Edwards AF 1979. Classification and genesis of biogenic sulfur deposits. Econ. Geol. 74:469-474.

Rueter P, Rabus R, Wilkes H, Aeckersberg F, Rainey FA, Jannasch HW, Widdel F 1994. Anaerobic oxidation of hydrocarbons in crude oil by new types of sulphate-reducing bacteria. Nature (London) 372:455-458.

Schmidt K-H, Romey I 1981. Kohle - Erdöl - Erdgas. Vogel-Verlag, Würzburg.

Schocher RJ, Seyfried B, Vazquez F, Zeyer J 1991. Anaerobic degradation of toluene by pure cultures of denitrifying bacteria. Arch. Microbiol. 157:7-12.

Stetter KO, Huber R, Blöchl E, Kurr M, Eden RD, Fielder M, Cash H, Vance I 1993. Hyperthermophilic archaea are thriving in deep North Sea and Alaskan oil reservoirs. Nature (London) 365:743-745.

Tissot BP, Welte DH 1984. Petroleum Formation and Occurence, 2nd edn. New York: Springer-Verlag.

Zhang X, Young LY 1997. Carboxylation as an initial reaction in the anaerobic metabolism of naphthalene and phenanthrene by sulfidogenic consortia. Appl. Environ. Microbiol. 63:4759-4764.

ZoBell CE 1958. Ecology of sulfate reducing bacteria. Prod. Monthly 22:12-29.

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Anaerobic degradation and carbon isotopic fractionation of alkylbenzenes in crude oil by sulphate-reducing bacteria

Heinz Wilkes[1*], Chris Boreham[2], Gerda Harms[3], Karsten Zengler[3], and Ralf Rabus[3]

Geochimica et Cosmochimica Acta, submitted

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[1] Institut für Erdöl und Organische Geochemie, Forschungszentrum Jülich GmbH, D-52425 Jülich, Germany

[2] Australian Geological Survey Organisation, Canberra, Australia

[3] Max-Planck-Institute for Marine Microbiology, Celsiusstr. 1, D-28359 Bremen, Germany

* Corresponding author

Abstract

A mesophilic enrichment culture of sulphate-reducing bacteria isolated from the water phase of a North Sea oil tank using oil from the same tank as sole source of carbon and energy specifically depletes certain C_1 - C_5 alkylbenzenes in crude oil during growth. The enrichment culture grows on oils of different origin and composition resulting in similar patterns of alkylbenzene depletion. Two pure cultures of sulphate-reducing bacteria, strain oXyS1 and mXyS1 which were isolated on *o*-xylene and *m*-xylene, respectively, also grow on crude oil. Strain oXyS1 preferentially depletes *o*-xylene and *o*-ethyltoluene while strain mXyS1 preferentially depletes *m*-xylene and *m*-ethyltoluene. Both strains also utilise toluene. The degradative patterns of the pure cultures are complementary and their combination results in the degradative pattern of the enrichment culture. During growth of the enrichment culture and the pure strains on crude oil mainly alkylated benzoic acids are produced as metabolites, which were isolated from the water phases of the incubation experiments. The patterns of alkylated benzoic acids produced by the pure cultures again are complementary with respect to the pattern observed for the enrichment culture. The spectrum of alkylated benzoic acids suggests that partial oxidation of alkylbenzenes, which do not support growth, takes place resulting in the formation of dead-end metabolites. Alkylphenylsuccinic and -fumaric acids were produced in trace amounts only. The remaining portion of alkylbenzenes depleted in crude oil becomes enriched in ^{13}C during growth of bacteria. From the data obtained in this study it can be estimated that the carbon isotopic fractionation of the initial reaction of alkylbenzene degradation by the present bacteria is between -26 and -33‰. We suggest that the variability of alkylbenzene concentrations and their carbon isotopic signature together with the occurrence of alkylated benzoic acids may be used as a specific indicator of initial biodegradation of crude oils and fossil fuel products by sulphate-reducing bacteria in various environments.

Introduction

Biodegradation of crude oil in reservoirs is an important phenomenon which has attracted much interest of organic geochemists (for reviews see Connan 1984, and Palmer 1993). Based on observations on compositional changes in crude oils, classification systems describing the extent of biodegradation have been suggested (e.g. Volkman et al. 1984; Peters and Moldowan 1993; Fisher et al. 1998). According to these systems, different types of oil constituents are degraded seqentiallly (*n*-alkanes > monocyclic alkanes > alkylbenzenes > isoprenoid alkanes > alkylnaphthalenes > bicyclic alkanes > steranes > hopanes). Until very recently it was generally accepted that these effects are mainly caused by aerobic bacteria (e.g. Palmer 1993). Sufficient oxygen and nutrient supply for the action of aerobic bacteria in undeveloped reservoirs was assumed to be provided by meteoric waters. However, biodegradation of crude oil is also observed in reservoirs where oxygen is not available but anoxic conditions prevail (Connan et al. 1996).

Anaerobic bacteria which might contribute to oil alteration during biodegradation are frequently encountered in oil and gas fields (e.g. Bernard et al. 1992; Scott and Davies 1993; Bodrossy et al. 1995 Nilsen and Torsvik, 1996). Of particular interest for this study is the widespread occurrence of sulphate-reducing bacteria in oil reservoirs (e.g. Cord-Ruwisch et al. 1986; Rosnes et al. 1991; Stetter et al. 1993; Beeder et al. 1994, 1996; L'Haridon et al. 1995; Mueller and Nielsen 1996; Nilsen et al. 1996). The first recognition of sulphate-reducing bacteria in oil field waters even dates back to the 1920s (Bastin et al. 1926). Sulphate reduction in oil reservoirs is often observed when sulphate-rich sea waters are injected into the reservoir during secondary oil production. Stetter et al. (1993) demonstrated that hyperthermophilic sulphate-reducers from a North Sea oil reservoir were capable of growth in the presence of crude oil as the sole source of carbon and energy.The actual electron donors, however, in their experiments remained unknown. In an earlier study, Jobson et al. (1979) had shown that sulphate-reducing bacteria grew on oils that were pre-incubated with aerobic bacteria. They concluded that the sulphate-reducers utilized products of the aerobic oxidation of oil constituents. Among these might be typical electron donors for bacterial sulphate reduction such as acetate, lactate or benzoate which in many studies were used for the isolation of sulphate-reducing bacteria from oil field waters (Nazina et al. 1985; Rozanova et al. 1991). These studies did not consider hydrocarbons, which are the major constituents of oil, as potential source of carbon and energy for sulphate-reducing bacteria. In fact, the capacity of sulphate-reducing bacteria to utilize hydrocarbons as sole source of carbon and energy under anoxic conditions has been established only in the recent years.

Evidence for the utilization of *n*-alkanes as electron donors by sulphate-reducing bacteria of the genus *Desulfovibrio* has been provided occasionally since the 1940s (Novelli and ZoBell 1944; Rosenfeld 1947; Davis and Yarbrough, 1966). Aeckersberg et al. (1991) isolated a pure culture of sulphate-reducing bacteria, strain Hxd3, from an enrichment culture obtained from an oil production plant. This bacterium can anaerobically utilize *n*-alkanes from C_{12} to C_{20} which

are completely oxidized to CO_2. The moderately thermophilic sulphate-reducing bacterium strain Tdx3 which was isolated from Guaymas Basin sediments grows on *n*-alkanes from C_6 to C_{16} and preferentially removes the C_8 - C_{11} *n*-alkanes from crude oil (Rueter et al. 1994). Depletion of *n*-alkanes in diesel fuel and weathered oil under sulphate-reducing conditions has also been reported to occur in incubation experiments with hydrocarbon-contaminated marine sediments as the sources of inoculum (Coates et al. 1997; Caldwell et al. 1998).

Degradation of monoaromatic hydrocarbons under sulphate-reducing conditions has been intensively studied during the last years. Several pure cultures of sulphate-reducing bacteria that have the capacity to degrade alkylbenzenes have been reported. Strain Tol2 which was isolated from marine sediment, was the first pure culture of sulphate-reducing bacteria that was demonstrated to completely oxidize toluene to carbon dioxide under strictly anoxic conditions (Rabus et al. 1993). Strain PRTOL1 was isolated from fuel-contaminated subsurface soil and shown to mineralize 80% of toluene carbon to carbon dioxide whereas 15% of toluene carbon were converted to biomass and nonvolatile metabolic by-products (Beller et al. 1996). *o*-Xylene degrading strain oXyS1 and *m*-xylene degrading strain mXyS1 were isolated (Harms et al. 1999) from a sulphate-reducing crude oil-utilizing enrichment culture (Rabus et al. 1996). Anaerobic degradation of benzene coupled to sulphate reduction has been observed in sediment studies (Edwards and Grbic-Galic, 1992; Lovley et al. 1995; Coates et al. 1996a,b; Kazumi et al. 1997; Reinhard et al. 1997). Also polycyclic aromatic hydrocarbons like naphthalene (Thierrin et al. 1992; Coates et al. 1996a,b, 1997; Zhang and Young 1997), methylnaphthalene (isomer not specified; Coates et al. 1997), phenanthrene (Coates et al. 1996a, 1997; Zhang and Young 1997), fluorene (Coates et al. 1997) and fluoranthene (Coates et al. 1997) were found to be degradable under sulphate-reducing conditions.

Aerobic organisms employ molecular oxygen as a highly reactive cosubstrate during degradation of hydrocarbons. In contrast, under anoxic conditions, the reactions of hydrocarbon degradation have to proceed via oxygen-independent steps. Reactions involved in anaerobic degradation of aromatic compounds were summarized by Heider and Fuchs (1997 a,b). The mechanism of anaerobic hydrocarbon degradation has been studied most intensively with toluene. Initial experiments with hypothetical intermediates did not support degradation of toluene via one of the cresols or methylbenzoates, benzyl alcohol, or phenylacetate as free intermediates. Strain Tol2 did not utilize any of the three xylene isomers as growth substrate, but nevertheless oxidized *p*-xylene to 4-methylbenzoic acid as a dead-end metabolite (Rabus and Widdel 1995). Benzylsuccinate was identified as a by-product of toluene oxidation in strains Tol2 (Rabus and Widdel 1995) and PRTOL1 (Beller et al. 1997b); strain PRTOL1 also formed trace amounts of benzylfumaric acid. This type of metabolites is also produced by nitrate-reducing bacteria during anaerobic degradation of alkylbenzenes (Evans et al. 1992; Biegert et al. 1996; Migaud et al. 1996; Beller and Spormann 1997a). Benzylsuccinic and benzylfumaric acids have also been identified in aquifer samples and it has been suggested to use them as indicators of anaerobic biodegradation in natural environments (Beller et al. 1995). Studies with

the denitrifying bacterium *Thauera aromatica* demonstrated for the first time the formation of benzylsuccinate from toluene in a fumarate-dependent reaction in cell free extracts (Biegert et al. 1996). The same reaction was identified in permeabilized cells of sulphate-reducing strain PRTOL1 (Beller and Spormann 1997b) and cell-free extracts of sulphate-reducing strain Tol2 (Rabus and Heider 1998), indicating that benzylsuccinate formation might be a general reaction in anaerobic toluene metabolism. Zhang and Young (1997) showed that anaerobic mineralization of naphthalene and phenanthrene in sulphidogenic consortia may be initiated by carboxylation of the aromatic hydrocarbons to form the corresponding carboxylic acids. Recent studies with sulphate-reducing strain Hxd3 showed that cellular fatty acids were C-even, if cells were grown on C-odd chain *n*-alkanes, and, reversely, C-odd, if cells were grown on C-even chain *n*-alkanes. These results suggest that a terminal addition of a C_1-unit could be a possible initial reaction of *n*-alkane degradation in strain Hxd3 (Aeckersberg et al. 1998).

In a previous paper we have demonstrated that an enrichment culture of sulphate-reducing bacteria obtained from a North Sea oil tank efficiently removes certain alkylbenzenes including toluene, *o*- and *m*-xylene, *o*- and *m*-ethyltoluene, *m*-propyltoluene and *m*-cymene from crude oil. Benzene, *p*-xylene and *p*-ethyltoluene were not affected. Using molecular biological methods, it was shown that the enrichment culture mainly consists of completely oxidizing sulphate-reducing bacteria (Rabus et al. 1996). Individual alkylbenzenes were depleted at different rates from crude oil indicating variable substrate specificities within the enrichment culture. The present paper describes a more detailed investigation of alkylbenzene degradation in crude oils by the enrichment culture and two pure strains of sulphate-reducing bacteria isolated from this enrichment culture. Also included is the investigation of carbon isotopic fractionation of alkylbenzenes and the formation of oxidation products during incubation of crude oil with sulphate-reducing bacteria.

Methods

Sources of Organisms. The mesophilic enrichment culture of sulphate-reducing bacteria growing on crude oil as the only source of organic carbon and energy originated from the water phase of a North Sea oil tank, Wilhelmshaven, Germany (Rueter et al. 1994; Rabus et al. 1996). Oil from the same oil tank was used as the cultivation substrate for the enrichment culture (North Sea 1; Table 1). Pure cultures of alkylbenzene utilizing sulphate-reducing bacteria were isolated from the enrichment culture by using repeated agar dilution series (Widdel and Bak 1992) overlaid with the alkylbenzene of interest in 2,2,4,4,6,8,8-heptamethylnonane as an inert carrier phase (Rabus et al. 1993). Strains oXyS1 and mXyS1 were isolated on *o*-xylene and *m*-xylene, respectively, as described by Harms et al. (1999).

Incubation Experiments. An overview of the origin and selected properties of crude oils used for incubation experiments is given in Table 1. The enrichment culture and the pure cultures, strains oXyS1 and mXyS1, were grown in defined, bicarbonate-buffered, sulphide-

reduced mineral medium, that essentially had the same salt composition as natural sea water (Widdel and Bak 1992). For maintenance, the enrichment culture was grown on crude oil for 6 weeks at 28°C and then stored at 4°C for no longer than 8 weeks. No organic substrate other than crude oil was used. Strains oXyS1 and mXyS1 were grown on *o*-xylene and *m*-xylene, respectively.

Incubation experiments of the enrichment culture and the strains oXyS1 and mXyS1 were carried out in flat glass bottles (250 ml) with 200 ml medium, crude oil and an anoxic head space of N_2-CO_2 (90:10[vol/vol]). The flasks were sealed with rubber stoppers and incubated horizontally on a shaker to maximize the contact area between the medium and the oil, thereby enhancing the diffusion of oil constituents into the medium. In order to avoid contact of the oil with the rubber stoppers, the orifice of the flasks was kept below the surface level of the medium. Depending on the viscosity, the crude oils were applied to the incubation flasks in different ways. Liquid crude oils (North Sea 1 - 4, Mississippi; Table 1) were deaerated and stored in special 300 ml flasks (Rabus and Widdel 1996). These oils (5 ml) were transferred to the incubation flasks under a constant anoxic atmosphere using N_2-flushed syringes. Highly viscous oils (Mahakam, Tualang; Table 1) instead were transfered directly into the incubation flasks under a constant stream of N_2. After the flasks were anoxically sealed with rubber stoppers they were warmed up under a jet of hot water in order to solubilize the oil. The flasks were then turned to cover the inner side of the incubation flasks with the solubilized oil. By cooling the flasks under a jet of cold water, the oil solidified as a thin layer on the inner side of the incubation flasks. During this procedure contact of the oil with the rubber stoppers was avoided. The incubation flasks were then filled with sulphide-reduced medium and incubated prior to inoculation over night to remove possible traces of oxygen. The inocula (5%) were injected into the flasks using N_2-flushed syringes.

Growth of the enrichment culture and the strains oXyS1 and mXyS1 during incubation was monitored by determining the amount of sulphide produced. Sulphide was determined by the methylene blue formation reaction in a colorimetric microassay as previously described (Aeckersberg et al. 1991).

Sample Preparation. The way of obtaining oil sub-samples for chemical analysis depended on the viscosity of the crude oil used for the incubation experiment. The liquid North Sea crude oils were withdrawn from the incubation flasks with N_2-flushed syringes. Samples from the highly viscous oil were removed from the inner side of the incubation flasks with a spatula. All oil samples were transferred to vials with teflon sealed screw caps and a small headspace. Oil samples where directly used for whole oil gas chromatography-mass spectrometry. Aromatic hydrocarbon fractions for GC analysis were obtained by medium-pressure liquid chromatography (MPLC) according to the method of Radke et al. (1980). The aromatic hydrocarbon fractions were concentrated to a final volume of approximately 1 ml without evaporation to dryness to avoid loss of volatile alkylbenzenes.

For analysis of metabolites, samples from the aqueous growth medium were purged with nitrogen to remove hydrogen sulphide, acidified with HCl to yield pH 1.5, and successively extracted with dichloromethane. The extracts were evaporated to dryness, methylated using a solution of diazomethane in diethyl ether and then directly analysed by GC-MS.

Gas Chromatography (GC). GC analysis of aromatic hydrocarbon fractions was performed on a HP 5890 instrument which was equipped with an on-column injector with electronic pressure control, a flame ionisation detector (FID), a sulphur-selective Hall electrolytic conductivity detector and an BPX5 fused silica capillary column (SGE) of 50 m length, 0.15 mm i.d., and 0.25 mm film thickness. Helium was used as the carrier gas. The oven temperature was programmed from 90°C (4 min isothermal) to 120°C at a heating rate of 50°C/min and from 120°C (1 min isothermal) to 310°C (50 min isothermal) at a heating rate of 3°C/min. In addition, for the separation of *m*-xylene and *p*-xylene, the aromatic hydrocarbon fraction was analysed using a Varian 3700 gas chromatograph which was equipped with an on column injector, a FID and a FS-FFAP-CB-fused silica capillary column (CS) of 50 m length, 0.2 mm i.d. and 0.22 mm film thickness. Helium was used as the carrier gas. The oven temperature was programmed from 70°C (5 min isothermal) to 200°C at a heating rate of 3°C/min. The relative concentrations of selected alkylbenzenes were determined with reference to naphthalene which was not affected by the present microorganisms.

Gas Chromatography-Mass Spectrometry (GC-MS). Whole oil GC-MS was performed on a Fisons GC 8000 gas chromatograph that was coupled to a Fisons MD 800 mass spectrometer. A volume of 1 ml of undiluted oil was injected for each run. Helium was used as the carrier gas. The gas chromatograph was equipped with a split/splitless injector and a HP-1 fused silica capillary column (Hewlett Packard) of 50 m length, 0.31 mm i.d. and 0.52 mm film thickness. The oven temperature was programmed from 40°C (3 min isothermal) to 300°C (25 min isothermal) at a heating rate of 5°C/min. The mass spectrometer was operated in EI mode at an electron energy of 70 eV and a source temperature of 240°C. Full scan mass spectra were recorded over the mass range from 10 to 420 Da at a scan rate of 0.073 s per 100 amu with an inter scan delay of 0.1 s and a scan cycle time of 0.4 s.

GC-MS of aromatic hydrocarbon fractions was performed on a HP 5890B gas chromatograph that was coupled to a Finnigan MAT 95SQ mass spectrometer. The gas chromatograph was equipped with a Gerstel KAS 3 injection system and an Ultra 2 fused silica capillary column (Hewlett Packard) of 50 m length, 0.2 mm i.d., and 0.33 mm film thickness. Helium was used as the carrier gas. The oven temperature was programmed from 60°C (5 min isothermal) to 310°C (34 min isothermal) at a heating rate of 3°C/min. The mass spectrometer was operated in EI mode at an electron energy of 70 eV and a source temperature of 240°C. Full scan mass spectra were recorded over the mass range of 35 to 600 Da at a scan rate of 1 s per decade with an inter scan delay of 0.2 s and a scan cycle time of 1.434 s.

GC-MS of derivatized extracts from the aqueous growth medium was performed on the same instrument. The gas chromatograph was equipped with a Gerstel KAS 3 injection system and a BPX5 fused silica capillary column of 50 m length, 0.22 mm i.d., and 0.25 mm film thickness. Helium was used as the carrier gas. The oven temperature was programmed from 60°C to 340°C (8 min isothermal) at a heating rate of 3°C/min. The mass spectrometer was operated in EI mode at an electron energy of 70 eV and a source temperature of 240°C. Full scan mass spectra were recorded over the mass range of 35 to 800 Da at a scan rate of 1 s per decade with an inter scan delay of 0.2 s and a scan cycle time of 1.559 s. Assignment of benzoic acid methyl esters was based on relative retention times, mass spectra and comparison with authentic standards.

Gas Chromatography-Combustion-Isotope Ratio Mass Spectrometry (GC-C-IRMS). Compound specific isotopic analysis of the alkylbenzenes in the aromatic hydrocarbon fractions was undertaken using the GC-C-IRMS methodology. The instrument consisted of a Varian 3400 gas chromatograph connected to a Finnigan MAT 252 isotope-ratio mass spectrometer via a micro-volume combustion system. The gas chromatograph was equipped with a SPI injection system and a DB1 fused silica capillary column of 30 m length, 0.25 mm i.d., and 0.25 mm film thickness. Helium was used as the carrier gas. The oven temperature was programmed from 40°C (10 min isothermal) to 310°C (8 min isothermal) at a heating rate of 4°C/min. During the initial GC oven hold time the injector was heated to 300°C at 150°C/min and then held at this temperature for the duration of the GC run. Perdeuterated C_{16} internal standard of known ^{13}C isotopic composition, was used as additional calibration checks. The individual alkylbenzenes were quantitatively converted to CO_2 and H_2O in the combustion reactor (CuO, 900°C), and following removal of water, the isotopic composition of CO_2 was measured. d values were determined using the manufacture's software which corrected for background contributions, partial separation of isotopic species on the chromatographic column, and contributions of ^{17}O.

Results and Discussion

Depletion of Alkylbenzenes in Crude Oils. The enrichment culture of sulphate-reducing bacteria used in this study was obtained from the water phase of a North Sea oil tank using crude oil from the same tank (North Sea 1; Table 1) as the growth substrate. We have demonstrated previously that during incubation experiments with the enrichment culture formation of hydrogen sulphide from sulphate is coupled to the specific depletion of certain alkylbenzenes in the crude oil (Rueter et al. 1994; Wilkes et al. 1995; Rabus et al. 1996). Fig. 1A and B display the distribution of the C_0 - C_3 alkylbenzenes in the North Sea 1 oil after incubation without inoculum (control experiment) and incubation with the enrichment culture for

Table 1 Selected properties of crude oils used for degradation experiments and % recovery of selected alkylbenzenes after incubation with an enrichment culture of sulphate-reducing bacteria for 60 days.

Oil	**North Sea 1**	**North Sea 2**	**North Sea 3**	**North Sea 4**	**Mississippi**	**Mahakam**	**Tualang**
Type	marine-clastic	marine-clastic	marine-clastic	marine-clastic	carbonate	fluviodeltaic	lacustrine
Origin	unknown	Haltenbanken	Snorre Field	Oseberg Field	U.S. Gulf Coast	Kalimantan	Sumatra
°API Gravity	38	29	33	31	11	30	25
% Sulphur	0.33	0.41	0.33	0.35	5.2	0.08	0.05
% Saturates	53	51	46	35	11	71	71
% Aromatics	31	38	26	44	36	24	18
% Resins	15	8	26	16	27	5	11
% Asphaltenes	1	3	3	5	27	<1	<1
Pristane/Phytane	1.4	1.5	1.3-1.4	1.75	0.4	6.61	2.52
				% recovery of alkylbenzenes			
Toluene	0.7	0	0	0	15	26	72
m-Xylene	1.2	3.9	8.7	11	35	nd*	nd**
o-Xylene	0.2	1.1	1.6	0.3	2.4	43	68
m-Ethyltoluene	0	0	0	0	0	79	105
o-Ethyltoluene	18	16	41	25	40	83	98
m-Cymene	5.4	1.8	0.5	0.9	5	nd	nd
m-Propyltoluene	4.3	0	0	0	0	nd	nd

nd, not determined

*recovery of *m*- + *p*-xylene = 87%

**recovery of *m*- + *p*-xylene = 99%

60 days, respectively. In the experiment with the enrichment culture, toluene, *m*-xylene and *o*-xylene were completely lost from the oil (separation of *m*- and *p*-xylene was achieved using a polar GC column as described in Methods) whereas a significant but not complete depletion of *m*- and *o*-ethyltoluene was observed. In the course of the present study, North Sea 1 oil samples from more than ten incubation experiments have been analysed during the last five years revealing stability of the degradative potential of the enrichment culture and reproducibility of the compositional changes. Control experiments showed that the presence of both inoculum and sulphate was necessary for the degradation of alkylbenzenes in crude oil. During these experiments, compositional changes of the aliphatic hydrocarbons including specific compound classes such as biomarkers (steranes and hopanes) could not be detected. Also, no variation of the polycyclic aromatic hydrocarbon distributions has been observed. Despite the general reproducibility, smaller variations of the compositional changes of alkylbenzenes have been observed for different experiments. For example, a slight depletion of *p*-xylene and 1,2,4-trimethylbenzene was observed in some experiments. The overall degradative potential of the enrichment culture with respect to the C_0 - C_3 alkylbenzenes is summarised in column 1 of Table 2. Importantly, no loss of benzene, ethylbenzene, *i*- and *n*-propylbenzene, *p*-ethyltoluene and 1,3,5- and 1,2,3-trimethylbenzene takes place. Toluene, *m*- and *o*-xylene and *m*- and *o*-ethyltoluene are lost completely in most but not all experiments (regarding the North Sea 1 oil, please note that the data in Table 1 and the chromatograms displayed in Fig. 1A and B derive from different sets of experiments).

All oil samples from incubation experiments were carefully checked for the possible depletion of alkylbenzenes of higher molecular weight. By now, a loss has only been observed for two C_4 and one C_5 alkylbenzenes whose mass spectra are shown in Fig. 2 along with partial extracted ion chromatograms from an incubation experiment of North Sea 1 crude oil with sulphate (lower traces) and the corresponding control without sulphate (upper traces). The depletion of these compounds was not observed in all experiments. *m*-Cymene (Fig. 2A-C) was identified by comparison with an authentic standard, whereas the assignment of *m*-propyltoluene and *m*-butyltoluene is based solely on mass spectra and relative retention times. The identification of *m*-propyltoluene is also supported by the analytical data provided by Hartgers et al. (1992). The peak depleted in Fig. 2H relative to Fig. 2G appears to represent the coelution of at least three different compounds. Comparison of individual extracted ion chromatograms reveals that *m/z* 106 is reduced in the degraded oil to a much lesser extent than *m/z* 105 (data not shown). Based on the analytical data and the overall degradative pattern, we hypothesize that this peak is composed of *m*-butyltoluene and a second C_5 alkylbenzene isomer. A comparison of the peak shapes reveals that the signals at *m/z* 132 and *m/z* 117 in the mass spectrum derive from another coeluting compound.

Table 2 Depletion of C_0 - C_3 alkylbenzenes in crude oil by sulphate-reducing bacteria.

Substrate	β4 enrichment	oXyS1	mXyS1
Benzene	-	-	-
Toluene	+++	+++*	+++*
Ethylbenzene	-	-	-
p-Xylene	+	-	+
m-Xylene	+++	+	+++*
o-Xylene	+++	+++*	-
i-Propylbenzene	-	-	-
n-Propylbenzene	-	-	-
m-Ethyltoluene	++	-	++
p-Ethyltoluene	-	-	-
1,3,5-Trimethylbenzene	-	-	+
o-Ethyltoluene	++	++	-
1,2,4-Trimethylbenzene	+	+	+
1,2,3-Trimethylbenzene	-	-	-

+++, completely removed
++, significantly depleted
+, only slightly depleted
-, not affected
*growth substrate

All results described so far were obtained with the crude oil originating from the same North Sea oil tank from which the enrichment culture was obtained. In addition, a set of experiments was performed in order to investigate the degradability of alkylbenzenes in crude oils of different origin and composition. Table 1 summarises selected properties of the crude oils tested and the recoveries of selected alkylbenzenes after 60 days of incubation. All four marine-clastic North Sea crude oils, which originated from different oil fields, proved to be good growth substrates. While the overall degradation patterns were quite similar some variation of the recovery was observed in particular for *m*-xylene and *o*-ethyltoluene. The carbonate-sourced Mississippi crude oil, which is extremely sulphur-rich, also supported active growth of the enrichment culture. However, the alkylbenzenes were depleted to a significantly lesser extent than in the four North Sea crude oils (Table 1). Both the fluviodeltaic-sourced Mahakam and the lacustrine-sourced Tualang oils are paraffinic and rich in waxes. The Mahakam oil supported growth but the recoveries of degradable alkylbenzenes were high in comparison to the North Sea and the Mississippi oils (Table 1). No growth was observed with the Tualong oil which is of very high viscosity and of low abundance in light hydrocarbons. This contrasts with the Mahakam oil in which light hydrocarbons are present in significant amounts and are dominanted by alkylbenzenes (Schenk et al. 1997). Nevertheless, a slight depletion of toluene and *o*-xylene was observed even for the Tualang oil (Table 1) suggesting

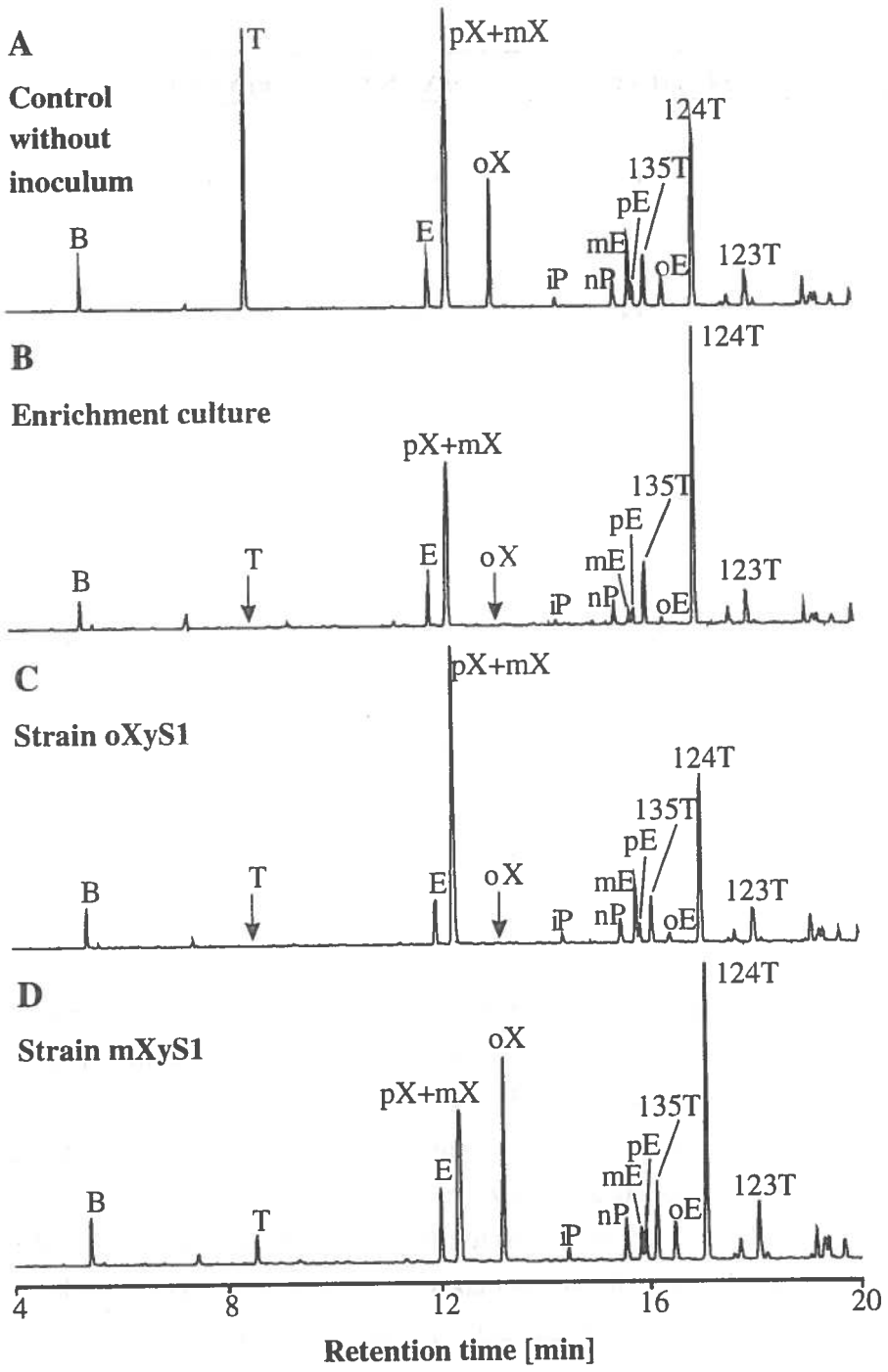

Fig. 1 Partial gas chromatograms of the aromatic hydrocarbon fraction of North Sea 1 crude oil **A** after incubation without inoculum (control) for 60 days, **B** after incubation with an enrichment culture of sulphate-reducing bacteria for 60 days, **C** after incubation with strain oXyS1 for 60 days, **D** after incubation with strain mXyS1 for 60 days. B, benzene; T, toluene; E, ethylbenzene; *p*X, *p*-xylene; *m*X, *m*-xylene; *o*X, *o*-xylene; *i*P, *i*-propylbenzene; *n*P, *n*-propylbenzene; *m*E, m-ethyltoluene; *p*E, *p*-ethyltoluene; 135T, 1,3,5-trimethylbenzene; *o*E, *o*-ethyltoluene; 124T, 1,2,4-trimethylbenzene; 123T, 1,2,3-trimethylbenzene.

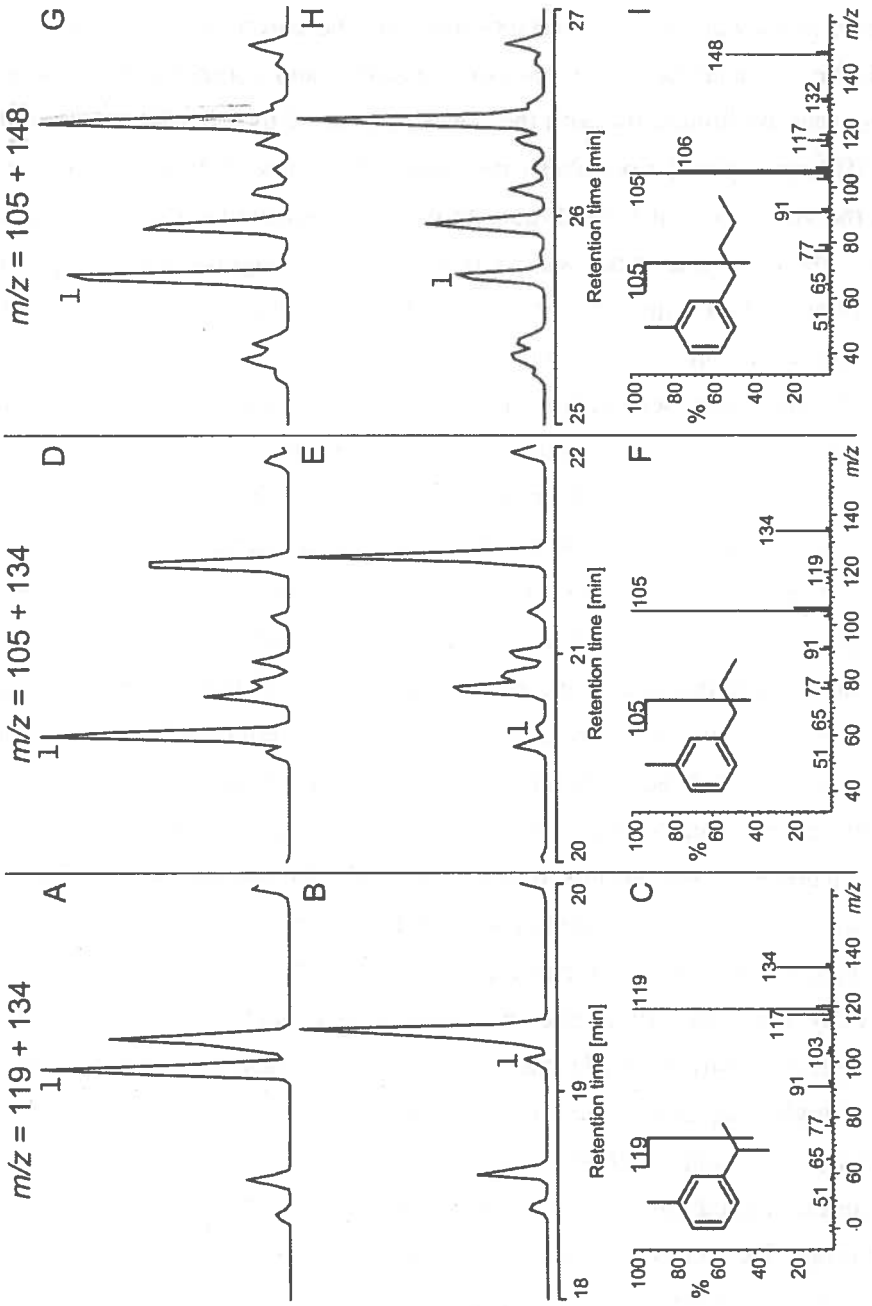

Fig. 2 Partial extracted ion chromatograms of the aromatic hydrocarbon fraction of North Sea 1 crude oil **A, D, G** after incubation without inoculum (control) for 60 days, **B, E, H** after incubation with an enrichment culture of sulphate-reducing bacteria for 60 days and mass spectra of **C** *m*-cymene (*m*-isopropyltoluene), **F** *m*-propyltoluene and **I** *m*-butyltoluene + unknown coeluting compounds. Peaks indicated by (1) correspond to the mass spectra shown at the bottom of each column.

that the minor biological activity has occurred even though growth was undetected.

It appears that availability of alkylbenzenes as the essential growth substrates in the crude oils might be a crucial factor for growth of bacteria and compositional changes of crude oils. Availability may be limited by both the concentration of the alkylbenzenes in the oils and their rates of diffusion from the oil phase into the water phase which is expected to depend particularly on the viscosity of the oil. It must be noted that growth on the Tualong oil was not observed even when benzoic acid was added, which is significantly water soluble and represents a proven growth substrate of the enrichment culture. Therefore, a toxic effect of certain constituents of this oil on the enrichment culture is likely.

Among the alkylbenzenes depleted in crude oil, only toluene, *o*-xylene and *m*-xylene yielded significant growth when used as pure compounds whereas *o*-ethyltoluene and *m*-ethyltoluene were poorer growth substrates (Rabus et al. 1996). We have previously shown that individual alkylbenzenes in the North Sea 1 crude oil are depleted at different rates by the enrichment culture (Rabus et al. 1996). In particular, toluene and *o*-xylene were depleted at significantly higher rates than *m*-xylene. Already these results pointed to the presence of different substrate specificities within the enrichment culture. Using *o*-xylene and *m*-xylene as pure substrates for selection, two pure cultures of sulphate-reducing bacteria, strains oXyS1 and mXyS1, respectively, whose properties are described in detail by Harms et al. (1999) were isolated from the enrichment culture. Typical distributions of alkylbenzenes in North Sea 1 crude oil after incubation with strains oXyS1 and mXyS1 are shown in Fig. 1C and D, respectively. Toluene is more or less completely consumed by both strains, while strain oXyS1 in addition completely consumes *o*-xylene and significantly depletes *o*-ethyltoluene while strain mXyS1 completely consumes *m*-xylene and significantly depletes *m*-ethyltoluene. A slight depletion of *m*-xylene by strain oXyS1 was also oserved. In these experiments, no decrease of alkylbenzenes of higher molecular weight was observed. The degradative patterns of strains oXyS1 and mXyS1 are complementary and their combination results in the above discussed pattern of the enrichment culture (Fig. 1B). It can be deduced, that in the present enrichment culture *o*- and *m*-alkylbenzenes have their specific degraders while no degradative potential towards *p*-alkylbenzenes is present.

Formation of Metabolites. Samples of the aqueous growth medium from various incubation experiments have been analysed for possible metabolites formed during degradation of alkylbenzenes from crude oils. Since it was assumed that the principal metabolites might be carboxylic acids formed by oxidation of the alkylbenzenes, extracts of the water phases were methylated prior to further characterization. GC-MS analyses of the methylated extracts revealed that benzoic acids were the main oxidation products formed during the incubation experiments. Typical distributions in the water phases after incubation with the enrichment culture and strains oXyS1 and mXyS1 for 60 days are shown in Fig. 3 in comparison with a control in which the inoculum was omitted. Benzoic acid was detected as a trace constituent in the water phases from all experiments with inoculum. Strain oXyS1 mainly accumulated 3-methylbenzoic acid which was accompanied by smaller amounts of 2- and 4-methyl- as well as 2,4-dimethylbenzoic acid. In addition, trace amounts of 2- and 3-ethyl and 2,3-, 2,5- and 3,5-dimethylbenzoic acid were

also formed. Strain mXyS1 produced 4-methyl- and 3,4- and 3,5-dimethylbenzoic acid as the major metabolites and trace amounts of 3-methylbenzoic acid. All of these benzoic acids with the exception of 3-ethylbenzoic acid were also detected in the water phase extract from the incubation experiment with the enrichment culture while they were essentially absent in the control experiment. The described incubation experiments were carried out in duplicate which demonstrated the reproducibility of these observations.

Significant amounts of benzylsuccinic and benzylfumaric acids were only detected in the methylated extracts from incubation experiments with strain mXyS1, however, their relative concentrations were approximately two orders of magnitude lower than those of the benzoic acids. The amounts of these compounds in extracts from experiments with the enrichment culture were extremely low while they were absent in extracts obtained from incubation experiments with strain oXyS1 and from control experiments without inoculum. The extracted ion chromatogram in Fig. 4A displays their distribution in the methylated water phase extract after incubation of North Sea 1 crude oil with strain mXyS1 for 60 days. The mass spectra of peaks MS1-3 (Fig 4B-D) show very similar fragmentation patterns and are in principal agreement with the mass spectra previously published for the dimethyl esters of (2-methylbenzyl)succinic acid (Beller et al. 1995, 1996; Beller and Spormann 1997a; Evans et al. 1992), (3-methylbenzyl)succinic acid (Beller et al. 1995) and (4-methylbenzyl)succinic acid (Beller et al. 1996). Due to the lack of authentic standards and available data on relative retention times, the assignment of isomers was not yet possible.

Benzylsuccinic acids are assumed to be formed by addition of toluenes to fumaric acid (see Introduction). The presence of all three possible isomers of (methylbenzyl)succinic acid shows that strain mXyS1 at least to some extent is able to transform all three xylene isomers by this reaction pathway. On the other hand, only one isomer of (methylbenzyl)fumaric acid was identified based on the similarity of its mass spectrum (Fig. 4E) with literature spectra of previously reported anaerobic transformation products of xylenes (Evans et al. 1992; Beller et al. 1995, 1996). Peaks MS5-7 in Fig 4A are significantly more abundant than the above discussed peaks MS1-3. The corresponding mass spectra (Fig. 4F-H) show similar fragmentation patterns as those of the (methylbenzyl)succinic acid dimethyl esters (Fig. 4B-D), however, all major fragment ions are shifted by 14 amu towards higher masses. We therefore assume, that these compounds are (dimethylbenzyl)- or (ethylbenzyl)succinic acids which could be formed upon transformation of trimethylbenzenes or ethyltoluenes, respectively. In analogy, peak MS8 (Fig. 4I) is assumed to represent a (dimethylbenzyl)- or (ethylbenzyl)fumaric acid. Benzylsuccinic or benzylfumaric acids derived from C_3 alkylbenzenes have not been reported so far. Non-alkylated benzylsuccinic or benzylfumaric acid which have been described as anaerobic transformation products of toluene (Beller et al. 1992, 1995; Beller and Spormann 1997a,b; Biegert et al. 1996; Evans et al. 1992; Rabus and Widdel 1995; Seyfried et al. 1994) could not be detected in our experiments.

There is increasing evidence that initial activation of toluene during anaerobic biodegradation both under nitrate-reducing (e.g. Biegert et al. 1996; Rabus and Heider 1998) and sulphate-reducing (e.g. Beller and Spormann 1997b; Rabus and Heider 1998) conditions

may proceed via formation of benzylsuccinic acid by addition of the methyl group of toluene to fumaric acid. Similar activation reactions might take place during the degradation of other alkylbenzenes containing at least one methyl group such as the xylenes, ethyltoluenes or trimethylbenzenes. It has also been proposed that toluene might be added to acetyl coenzyme A rather than to fumaric acid (Chee-Sanford et al. 1996) yielding cinnamoyl coenzyme A as the initial intermediate. The proposed degradation pathways of both benzylsuccinic acid (Biegert et al. 1996) and cinnamoyl coenzyme A (Chee-Sanford et al. 1996) which are not yet established involve complex reaction sequences finally resulting in the formation of benzoic acid or benzoyl coenzyme A. For the sulphate-reducing bacteria used in this study, utilization of *p*-alkyltoluenes for growth can be excluded. However, strain mXyS1 and the enrichment culture produce significant amounts of 4-methylbenzoic acid (Fig. 3) which is a transformation product of *p*-xylene if the above discussed degradation pathways are correct. It is noteworthy that this would require activity of all enzymes necessary for the degradation of the initial transformation product of *p*-xylene into 4-methylbenzoic. Since there seems to be no benefit for the organism and moreover energy must be supplied for these enzyme reactions, it may be speculated that the initial reaction sequence of alkylbenzene degradation by the present bacteria is rather unspecific.It would turn out that the substrate selectivity exhibited by strains oXyS1 and mXyS1 is located at the stage of the benzoic acids/benzoyl coenzyme A esters. This is also supported by the large amounts of 3-methylbenzoic acid produced by strain oXyS1 which is not capable of utilizing *m*-xylene as a growth substrate. Alternatively, other degradation pathways of alkylbenzenes may be taken into account, e.g. the direct oxidation of methyl groups as has been proposed by Seyfried et al. (1994). In this case, the small amounts of benzylsuccinic and benzylfumaric acids identified in this study might represent unproductive dead-end metabolites.

Carbon Isotopic Fractionation of Alkylbenzenes. $\delta^{13}C$ values of alkylbenzenes in the original North Sea 1 oil vary between -29 and -25‰ (Fig. 5A). In addition to the original oil, the carbon isotopic composition of alkylbenzenes in two oil samples incubated with the enrichment culture for 28 and 56 days, respectively, has been determined. Only minor variations of the $\delta^{13}C$ values are observed for most of the alkylbenzenes which are not depleted during the incubation experiment. On the other hand, a significant enrichment of ^{13}C is observed for the remaining portion of those alkylbenzenes which are depleted by the enrichment culture. This is most pronounced for *o*-xylene and *o*-ethyltoluene but can also be seen for the coeluting *m*- and *p*-xylenes and *m*- and *p*-ethyltoluenes which could not be resolved gas chromatographically during the GC-IRMS measurements (Fig. 5A). It was not possible to determine the $\delta^{13}C$ value of toluene in the incubated samples due to the very low remaining concentration even after 28 days of incubation. Figure 5B displays the carbon isotopic composition of selected degradable alkylbenzenes as a function of the percentage depleted in comparison to the original oil. For *o*-xylene, a shift of the $\delta^{13}C$ value by 4‰ (from -28‰ to -24‰) after loss of 70% of the original amount is observed while the shift for *o*-ethyltoluene (6‰ from -27‰ to -21‰ after 80% loss) is even stronger. The shifts for the coeluting pairs of *m*- and *p*-alkylbenzenes are less pronounced which most probably reflects the contribution of compounds which are not significantly depleted during incubation, i.e. the *p*-isomers. Based on

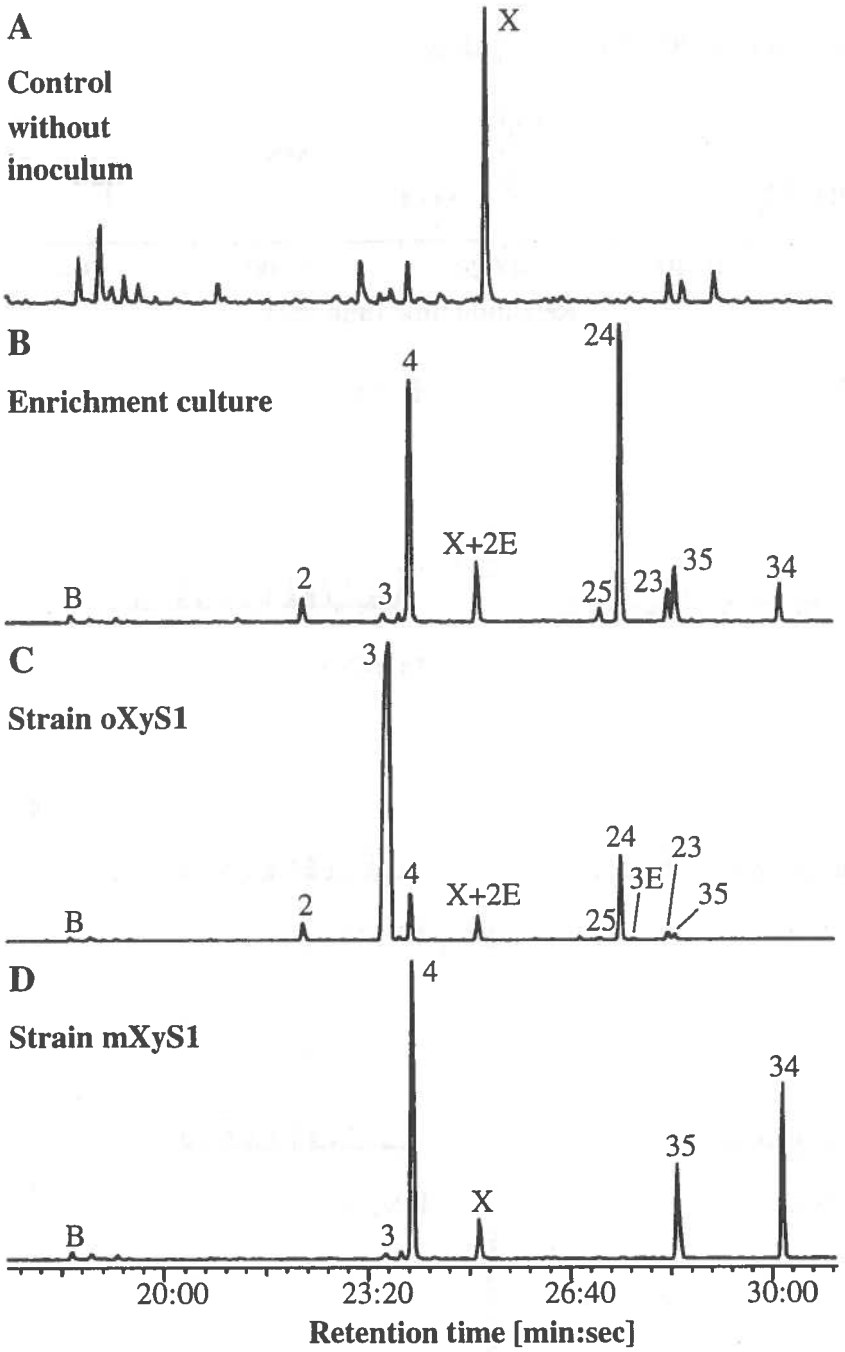

Fig. 3 Partial total ion chromatograms of the methylated extracts of the water phase recovered from incubation experiments of North Sea 1 crude oil **A** after incubation without inoculum (control) for 60 days, **B** after incubation with an enrichment culture of sulphate-reducing bacteria for 60 days, **C** after incubation with strain oXyS1 for 60 days, **D** after incubation with strain mXyS1 for 60 days. B, benzoic acid; 2, 2-methylbenzoic acid; 3, 3-methylbenzoic acid; 4, 4-methylbenzoic acid; X, benzoisothiazole (contamination?); 2E, 2-ethylbenzoic acid; 25, 2,5-dimethylbenzoic acid; 24, 2,4-dimethylbenzoic acid; 3E, 3-ethylbenzoic acid; 23, 2,3-dimethylbenzoic acid; 35, 3,5-dimethylbenzoic acid; 34, 3,4-dimethylbenzoic acid.

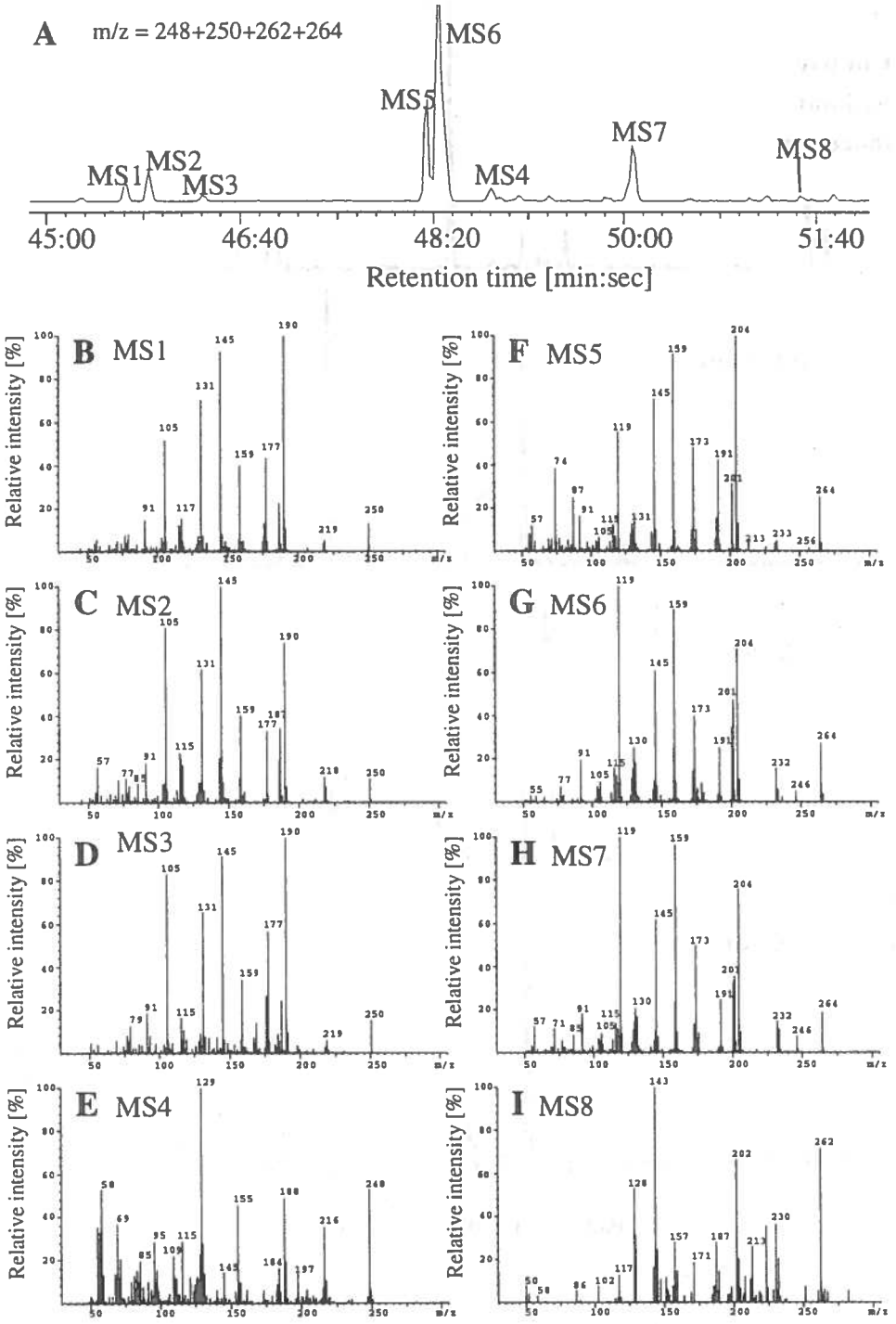

Fig. 4 A Partial extracted ion chromatogram (*m/z* = 248 + 250 + 262 + 264) of the methylated extract of the water phase recovered from an incubation experiment of North Sea 1 crude oil after incubation with strain mXyS1 for 60 days. **B - I** Mass spectra of peaks MS1 - MS8 indicated in Fig. 4A; MS1, (methylphenyl)succinic acid isomer; MS2, (methylphenyl)succinic acid isomer; MS3, (methylphenyl)succinic acid isomer; MS4, (methylphenyl)fumaric acid isomer; MS5, (C_2-phenyl)succinic acid isomer; MS6, (C_2-phenyl)succinic acid isomer; MS7, (C_2-phenyl)succinic acid isomer; MS8, (C_2-phenyl)fumaric acid isomer.

these data, the kinetic isotope effect (e) between product and reactant is calculated to be -3.2‰ and -3.7‰ for *o*-xylene and *o*-ethyltoluene, respectively, using the Rayleigh model (Mariotti et al. 1981). From this a estimation can be made of the carbon isotopic fractionation for the initial reaction of alkylbenzene degradation by the present sulphate-reducing bacteria independently from the actual mechanism of this initial reaction. Both the patterns of alkylbenzene depletion and formation of metabolites point to only aromatic methyl groups playing a role in the reaction influencing the carbon isotopic fractionation process. Hence, for an alkylbenzene with 8 carbon atoms (i.e. a xylene) the measured data would translate to a kinetic isotope effect of approximately -26‰ (-33‰ for an ethyltoluene with 9 carbon atoms) at the reaction site.

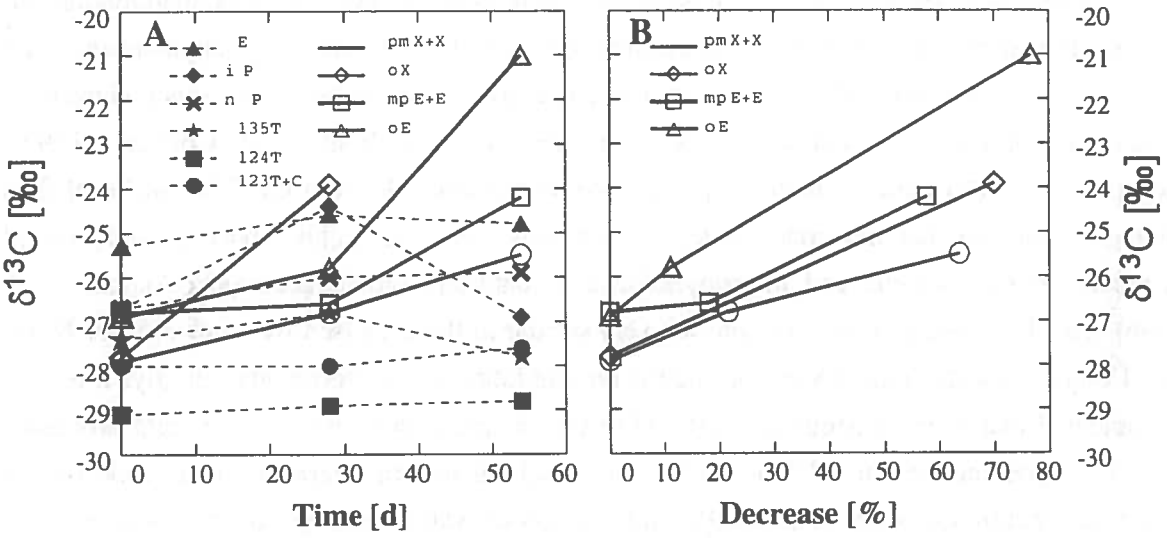

Fig. 5 $\delta^{13}C$ of selected alkylbenzenes in a North Sea crude oil during incubation with an enrichment culture of sulphate-reducing bacteria *versus* incubation time **A** and relative decrease **B**. For a definition of compound abbreviations see legend for Fig. 1; **C**, cymene.

Geochemical Implications. The findings presented in the previous section suggest that the carbon isotopic composition of alkylbenzenes might be a useful tracer of biogeochemical processes in natural environments. There is almost no information available from the literature on the carbon isotopic composition of alkylbenzenes. Dempster et al. (1997) described a method for the determination of $\delta^{13}C$ values of BTEX aromatics in water using GC-IRMS and also showed that the isotopic signature of BTEX aromatics supplied by different manufacturers show variations in the order of 0.5‰ to 2.5‰. They therefore suggested that compound-specific isotopic analysis might be a useful tool for tracing organic contaminants in groundwater. The strong isotopic enrichments observed for alkylbenzenes in the present experiments indicate that biodegradation (but probably also other alteration processes) have to be taken into account if $\delta^{13}C$ values are used in geochemical studies.

This study for the first time demonstrates the production of benzoic acids by sulphate-reducing bacteria during utilization of alkylbenzenes from crude oil. Several of these alkylbenzoic acids appear to be dead-end metabolites whose formation seems not to be of any

benefit for the bacteria. Benzoic acids occur in fossil fuel contaminated anoxic aquifers where they are assumed to be anaerobic oxidation products of alkylbenzenes (Cozzarelli et al. 1990, 1994, 1995; Schmitt et al. 1996). In contrast, benzoic acids have not yet been described to occur in oil field waters and other deep subsurface brines where short-chain aliphatic acids are ubiquitously present which are attributed to thermal and/or bacterial decomposition of sedimentary organic matter and crude oil (Barth et al. 1996; Borgund and Barth 1994; Means and Hubbard 1987). It is of interest that the short-chain aliphatic acids are particularly common in anaerobic brines which have not been flushed by recent meteoric waters (Means and Hubbard 1987). At the moment it remains unclear if benzoic acids occur in oil fields as a result of anaerobic biodegradation of crude oil. The present results from laboratory experiments, however, give strong evidence that they might be useful tracers of such processes.

This might particularly be the case in shallow reservoirs where initial biodegradation has only slightly altered the original oil composition. Selective loss of long-chain alkylbenzenes in a heavy oil from the Pécorade oil field in the Aquitaine Basin without any other significant molecular compositional alteration has been described by Blanc and Connan (1993). Comparison of alkylbenzene distributions in two reservoired oils from the Tchibouela oil field (Kongo) revealed that moderate biodegradation may result in highly specific depletion of *m*-xylene, *m*-ethyltoluene and *m*-propyltoluene in naturally biodegraded oil (Connan et al. 1996). The described pattern variations are very similar to those caused by incubation of North Sea 1 crude oil with strain mXyS1 during the present laboratory experiments. Finally, it is well documented that water washing of crude oil results in preferential removal of light aromatics (e.g. Lafargue and Le Thiez 1996). Both water washing and biodegradation of crude oils in reservoirs require oil-water contacts. Possible effects of water washing on oil composition on the one hand are mainly controlled by the physicochemical properties of individual oil constituents whereas possible effects of anaerobic biodegradation on the other hand are controlled by the substrate specificities of the involved bacteria. Highly specific differences of compositional changes going along with laboratory simulation of water washing (Lafargue and Le Thiez 1996) and anaerobic biodegradation (this study) of crude oils show that careful analysis of alkylbenzene distributions in reservoired oils may aid assessment of alteration processes in petroleum reservoirs.

Conclusions

Laboratory experiments have revealed that (1) alkylbenzenes may selectively be removed from crude oil by sulphate-reducing bacteria, (2) depletion of the alkylbenzenes goes along with a carbon isotopic enrichment of the target compounds and (3) alkylated benzoic acids are produced as the main metabolic byproducts detected in the water phase. It is probable that the same processes occur in natural environments such as fossil fuel contaminated soils and aquifers as well as shallow oil reservoirs under sulphate-reducing conditions. We therefore suggest that the variability of alkylbenzene concentrations and their carbon isotopic signature together with the occurrence of alkylated benzoic acids may be used as a specific indicator of

initial biodegradation of crude oils and fossil fuel products by sulphate-reducing bacteria in various environments.

Acknowledgements. The authors wish to thank U. Disko, F. J. Keller, W. Laumer, F. Leistner, A. Ropertz and H. Willsch for their expert technical contributions. Arco Exploration and Production Technology (Plano), Exxon Production Research Company (Houston), Saga Petroleum (Sandvika) and Total Exploration Production (Paris La Défense) provided oil samples for incubation experiments. This research was supported by the Max-Planck-Gesellschaft.

References

Aeckersberg F, Bak F, Widdel F 1991. Anaerobic oxidation of saturated hydrocarbons to CO_2 by a new type of sulfate-reducing bacterium. Arch. Microbiol. 156:5-14.

Aeckersberg F, Rainey FA, Widdel F 1998. Growth, natural relationships, cellular fatty acids and metabolic adaptation of sulfate-reducing bacteria that utilize long-chain alkanes under anoxic conditions. Arch. Microbiol. 170:361-369.

Barth T, Andresen B, Iden K, Johansen H. 1996. Modelling source rock production potentials for short-chain organic acids and CO_2 - a multivariate approach. Org. Geochem. 25:427-438.

Bastin ES, Greer FE, Merritt CA, Moulton G. 1926. The presence of sulphate reducing bacteria in oil field waters. Science 63:21-24.

Beeder J, Nilsen RK, Bosnes JT, Torsvik T; Lien T 1994. *Archaeoglobus fulgidus* isolated from hot north sea oil field waters. Appl. Environ. Microbiol. 60:1227-1231.

Beeder J, Nilsen RK, Thorstenson T, Torsvik T 1996. Penetration of sulfate reducers through a porous North Sea oil reservoir. Appl. Environ. Microbiol. 62:3551-3553.

Beller HR, Spormann AM 1997a. Anaerobic activation of toluene and *o*-xylene by addition to fumarate in denitrifying strain T. J. Bacteriol. 179:670-676.

Beller HR, Spormann AM 1997b. Benzylsuccinate formation as a means of anaerobic toluene activation by sulfate-reducing strain PRTOL1. Appl. Environ. Microbiol. 63:3729-3731.

Beller HR, Ding W-H, Reinhard M 1995. Byproducts of anaerobic alkylbenzene metabolism useful as indicators of in situ bioremediation. Environ. Sci. Technol. 29:2864-2870.

Beller HR, Reinhard M, Grbic-Galic D 1992. Metabolic by-products of anaerobic toluene degradation by sulfate-reducing enrichment cultures. Appl. Environ. Microbiol. 58:3192-3195.

Beller HR, Spormann M, Sharma PK, Cole JR, Reinhard M 1996. Isolation and characterization of a novel toluene-degrading, sulfate-reducing bacterium. Appl. Environ. Microbiol. 62:1188-1196.

Bernard FP, Connan J, Magot M 1992. Indigenous microorganisms in connate water of many oil fields: A new tool in exploration and production techniques. In Proc. 67th SPE Conf. Exhibition, Vol. 2, pp. 467-476. SPE.

Biegert T, Fuchs G, Heider J 1996. Evidence that anaerobic oxidation of toluene in the denitrifying bacterium *Thauera aromatica* is initiated by formation of benzylsuccinate from toluene and fumarate. Eur. J. Biochem. 238:661-668.

Blanc P, Connan J 1993. Crude oils in reservoirs: the factors influencing their composition. In Applied Petroleum Geochemistry (ed. M. L. Bordenave), pp. 151-174. Éditions Technip.

Bodrossy L, Murrell JC, Dalton H, Kalman M, Puskas LG, Kovacs KL 1995. Heat-tolerant methanotrophic bacteria from the hot water effluent of a natural gas field. Appl. Environ. Microbiol. 61:3549-3555.

Borgund AE, Barth T 1994. Generation of short-chain organic acids from crude oils by hydrous pyrolysis. Org. Geochem. 21:943-952.

Caldwell ME, Garrett RM, Prince RC, Suflita JM 1998. Anaerobic biodegradation of long-chain *n*-alkanes under sulfate-reducing condition. Environ. Sci. Technol. 32:2191-2195.

Chee-Sanford JC, Frost JW, Fries MR, Zhou J, Tiedje JM 1996. Evidence for acetyl coenzyme A and cinnamoyl coenzyme A in the anaerobic toluene mineralization pathway in *Azoarcus tolulyticus* Tol-4. Appl. Environ. Microbiol. 62:964-973.

Coates JD, Anderson RT, Lovley DR 1996a. Oxidation of polycyclic aromatic hydrocarbons under sulfate-reducing conditions. Appl. Environ. Microbiol. 62:1099-1101.

Coates JD, Anderson RT, Woodward JC, Phillips EJP, Lovley DR 1996b. Anaerobic hydrocarbon degradation in petroleum-contamianted harbor sediments under sulfate-reducing and artificially, imposed iron-reducing conditions. Environ. Sci. Technol. 30:2784-2789.

Coates JD, Woodward J, Allen J, Philp P, Lovley DR 1997. Anaerobic degradation of polycyclic aromatic hydrocarbons and alkanes in petroleum-contaminated harbor-sediments. Appl. Environ. Microbiol. 63:3589-3593.

Connan J. 1984. Biodegradation of crude oils in reservoirs. In Advances in Petroleum Geochemistry (eds. J. Brooks and D. H. Welte), pp. 299-330. Academic Press.

Connan J, Lacrampe-Coulome G, Magot M 1996. Origin of gases in reservoirs. In Proceedings of the 1995 International Gas Research Conference, Vol. 1 (ed. D. Dolenc), pp. 21-62. Government Institutes.

Cord-Ruwisch R, Kleinitz W,Widdel F 1986. Sulfatreduzierende Bakterien in einem Erdölfeld - Arten und Wachstumsbedingungen. Erdöl, Erdgas, Kohle 102:281-289.

Cozzarelli I, Baedecker MJ, Eganhouse RP, Goerltz DF 1994. The geochemical evolution of low-molecular-weight organic acids derived from the degradation of petroleum contaminants in groundwater. Geochim. Cosmochim. Acta 58:863-877.

Cozzarelli IM, Eganhouse RP, Baedecker MJ 1990. Transformation of monoaromatic hydrocarbons to organic acids in anoxic groundwater environment. Environ. Geol. Water Sci. 16:135-141.

Cozzarelli IM, Herman JS, Baedecker MJ 1995. Fate of microbial metabolites of hydrocarbons in a coastal plain aquifer: The role of electron acceptors. Environ. Sci. Technol. 29:458-469.

Davis JB, Yarbrough HF 1966. Anaerobic oxidation of hydrocarbons by *Desulfovibrio desulfuricans*. Chem. Geol. 1:137-144.

Dempster HS, Sherwood Lollar B, Feenstra S 1997. Tracing organic contaminants in groundwater: A new methodology using compound-specific isotope analysis. Environ. Sci. Technol. 31:3193-3197.

Edwards EA, Grbic-Galic D 1992. Complete mineralization of benzene by aquifer microorganisms under strictly anaerobic conditions. Appl. Environ. Microbiol. 58:2663-2666.

Evans PJ, Ling W, Goldschmidt B, Ritter ER, Young LY 1992. Metabolites formed during anaerobic transformation of toluene and o-xylene and their proposed relationship to the initial steps of toluene mineralization. Appl. Environ. Microbiol. 58:496-501.

Fisher SJ, Alexander R, Kagi RI, Oliver GA 1998. Aromatic hydrocarbons as indicators of biodegradation in north Western Australian reservoirs. In The Sedimentary Basins of Western Australia 2: Proceedings of Petroleum Exploration Society of Australia Symposium, Perth, WA, 1998 (eds P. G. Purcell and R. R. Purcell), pp. 185-194.

Harms G, Zengler K, Rabus R, Aeckersberg F, Minz D, Roselló-Mora R, Widdel F 1999. Anaerobic oxidation of *o*-xylene, *m*-xylene, and homologous alkylbenzenes by new types of sulfate-reducing bacteria. Appl. Environ. Microbiol. 65:999-1004

Hartgers WA, Sinninghe Damsté JS, de Leeuw JW 1992. The identification of C_2-C_4 alkylated benzenes in flash pyrolysates of kerogens, coals and asphaltenes. J. Chromatogr. 606:211-220.

Heider J, Fuchs G 1997a. Anaerobic metabolism of aromatic compounds. Eur. J. Biochem. 243:577-596.

Heider J, Fuchs G 1997. Microbial anaerobic metabolism. Anaerobe 3, 1-22.

Jobson AM, Cook FD, Westlake DWS 1979. Interaction of aerobic and anaerobic bacteria in petroleum biodegradation. Chem. Geol. 24:355-365.

Kazumi J, Caldwell ME, Suflita JM, Lovley DR, Young LY 1997. Anaerobic degradation of benzene, in diverse anoxic environments. Environ. Sci. Technol. 31:813-818.

Lafargue E, le Thiez P 1996. Effect of waterwashing on light ends compositional heterogeneity. Org. Geochem. 24:1141-1150.

L'Haridon S, Reysenbach A-L, Glénat P, Prieur D, Jeanthon C 1995. Hot subterranean biosphere in a continental oil reservoir. Nature (London) (London) 377:223-224.

Lovley DR, Coates JD, Woodward JC, Philipps EJP 1995. Benzene oxidation coupled to sulfate reduction. Appl. Environ. Microbiol. 61:953-958.

Mariotti A, Germon JJ, Hubert P, Kaiser P, Letolle R, Tardieux A, Tardieux, P 1981. Experimental determination of nitrogen kinetic isotope fractionation: illustration for the denitrification and nitrification processes. Plant and Soil 62:413-430.

Means JL, Hubbard N 1987. Short-chain aliphatic acid anions in deep subsurface brines: A review of their origin, occurrence, properties, and importance and new data on their distribtuion and geochemical implications in the Palo Duro Basin, Texas. Org. Geochem. 11:177-191.

Migaud ME, Chee-Sanford JC, Frost J W 1996. Benzylfumaric, benzylmaleic, and Z- and E-phenylitaconic acids: Synthesis, characterization, and correlation with a metabolite generated by *Azoarcus tolulyticus* Tol-4 during anaerobic toluene degaradation. Appl. Environ. Microbiol. 62:974-978.

Mueller RF, Nielsen PH 1996. Characterization of thermophilic consortia from two souring oil reservoirs. Appl. Environ. Microbiol. 62:3083-3087.

Nazina TN, Rozanova EP, Kuznetsov SI 1985. Microbial oil transformation processes accompanied by methane and hydrogen sulfide formation. Geomicrobiol. J. 4:103-130.

Nilsen RK, Torsvik T 1996. *Methanococcus thermolithotrophicus* isolated from North sea oil field reservoir water. Appl. Environ. Microbiol. 62:728-731.

Nilsen R.K, Beeder J, Thorstensen T, Torsvik T 1996. Distribution of thermophilic marine sulfate reducers in North Sea oil field waters and oil reservoirs. Appl. Environ. Microbiol. 62:1793-1798.

Novelli GD, ZoBell CE 1944. Assimilation of petroleum hydrocarbons by sulfate-reducing bacteria. J. Bacteriol. 47:447-448.

Palmer SE 1993. Effect of biodegradation and water washing on crude oil composition. In Organic Geochemistry (eds. M. H. Engel and S. A. Macko), pp. 511-533. Plenum Press.

Peters K. E. and Moldowan J. M. 1993 The Biomarker Guide. Prentice Hall.

Rabus R, Heider J 1998. Initial reactions of anaerobic metabolism of alkylbenzenes in denitrifying and sulfate-reducing bacteria. Arch. Microbiol. 170:377-384.

Rabus R, Widdel F 1995. Conversion studies with substrate analogues of toluene in a sulfate-reducing bacterium, strain Tol2. Arch. Microbiol. 164:448-451.

Rabus R, Widdel F 1996. Utilization of alkylbenzenes during anaerobic growth of pure cultures of dentirifying bacteria on crude oil. Appl. Environ. Microbiol. 62:1238-1241.

Rabus R, Fukui M, Wilkes H, Widdel F 1996. Degradative capacities and 16S rRNA-targeted whole-cell hybridization of sulfate-reducing bacteria in an anaerobic enrichment culture utilizing alkylbenzenes from crude oil. Appl. Environ. Microbiol. 62:3605-3613.

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

Radke M, Willsch H, Welte DH 1980. Preparative hydrocarbon group determination by automated medium pressure liquid chromatography. Anal. Chem. 52:406-411.

Reinhard M, Shang S, Kitanidis PK, Orwin E, Hopkins GD, Lebron CA 1997. In situ BTEX biotransformation under enhanced nitrate- and sulfate-reducing conditions. Environ. Sci. Technol. 31:28-36.

Rosenfeld WD 1947. Anaerobic oxidation of hydrocarbons by sulfate-reducing bacteria. J. Bacteriol. 54:664-665.

Rosnes JT, Torsvik T, Lien T 1991. Spore-forming thermophilic sulfate-reducing bacteria isolated from North Sea oil field waters. Appl. Environ. Microbiol. 57:2302-2307.

Rozanova EP, Galushko AS, Ivanova AE 1991. Distribution of sulfate-reducing bacteria utilizing lactate and fatty acids in anaerobic ecotopes of flooded petroleum reservoirs. Microbiology 60:251-256.

Rueter P, Rabus R, Wilkes H, Aeckersberg F, Rainey FA, Jannasch HW, Widdel F 1994. Anaerobic oxidation of hydrocarbons in crude oil by new types of sulphate-reducing bacteria. Nature (London) 372:455-458.

Schenk HJ, di Primio R, Horsfield B 1997. The conversion of oil into gas in petroleum reservoirs. Part 1: Comparative kinetic investigation of gas generation from crude oils of lacustrine, marine and fluviodeltaic origin by programmed-temperature closed-system pyrolysis. Org. Geochem. 26:467-481.

Schmitt R, Langguth H-R, Püttmann W, Rohns HP, Eckert P, Schubert J 1996. Biodegradation of aromatic hydrocarbons under anoxic conditions in a shallow sand and gravel aquifer of the Lower Rhine Valley, Germany. Org. Geochem. 25:41-50.

Scott PJB, Davies M 1993. Souring of new Irian Jaya wells traced to indigenous bacteria. Oil & Gas J.: 47-50.

Seyfried B, Glod G, Schocher R, Tschech A, Zeyer J 1994. Initial reactions in the anaerobic oxidation of toluene and m-xylene by denitrifying bacteria. Appl. Environ. Microbiol. 60, 4047-4052.

Stetter KO, Huber R, Blöchl E, Kurr M, Eden RD, Fielder M, Cash H, Vance I 1993. Hyperthermophilic archaea are thriving in deep North Sea and Alaskan oil reservoirs. Nature (London) 365:743-745.

Thierrin J, Davis GB, Barber C, Patterson BM, Pribac F, Power TR, Lambert M 1993. Natural degradation rates of BTEX compounds and naphthalene in a sulfate-reducing ground water environment. Hydrol. Sci. J. 38:309-332.

Volkman JK, Alexander R, Kagi RI, Rowland SJ, Sheppard PN 1984. Biodegradation of aromatic hydrocarbons in crude oils from the Barrow Sub-basin of Western Australia. Org. Geochem. 6:619-632.

Widdel F, Bak F 1992. Gram-negative mesophilic sulfate-reducing bacteria. In The Prokaryotes, 2nd ed, vol. 4 (eds. A. Balows, H. G. Trüper, M. Dworkin, W. Harder, and K. H. Schleifer), pp. 3352-3378. Springer-Verlag.

Wilkes H, Willsch H, Rabus R, Aeckersberg F, Rueter P, Widdel F 1995. Compositional changes of crude oils upon anaerobic degradation by sulphate-reducing bacteria. In Organic Geochemistry: Developments and Applications to Energy, Climate, Environment and Human History (eds. J. O. Grimalt and C. Dorronsoro), pp. 321-323. A.I.G.O.A.

Zhang X, Young LY 1997. Carboxylation as an initial reaction in the anaerobic metabolism of naphthalene and phenanthrene by sulfidogenic consortia. Appl. Environ. Microbiol. 63:4759-4764.

C Weitere Publikationen

1 **Anaerobic degradation of ethylbenzene and toluene in denitrifying strain EbN1 proceeds via independent substrate-induced pathways**

Kathleen M. Champion, Karsten Zengler, and Ralf Rabus

Journal of Molecular Microbiology and Biotechnology, 1: 157-164 (1999)

Danksagung

Zuerst möchte ich mich bei Herrn Prof. Fritz Widdel für die Betreuung der Arbeit und den vielen anregenden Diskussionen bedanken. Sätze wie „jetzt paßt es gerade nicht" scheinen nicht zu seinem aktiven Wortschatz zu gehören.

Herrn Prof. Ulrich Fischer gilt mein Dank für die bereitwillige Übernahme des Zweitgutachtens.

Ein großer Dank gebührt auch all den Mitarbeitern inner- und außerhalb des Instituts durch die diese Arbeit erst ermöglicht wurde. Hier seien besonders die beiden Molekular-Mallorquiner Enric Llobet-Brossa und Ramon Rosselló-Mora (venga-venga!) erwähnt. Aber auch Hans Hermann Richnow von der Universität Hamburg gilt mein besonderer Dank für die Isotopen-Analysen. Hans Heider danke ich für seine große Hilfsbereitschaft („man kann sich das Leben auch schwer machen") während meines Aufenthalts in Freiburg.

Der Abteilung Mikrobiologie des MPIs gilt ebenfalls ein großer Dank. Besonders möchte ich mich bei Olaf Ellenbogen-, Pardon, Kniemeyer, Jens Harder (war doch kein Firlefanz) und Martin Könneke (die Heide brennt) für die nette Zeit bedanken (was wäre ein Leben ohne Lachen). Des weiteren gilt mein Dank dem Komma-König und Udo Heyen für das Korrekturlesen der Arbeit.

Zuletzt möchte ich mich noch bei allen Teilnehmern der „Black Sea Cruise" und der „Alvin-Atlantis-Cruise" bedanken. Erst durch sie sind diese Ausfahrten zu einem unvergeßlichen Erlebnis für mich geworden.