Untersuchung der zellularen Fettsäuren von sulfatreduzierenden Bakterien aus kalten, marinen Sedimenten

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Abkürzungen

A	Arrheniuskonstante
ACP	Acyl carrier protein
AVS	Acid-volatile sulfide
C-irm MS	Combastion interfaced isotope ratio mass spectrometry
CFA	Cyclopropanfettsäure(n)
d	Tag
D	Diffusionskoeffizient
DAPI	4´,6-Diamidino-2-phenylindol
DGGE	Denaturing gradient gel electrophoresis
DSMZ	Deutsche Sammlung von Mikroorganismen und Zellkulturen
dwt	dry weight
eV	Elektronenvolt
E_a	Aktivierungsenergie
FAME	Fettsäuremethylester
FID	Flammenionisationsdetektor
FISH	Fluoreszenz-*in situ*-Hybridisierung
GC	Gaschromatographie
GC-C-IRMS	Gaschromatography combustion interfaced isotope ratio mass spectrometry
GC/MS	Gaschromatographie/Massenspektroskopie
h	Stunde
HPLC	High performance liquid chromatography
ICBM	Institut für Chemie und Biologie des Meeres
ICP-OES	Inductively coupled plasma optical emission spectroscopy
kPa	Kilopascal
MPN	most-probable number
MS	Massenspektroskopie
m/z	Masse/Ladung
OD	Optische Dichte
PCR	Polymerase chain reaction
PLFA	Phospholipidfettsäuren

R	Allgemeine Gaskonstante = 8,31 $JK^{-1}mol^{-1}$
SRB	Sulfatreduzierende Bakterien
SRR	Sulfatreduktionsraten
T	Absolute Temperatur
T_{max}	Maximale Wachstumstemperatur
T_{min}	Minimale Wachstumstemperatur
T_{opt}	Optimale Wachstumstemperatur
TOC	Total organic carbon
V-CDT	Vienna Canyon Diablo Troilit
VFA	Volatile fatty acids
v	Reaktionsgeschwindigkeit oder Wachstumsrate

Zusammenfassung

Im Mittelpunkt der vorliegenden Dissertation standen Analysen der zellularen Fettsäurenzusammensetzung von verschiedenen sulfatreduzierenden Bakterien (SRB) aus marinen Habitaten. In vergleichenden Analysen wurde erstmals der Einfluß der Wachstumstemperatur und der Wachstumsphase auf die zellularen Fettsäurenzusammensetzungen von verschiedenen SRB in Reinkulturen untersucht. Ferner wurden *in situ* Analysen der Phospholipide an marinen Sedimenten der Arktis (Svalbard) und des Wattenmeers (Deutschland) durchgeführt, um durch den Nachweis von Biomarkern Hinweise über die Häufigkeit und die Verteilung unterschiedlicher Mikroorganismengruppen zu erhalten. Die Bestimmung der Lebendzellzahlen verschiedener stoffwechselphysiologischer Bakterien in Sedimenten aus Svalbard wurde mit der selektiven Isolierung von neuen Mikroorganismen in Reinkulturen bei konstant niedrigen Temperaturen verbunden.

1. Erstmalig konnte gezeigt werden, daß Angehörige der Gattung *Desulfobacter* die Fähigkeit besitzen, ihre Fettsäurenzusammensetzung an wechselnde Temperaturen anzupassen. Dadurch regulieren sie die temperaturabhängige Fluidität ihrer Lipidmembranen. Bei niedrigen Temperaturen (4–12 °C) besitzt *Desulfobacter hydrogenophilus*, wie die meisten psychrophilen oder moderat psychrophilen SRB, einen konstant hohen Anteil an fluiditäterhöhenden, *cis*-ungesättigten Fettsäuren. Dieser Anteil verringert sich bei der Erhöhung der Wachstumstemperatur auf Werte, wie sie in anderen mesophilen SRB gefunden wurden. Durch wachstumsabhängige Untersuchungen mit ^{13}C-markiertem Substrat wurde gezeigt, daß diese Temperaturanpassung durch *de novo* Synthese der zellularen Fettsäuren stattfindet. Bei niedrigen Temperaturen konnte neben einer Neusynthese von ungesättigten Fettsäuren, eine Inhibierung der Synthese von *cis*-9,10-Methylenhexadecansäure (cyc17:0) und 10-Methyl-hexadecansäure (10Me16:0) nachgewiesen werden.

2. Ein sulfatreduzierendes Bakterium, das aus marinem Küstensediment der Ostsee isoliert wurde, stellt hinsichtlich der 16S rRNA Gensequenz, der Morphologie und des zellularen Fettsäuremusters eine neue Gattung innerhalb der δ-Proteobakterien dar. Als Bezeichnung für den vollständig-oxidierenden Typenstamm wurde *Desulfotignum balticum* vorgeschlagen. Aufgrund der großen Ähnlichkeit der 16S rRNA Gensequenz, der Morphologie, der Physiologie und der Fettsäuremuster in *Desulfobacterium phenolicum* und *Desulfobacula*

toluolica wurden beide Stämme in der Gattung *Desulfobacula* vereinigt. Als neue Bezeichnung für *D. phenolicum* wurde *Desulfobacula phenolica* vorgeschlagen. In *Desulfobacula* und *Desulfotignum* konnte die für *Desulfobacter* Arten als spezifisch geltende Fettsäure 10Me16:0 nachgewiesen werden. Dieser Befund widerlegt die oft beschriebene Spezifität dieser Fettsäure als Biomarker für Angehörige der Gattung *Desulfobacter* in marinen Sedimenten.

3. Die *in situ* Analyse der Phospholipidfettsäuren in permanent kalten, marinen Sedimenten (Svalbard) zeigte ein für marines Sediment typisches Tiefenprofil. Durch den Nachweis spezifischer Fettsäuren konnte ein Wechsel einer von aeroben Mikroorganismen dominierten oberen Sedimentschicht zu einer von anaeroben Bakterien dominierten tieferen Schicht gezeigt werden. Die für SRB spezifischen Biomarker i17:1 und 10Me16:0 konnten nur in geringen Konzentrationen nachgewiesen werden und zeigten maximale Werte in der obersten Sedimentschicht. Die Phospholipidfettsäuren-Profile waren in guter Übereinstimmung mit der Tiefenverteilung von kultivierbaren aeroben Bakterien und SRB, die über die most-probable number (MPN) Methode ermittelt wurden. Aus den Verdünnungsserien konnten verschiedene Reinkulturen isoliert werden, die eine enge Verwandtschaft zu bekannten psychrophilen Bakterien zeigten. Drei sulfatreduzierende Reinkulturen, denen Wasserstoff als Elektronendonator diente, stellen aufgrund ihrer 16S rRNA Gensequenzen neue Gattungen innerhalb der δ-Proteobakterien dar. Die geringen Konzentrationen der für *Desulfovibrio* spezifischen Biomarker und deren Abwesenheit in den höchsten MPN Verdünnungsreihen weisen auf eine nur untergeordnete ökologische Bedeutung dieser Gattung *Desulfovibrio* in diesem kalten Habitat hin.

4. Aus permanent kaltem, marinem Sediment (Svalbard) konnten mit Methanol oder Trimethylamin methanogene Archaea selektiv bei niedrigen Temperaturen in Reinkultur isoliert werden. Zwei der sieben Isolate zeigen aufgrund ihrer 16S rRNA-Gensequenzen und ihrer Morphologie große Ähnlichkeiten zu den methylotrophen Archaea *Methanolobus taylorii* bzw. *Methanococcoides burtonii*. Die isolierten Stämme verwerten wie ihre nahen Verwandten lediglich Methanol und Trimethylamin. Die Reinkulturen sind die ersten beschriebenen Methanogenen, die aus permanent kalten, marinen Sedimenten der Arktis isoliert wurden.

Teil I: Darstellung der Ergebnisse im Gesamtzusammenhang

A Einleitung

1. Mikrobieller Abbau von organischem Material in marinen Sedimenten

In den Meeren findet die Primärproduktion von organischem Material durch phototrophe Organismen in den oberflächennahen Bereichen der Wassersäule statt, in denen ausreichend Licht für die Photosynthese eindringt. Während das für die Kohlenstofffixierung notwendige Kohlendioxid im Meerwasser in gelöster Form im Überschuß enthalten ist, limitiert meistens die Verfügbarkeit der Elemente Stickstoff, Phosphor und Eisen die Bildung von Biomasse (Sommer, 1998; Smetacek, 1998). Das in den euphotischen Zonen gebildete organische Material dient heterotrophen Organismen als Energie- und Kohlenstoffquelle und wird von diesen in unterschiedlichem Umfang beim Herabsinken in der Wassersäule abgebaut. Der wichtigste Elektronenakzeptor ist dabei Sauerstoff. Nur ein Teil des organischen Materials sedimentiert auf den Meeresboden und wird anschließend in den Sedimenten unter oxischen und anoxischen Bedingungen überwiegend durch Mikroorganismen remineralisiert (Jørgensen, 1983). Der Transport des in der Wassersäule nur in geringen Konzentrationen gelösten Sauerstoffs in die Sedimente verläuft überwiegend durch Diffusion. So ist in Schelfsedimenten die Verfügbarkeit von Sauerstoff infolge der Atmungsaktivität aerober Organismen und durch die chemische (abiotische) Reaktion mit Sulfid auf die oberste, nur wenige Millimeter starke Sedimentschicht begrenzt (Revsbech et al., 1980; Jørgensen, 1982). In den darunterliegenden anoxischen Sedimenten erfolgt der Abbau des organischen Materials schrittweise durch anaerobe Mikroorganismen unter Verwendung verschiedener terminaler Elektronenakzeptoren (Abb. 1). Die in diesen Schichten noch enthaltenen, komplexen organischen Polymere werden zuerst durch fermentierende Bakterien abgebaut. Die Endoxidation der Fermentationsprodukte findet durch Bakterien über die Reduktion von Nitrat, Eisen(III), Mangan(IV) und Sulfat statt (Canfield, 1993). Aufgrund der hohen Konzentration von Sulfat im Vergleich zu der von Nitrat und die an vielen Orten eingeschränkte Verwertbarkeit von schwerlöslichem Eisen(III) stellt die dissimilatorische Sulfatreduktion den wichtigsten anaeroben Prozeß für die Mineralisation von organischem Kohlenstoff in marinen Sedimenten dar (Jørgensen, 1982). Die methanogenen Archaea spielen infolge der Konkurrenz um Abbauprodukte der fermentierenden und acetogenen Bakterien (z.B. Acetat, Formiat, Wasserstoff) mit den energetisch begünstigten SRB in den sulfatreichen marinen Sedimentschichten eine untergeordnete Rolle. Ausnahmen bilden

Bereiche, in denen durch die Umsetzung großer Mengen an schnell abbaubarem Material (z.B. faulendes Pflanzenmaterial) die Sulfatverfügbarkeit limitiert ist. Einige methanogene Archaea können Methylgruppen enthaltende Verbindungen (Methanol, Methylamine oder Methylsulfide) verwerten, die von den SRB nicht oder nur sehr langsam als Substrat genutzt werden können. Dadurch können die methylotrophen methanogenen Archaeen in marinen Sedimenten mit den SRB koexistieren (Oremland et al., 1982; Zinder, 1993).

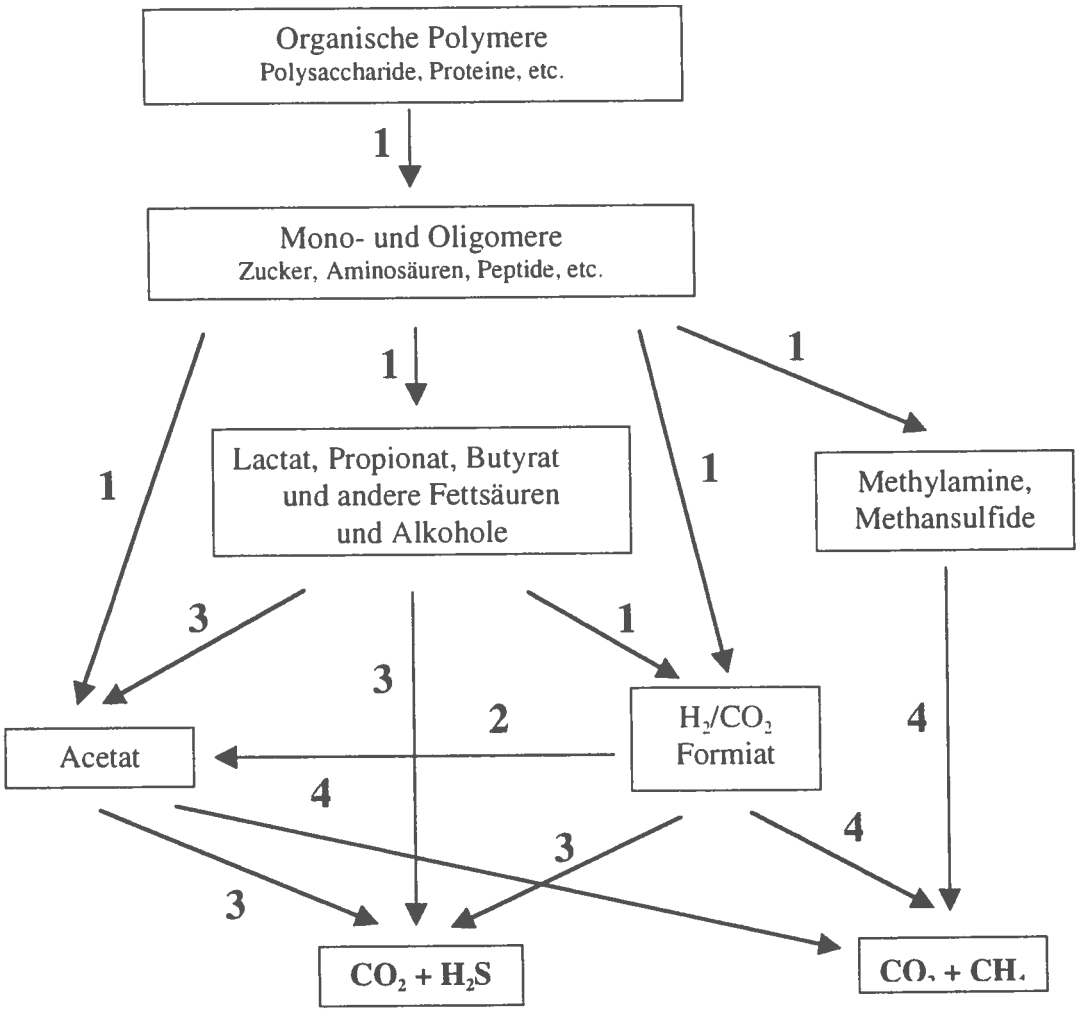


Abbildung 1: Vereinfachter Kohlenstoffffluß des anaeroben mikrobiellen Abbaus von organischem Material in marinen Sedimenten: (1) Fermentative Bakterien; (2) Homoacetogene Bakterien; (3) Sulfatreduzierende Bakterien; (4) Methanogene Bakterien.

2. SRB in marinen Sedimenten

SRB bilden innerhalb der Prokaryoten eine heterogene Organismengruppe, die die Fähigkeit besitzt, über Elektronentransport während der dissimilatorischen Reduktion von Sulfat zu Sulfid Energie zu konservieren. Sie sind in der Lage eine Vielzahl von niedermolekularen

organischen Verbindungen und auch Wasserstoff unter anoxischen Bedingungen als Elektronendonatoren zu nutzen (Rabus et al., 2000). In anoxischen marinen Habitaten, in denen Sulfat aufgrund der hohen gelösten Konzentration im Meerwasser (28 mM) meistens keinen limitierenden Faktor darstellt, wurden Acetat, Propionat und Butyrat sowie Wasserstoff als die wichtigsten Substrate von SRB bestimmt (Sørensen et al., 1981; Balba und Nedwell, 1982; Boschker et al., 2001).

Das Wachstum von SRB ist nach bisherigen Erkenntnissen an anoxische Bedingungen gebunden. Jedoch wurden wiederholt hohe Zahlen SRB und hohe Sulfatreduktionsraten (SRR) auch in oxischen Zonen von marinen Sedimenten und mikrobiellen Matten nachgewiesen (Teske et al., 1996, 1998; Wierenga et al., 2000), wofür es noch keine vollständige Erklärung gibt. In Reinkulturen der Gattung *Desulfovibrio* wurde die Reduktion von Sauerstoff zu Wasser festgestellt, jedoch konnte dabei nur ein sehr geringes oder gar kein Wachstum beobachtet werden. Es wird vermutet, daß diese aerobe Atmung eine Schutzfunktion gegen Sauerstoff für die dazu befähigten SRB darstellt (Cypionka, 2000).

Ein wichtiger Faktor, der alle biologischen und chemischen Reaktionen in einem Habitat beeinflußt, ist die Temperatur. Durch *in situ* Messungen konnte eine mikrobielle Sulfatreduktion in polaren marinen Sedimenten unterhalb von 0 °C (Sagemann et al., 1998) und in von geothermischen Quellen erhitzten Sedimenten oberhalb von 100 °C (Jørgensen et al., 1992) gemessen werden. Küstennahe Sedimente innerhalb der gemäßigten Breiten unterliegen jahreszeitlichen Temperaturschwankungen, die die Aktivität der dort vorkommenden mikrobiellen Gemeinschaften beeinflussen. Die Sulfatreduktionsraten liegen daher in den Wintermonaten unterhalb der Werte des wärmeren Sommers (Jørgensen, 1977; Abdollahi, 1979). Generell konnte in Inkubationsversuchen mit marinen Sedimenten verschiedener Herkunft bei unterschiedlichen Temperaturen gezeigt werden, daß die optimalen Temperaturen für die höchsten Sulfatreduktionsraten um etwa 20 °C oberhalb der *in situ* -Temperaturen der jeweiligen Sedimente liegen (Nedwell, 1989; Isaksen und Jørgensen, 1996; Sagemann et al., 1998). Im größten Teil des Meeresvolumens (90 %) herrschen permanent niedrige Temperaturen (≤ 5 °C) (Morita, 1975), da in den offenen Ozeanen nur ein sehr langsamer Austausch zwischen dem kalten Tiefenwasser und dem hauptsächlich von der Sonnenstrahlung erwärmten Oberflächenwasser stattfindet. Weitere permanent kalte, marine Habitate befinden sich in den Polarregionen, die etwa 14 % der Erdoberfläche einnehmen. Aufgrund der im Vergleich zu Sedimenten gemäßigter Breiten niedrigeren Temperaturoptima (18-28 °C) der Sulfatreduktion in der Arktis und Antarktis

vermutet man hier das Vorherrschen einer an die Kälte angepaßten mikrobiellen Lebensgemeinschaft (Isaksen und Jørgensen, 1996; Sagemann et al., 1998).

3. Psychrophile und psychrotolerante SRB

Obwohl die Sulfatreduktion in marinen Sedimenten der Polarregionen für bis zu 42 % des dortigen Abbaus von organischem Material verantwortlich ist (Glud et al., 1998), sind bisher nur wenige SRB bekannt, die noch bei einer Temperatur von ≤ 4 °C wachsen. Das erste sulfatreduzierende Bakterium, das noch bei 0 °C Wachstum zeigt, war *Desulfobacter hydrogenophilus*, eine vollständig-oxidierende Art, die aus marinem Schlamm des Mittelmeers isoliert wurde (Widdel, 1987). *D. hydrogenophilus* wächst optimal bei 28-30 °C und kann daher als psychrotolerant eingeordnet werden. Ein weiterer als moderat psychrophil eingeordneter Stamm wurde selektiv bei 10 °C aus der Ostsee isoliert (Isaksen und Teske, 1996). Dieses unvollständig-oxidierende, sulfatreduzierende Bakterium, *Desulforhopalus vacuolatus*, zeigt ebenfalls Wachstum bei 0 °C und besitzt eine optimale Wachstumstemperatur von 19 °C, wohingegen kein Wachstum oberhalb von 24 °C stattfindet. Erst 1999 gelang es Knoblauch und Mitarbeitern, obligat psychrophile SRB aus permanent kalten, marinen Sedimenten vor der Küste Svalbards zu isolieren (Knoblauch et al., 1999). Fünf neue Arten von SRB wurden mit bekannten Fermentationsprodukten (Acetat, Propionat, Lactat) in Reinkulturen isoliert. Organische Substrate werden unvollständig zu Acetat (*Desulfofaba gelida, Desulfofrigus fragile, Desulfotalea psychrophila* und *Desulfotalea arctica*) oder vollständig zu Kohlendioxid (*Desulfofrigus oceanense*) umgesetzt. Die große Abundanz dieser und noch anderer, bisher nicht kultivierter SRB in den arktischen Sedimenten wurde auch durch molekularbiologische Methoden nachgewiesen (Ravenschlag, 2000). Daneben gibt es nur sehr wenige Studien, in denen versucht wurde, SRB selektiv bei niedrigen Temperaturen zu isolieren. Lediglich drei Süßwasserisolate sind bisher bekannt, die ebenfalls noch bei niedrigen Temperaturen (4 °C) wachsen. Sie gehören alle der Gattung *Desulfovibrio* an (Sass et al., 1998; Montamedi und Petersen, 1998).

Noch geringer ist die Anzahl von bekannten psychrophilen methanogenen Archaea. In den letzten Jahren haben jedoch Studien mittels biochemischer und molekularbiologischer Methoden gezeigt, daß Archaea auch in kalten, marinen Standorten häufig vorkommen (DeLong et al., 1994, 1999; Sahm und Berninger, 1998; Boetius, 2000). Bisher ist es einzig Franzmann und Mitarbeitern gelungen, eine moderat psychrophile und eine psychrophile methanogene Reinkultur aus der Antarktis zu isolieren. Dabei handelt es sich um

Methanococcoides burtonii (T_{opt} = 23 °C) bzw. *Methanogenium frigidum* (T_{opt} = 15 °C). Sie wurden aus methanhaltigem, anoxischem Wasser des Ace Lake isoliert, das eine konstant niedrige Temperatur von 1-2 °C besitzt (Franzmann et al., 1992, 1997).

Tabelle 1: Psychrophile und psychrotolerante SRB und ihre beschriebenen Wachstumstemperaturbereiche

Stamm	Wachstumstemperatur (°C)		Habitat	Literaturverweis
	Bereich	Optimum		
Desulfobacter hydrogenophilus	0-32	28-30	Mariner Schlamm	Widdel, 1987
Desulforhopalus vacuolatus	0-24	19	Marines Sediment	Isaksen et al., 1996
Desulfovibrio aspoeensis	4-35	25-30	Grundwasser	Motamedi et al., 1998
Desulfovibrio cuneatus	0-33	28	Süßwassersee	Sass et al., 1998
Desulfovibrio litoralis	0-33	28	Süßwassersee	Sass et al., 1998
Desulfofaba gelida	-1,7-10	7	Marines Sediment	Knoblauch et al., 1999
Desulfofrigus fragile	-1,7-27	18	Marines Sediment	Knoblauch et al., 1999
Desulfofrigus oceanense	-1,7-16	10	Marines Sediment	Knoblauch et al., 1999
Desulfotalea arctica	-1,7-26	26	Marines Sediment	Knoblauch et al., 1999
Desulfotalea psychrophila	-1,7-19	10	Marines Sediment	Knoblauch et al., 1999

4. Physiologische Merkmale psychrophiler SRB

Bakterien können anhand von Temperaturkardinalpunkten (Temperaturbereich, in dem Wachstum erfolgt sowie optimale Wachstumstemperatur) in Gruppen eingeordnet werden. In der vorliegenden Arbeit wurde in Anlehnung an die Definition von Wiegel (1990) die in Tabelle 2 dargestellte Einteilung verwendet. Der Begriff "temperaturtolerante Mesophile", der als synonyme Bezeichnung zu "psychrotroph" verwendet wird, wurde durch den Begriff "psychrotolerant" ersetzt. Weiterhin wurde die Einteilung durch den Begriff "moderat psychrophil" ergänzt.

Tabelle 2: Einteilung von Bakterien hinsichtlich ihrer Temperaturanpassung aufgrund von Temperaturkardinalpunkten (in °C) (nach Wiegel, 1990, modifiziert).

	T_{min}	T_{opt}	T_{max}
Psychrophile Bakterien	≤ 0	< 15	< 20
Moderat psychrophile Bakterien	≤ 0	< 20	≈ 20
Psychrotolerante Bakterien	< 5	> 15	> 20
Mesophile Bakterien	> 5	< 45	< 50

Die optimale Wachstumstemperatur ist definiert als die Temperatur, bei der ein Organismus seine höchste Wachstumsrate besitzt. Die Beziehung zwischen der Wachstumsrate und der Temperatur wird häufig durch die Arrheniusgleichung beschrieben, die ursprünglich den Zusammenhang zwischen der Reaktionsgeschwindigkeit und der Temperatur bei chemischen Reaktionen darstellt:

$$v = A \cdot e^{-Ea/RT}$$

(v = Reaktionsgeschwindigkeit oder Wachstumsrate; A = Arrheniuskonstante; E_a = Aktivierungsenergie; R = Allgemeine Gaskonstante; T = absolute Temperatur)

Graphisch wird häufig der Logarithmus der Wachstumsrate als Funktion der reziproken absoluten Temperatur dargestellt.

$$\ln v = \ln A - \frac{E_a}{R} \cdot \frac{1}{T}$$

E_a aus diesem sogenannten "Arrheniusgraphen" stellt bei der Anwendung auf chemische Reaktionen die Aktivierungsenergie dar. In der Anwendung auf das Wachstum von Bakterien ist E_a, sofern der Wert im suboptimalen Temperaturbereich bestimmt wird, zwar eine organismenspezifische Größe, korreliert jedoch zu keiner physiologischen oder biochemisch definierbaren Eigenschaft. An verschiedenen Bakterien wurde gezeigt, daß sich der Wert von E_a für einen Stamm ändert, wenn die Zellen bei verschiedenen Temperaturen vorinkubiert werden, d.h. die Kulturen akklimatisierten sich an die Temperaturen (Reichert und Morita, 1982).

SRB, die aus kaltem arktischem Sediment isoliert wurden (Knoblauch et al., 1999), sowie *Desulforhopalus vacuolatus* besitzen ihre höchsten Wachstumsraten unterhalb von 20 °C und wurden somit als psychrophil bzw. moderat psychrophil eingeordnet. Jedoch lagen die optimalen Temperaturen ihrer Sulfatreduktionsraten um 2-9 °C über den optimalen Wachstumstemperaturen (Isaksen und Jørgensen, 1996; Knoblauch und Jørgensen, 1999). Die Wachstumsraten von Bakterien ergeben sich aus vielen geschwindigkeitsbestimmenden Mechanismen auf der Ebene biochemischer Reaktionen. Das Temperaturoptimum der Wachstumsrate fällt häufig nicht mit dem Temperaturoptimum einzelner enzymatischer Reaktionen zusammen (Feller et al., 1994).

Eine weitere physiologische Größe, die temperaturabhängig sein kann, ist der Wachstumsertrag. Der Wachstumsertrag ist die Biomasse, die pro umgesetzter Menge

Substrat von einem Organismus gebildet wird. Der Wachstumsertrag wird üblicherweise in der Einheit g Trockenmasse pro mol Substrat dargestellt. In den psychrophilen und moderat psychrophilen SRB sowie in den psychrotoleranten Süßwasserisolaten *D. cuneatus* und *D. litoralis* lagen die Temperaturen, bei denen der höchste Wachstumsertrag ermittelt wurde, unterhalb der jeweiligen optimalen Wachstumstemperaturen (Knoblauch und Jørgensen, 1999; Isaksen und Jørgensen, 1996; Sass et al., 1998). Die unterschiedlichen Kurvenverläufe des Wachstumsertrags in Abhängigkeit von der Temperatur einzelner SRB lassen jedoch kein generelles Prinzip erkennen.

5. Zellulare Fettsäuren von SRB

In Bakterien, die keine Neutralfette in Form von Trigylceriden enthalten, findet man zellulare Fettsäuren in den Lipiden der Cytoplasmamembran sowie in Gram-negativen Bakterien zusätzlich in geringeren Anteilen in den Lipopolysacchariden der äußeren Membran. Die Zusammensetzung dieser Fettsäuren bietet häufig ein Gattungs- oder sogar Art-spezifisches, chemotaxonomisches Merkmal, das bei der Einordnung und der Identifizierung von Reinkulturen oder bei der *in situ* Identifizierung von Bakterien hilfreich sein kann. Typische bakterielle Fettsäuren, ihre chemische Struktur und die in dieser Arbeit verwendeten Kurzbezeichnungen sind in Tabelle 3 dargestellt.

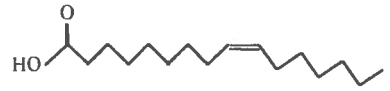
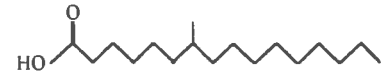
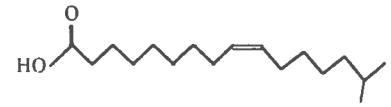
Die phylogenetische Einteilung von Bakterien beruht überwiegend auf vergleichenden Analysen der 16S rRNA Gensequenzen (Ludwig und Klenk, 2001). Innerhalb der SRB decken sich die über zellulare Fettsäuremuster ermittelten chemotaxonomischen Gruppen häufig mit den phylogenetischen Gruppen. Charakteristisch für viele SRB sind die oft hohen Anteile verzweigter Fettsäuren, die in relativen Anteilen von über 50 % gefunden wurden (Vainshtein, 1992). Die in iso-Position verzweigte, ungesättigte Fettsäure i17:1 wurde bisher nur in SRB detektiert. Den größten Anteil an i17:1 besitzen Angehörige der Gattung *Desulfovibrio*. So enthält *Desulfovibrio desulfuricans* beispielsweise einen relativen Anteil i17:1 von bis zu 33 % (Kohring et al., 1994). Weiterhin wurde i17:1 in einem Anteil von 11 % in Arten der Gattung *Desulfotomaculum* nachgewiesen. In *Desulfosarcina variabilis* und *Desulfococcus multivorans* wurde i17:1 nur in sehr geringen Anteilen gefunden (≈1 %), jedoch besitzen diese die anteiso-verzweigte Fettsäure a17:1 in Anteilen von 4 bzw. 10 % (Kohring et al., 1994). Eine weitere für einige SRB charakteristische Fettsäure ist 10Me16:0, die bisher nur in wenigen Bakterien gefunden wurde (Kroppenstedt und Kutzner, 1978; Tsitko et al., 1999). Die Fettsäure 10Me16:0 wurde innerhalb der SRB nur in einigen Angehörigen

der Familie *Desulfobacteriaceae* identifiziert. Während 10Me16:0 in *Desulfobacterium autotrophicum* nur einen Anteil von 7,6 % ausmacht, bildet sie in Arten der Gattung *Desulfobacter* in z.T. höheren Anteilen in Kombination mit cyc17:0 ein gattungsspezifisches Merkmal (Dowling et al., 1986; Parkes und Taylor, 1983, 1985). Weiterhin wurden in einigen SRB Fettsäuren nachgewiesen, die Hydroxygruppen enthalten (Boon et al., 1977; Edlund et al., 1985; Vainshtein et al., 1992). Diese sind charakteristisch für Gram-negative Bakterien und in den Lipopolysacchariden der äußeren Membran lokalisiert (Wilkenson, 1988). Einzigartig innerhalb der Bakterien ist das Vorkommen von nicht-isoprenoiden, verzweigten Glycerol-Diethern und Monoethern in den Lipidmembranen des thermophilen sulfatreduzierenden *Thermodesulfobacterium commune*. Die im Vergleich zu Estern (in Bakterien vorherrschend) stabilere Etherbindung, die sonst nur in den Membranen von Archaea vorkommt, wird als Anpassung an das Wachstum im thermophilen Temperaturbereich angesehen (Langworthy, 1983). Für Bakterien ungewöhnlich lange Fettsäuren mit Acylketten aus bis zu 34 Kohlenstoffatomen wurden in Gram-positiven SRB der Gattung *Desulfotomaculum* nachgewiesen (Rezanka et al., 1990).

Aufgrund der Seltenheit von 10Me16:0 und der noch höheren Spezifität von i17:1 wurden diese beiden Fettsäuren häufig als spezifische Biomarker für SRB beschrieben (Taylor und Parkes, 1985; Findlay und Dobbs, 1993). Als Biomarker bezeichnet man chemische Verbindungen, die direkt in den Lebensräumen nachgewiesen werden können und Aufschluß über die Art und die Häufigkeit vorkommender Organismengruppen geben. Das Vorkommen von 10Me16:0 in Actinomyceten als terrestrische Bakterien beschränkt den Anwendungsbereich dieser Fettsäure als Biomarker für *Desulfobacter* und *Desulfobacterium* auf marine Habitate (Dowling et al., 1986).

Aufgrund der unterschiedlichen Gehalte einzelner Fettsäuren in diversen Bakterien und der Tatsache, daß diese Gehalte von physikalischen und physiologischen Faktoren abhängig sein können, ist eine exakte Quantifizierung von Bakterienpopulationen durch *in situ* Analysen von Fettsäuren jedoch nicht möglich. So wurden zum Beispiel Veränderungen der Fettsäurezusammensetzung von SRB durch unterschiedliche Kohlenstoffquellen in Reinkulturen gemessen (Taylor und Parkes, 1983; Dowling et al., 1986; Aeckersberg et al., 1998). In vielen Bakterien, jedoch noch nicht in SRB, wurde nachgewiesen, daß sich die Fettsäurenzusammensetzung bei Änderung der Wachstumstemperatur oder sogar während des Wachstums bei konstanter Temperatur ändert (Gounot und Russel, 1999; Cronan Jr., 1968).

Tabelle 3: Nomenklatur und die in dieser Arbeit verwendeten Abkürzungen sowie Strukturformeln bakterieller Fettsäuren.

IUPAC Name (Trivialname in Klammern)	**Abkürzung***	**Strukturformel**
Hexadecansäure (Palmitinsäure)	16:0	
cis -9-Hexadecensäure (Palmitoleinsäure)	c9 16:1	
trans -9-Hexadecensäure	t9 16:1	
cis-9,10-Methylenhexadecansäure	cyc17:0	
14-Methylpentadecansäure	i16:0	
13-Methylpentadecansäure	a16:0	
10-Methylhexadecansäure	10Me16:0	
15-Methyl-*cis*9-hexadecensäure	i17:1	
2-Hydroxyhexadecansäure	2OH16:0	

*Die Abkürzung xn Y:Z enthält folgende Informationen: Y steht für die Anzahl der Kohlenstoffatome, Z für die Anzahl an Doppelbindungen, deren Stellung in der Acylkette, ausgehend von der Carboxylgruppe, durch n gekennzeichnet ist. Der Buchstabe x kennzeichnet eine *cis* (c)- oder *trans*(t)- Doppelbindung. Die Vorsilbe cyc steht für einen Cyclopropanring in der Fettsäurenkette, während i(iso), a(anteiso) bzw. 10Me(10-Methyl) die Stellung der Methylgruppe innerhalb verzweigter Fettsäuren angibt.

6. Einfluß der Temperatur auf die Lipidfettsäurenzusammensetzung von Bakterien

Biologische Membranen bestehen aus einer Doppelschicht von amphiphilen Phospholipiden. In dieser fluiden Lipidmatrix befinden sich mosaikartig verteilt integrale und periphere Proteine, die in lateraler Richtung beweglich sind (Singer und Nicolson, 1972). Zur Beschreibung der Beweglichkeit von Molekülen innerhalb von Membranen wird gewöhnlich der Ausdruck Membranfluidität verwendet (Russel, 1988).

Phospholipide liegen in der Natur in zwei unterschiedlichen Zuständen vor. Bei niedrigeren Temperaturen besitzen Phospholipide einen geordneten, gelartigen Zustand, während sie sich bei höheren Temperaturen in einem ungeordnetem, flüssig-kristallinen Zustand befinden. Die Temperatur, bei der die Phospholipide durch thermische Einwirkung von dem gelartigen in den flüssig-kristallinen Zustand übergehen, wird als Phasenübergangstemperatur bezeichnet (Silvius, 1982). Diese Phasenübergangstemperatur ist abhängig von der chemischen Zusammensetzung der Phospholipide. In stoffwechselaktiven Zellen, in denen eine fluide Membran essentiell ist, müssen die Phospholipide somit überwiegend im flüssig-kristallinen Zustand vorliegen, d.h. die Phasenübergangstemperaturen der vorherrschenden Lipide müssen unterhalb der Wachstumstemperatur liegen (Jackson und Cronan, 1978). Eine Veränderung der Fettsäurezusammensetzung wirkt sich deutlich stärker als eine Veränderung der polaren Kopfgruppe auf die Phasenübergangstemperatur von Phospholipiden aus (Russel, 1988). Die Phasenübergangstemperaturen künstlicher Lipide (Phosphatidylcholin), die aus unterschiedlichen Fettsäuren synthetisiert wurden, sind in Tabelle 4 dargestellt.

Viele Eukaryoten und Prokaryoten sind in der Lage, ihre Lipidfettsäurenzusammensetzung bei wechselnden Umgebungstemperaturen zu verändern. Dies befähigt sie, die stark temperaturabhängige Membranfluidität in einem gewissen Temperaturbereich konstant zu halten. Beispielsweise reagieren viele Bakterien auf Temperatursenkungen mit einer Erhöhung des Anteils von Fettsäuren, die zu geringeren Phasenübergangstemperaturen der Lipide führen (Abb. 2). Dadurch wirken sie einer Abnahme der Membranfluidität bei niedrigeren Temperaturen entgegen (Gounot und Russel, 1999).

Tabelle 4: Der Einfluß der Fettsäurenzusammensetzung in künstlich synthetisiertem Phosphatidylcholinen auf die Phasenübergangstemperatur. Das künstliche Phosphatidylcholin besitzt jeweils zwei identische Fettsäuren.

Fettsäure	**Phasenübergangstemperatur** (°C)*
i16:0	22,0
a16:0	-3,0
16:0	41,5
cis9 16:1	-35,5
cyc 17:0	-19,9
17:0	48,8
18:0	55,8

*Daten von Silvius (1982).

Die häufigste in Bakterien gefundene Regulation der Membranfluidität bei wechselnden Temperaturen ist eine Veränderung des Verhältnisses von gesättigten zu ungesättigten Fettsäuren (Gounot and Russel, 1999). Während die geraden Kohlenstoffketten gesättigter Fettsäuren optimal miteinander in hydrophobe Wechselwirkung treten können, verringern ungesättigte Fettsäuren mit *cis*-Doppelbindungen infolge des starren Knicks in den Kohlenstoffketten (Abb. 2) die Packungsdichte der Lipidschichten und erniedrigen so die Phasenübergangstemperatur. Einen ähnlichen, jedoch deutlich geringeren Effekt auf den Ordnungszustand der Lipide entsteht durch den Einbau von verzweigten Fettsäuren. Dabei besitzen Lipide mit anteiso-Methylverzweigungen niedrigere Phasenübergangstemperaturen als Fettsäuren mit iso-Methylverzweigungen (Tabelle 4; Russel, 1988). Ein weiterer temperaturabhängiger Regulationsmechanismus, der in einigen Bakterien zur Erhaltung der Membranfluidität dient, ist der Einbau von Fettsäuren mit unterschiedlich langen Kohlenstoffketten (Gounot und Russel, 1999). Kürzere Kohlenstoffketten besitzen untereinander geringere hydrophobe Wechselwirkungen als längere Ketten und führen somit zu geringeren Phasenübergangstemperaturen. Die Auswirkung der Kettenlänge von Lipidfettsäuren auf die Membranfluidität ist jedoch geringer als die Auswirkung des Sättigungs- oder Verzweigungsgrads (Tabelle 4).

Ob und in welcher Art eine temperaturabhängige Veränderung der Lipidfettsäuren-zusammensetzung in Bakterien stattfindet, hängt von der jeweiligen enzymatischen Ausstattung des Organismus ab. Viele aerobe Bakterien besitzen sauerstoffabhängige Desaturasen, wodurch sie in der Lage sind, gesättigte Phopholipidfettsäuren in der Membran zu oxidieren und somit Doppelbindungen in diese einzufügen (Schweizer, 1989). Durch diese postsynthetische Modifikation können sie den Anteil von ungesättigten Fettsäuren direkt in der Membran erhöhen. Eine weitere Regulation der Membranfluidität, die direkt in den Lipidschichten erfolgt, wurde in Angehörigen der Gattung *Pseudomonas* gefunden . Diese Bakterien besitzen Isomerasen, mit denen sie *trans*-Doppelbindungen in die fluiditätserhöhenden *cis*-Isomere umwandeln können (Okuyama, 1996). Isomerasen können die Reaktion auch von *cis*- zu *trans*-Doppelbindungen durchführen. Diese Umwandlung von *cis*- in *trans*- Isomere von ungesättigten Lipidfettsäuren wurde als Anpassung gegen sich in die Membran einlagernde, fluiditätserhöhende Substanzen beschrieben (Keweloh, 1995). Für die Regulation der Fettsäurenzusammensetzung durch Desaturasen oder Isomerasen ist kein Wachstum erforderlich.

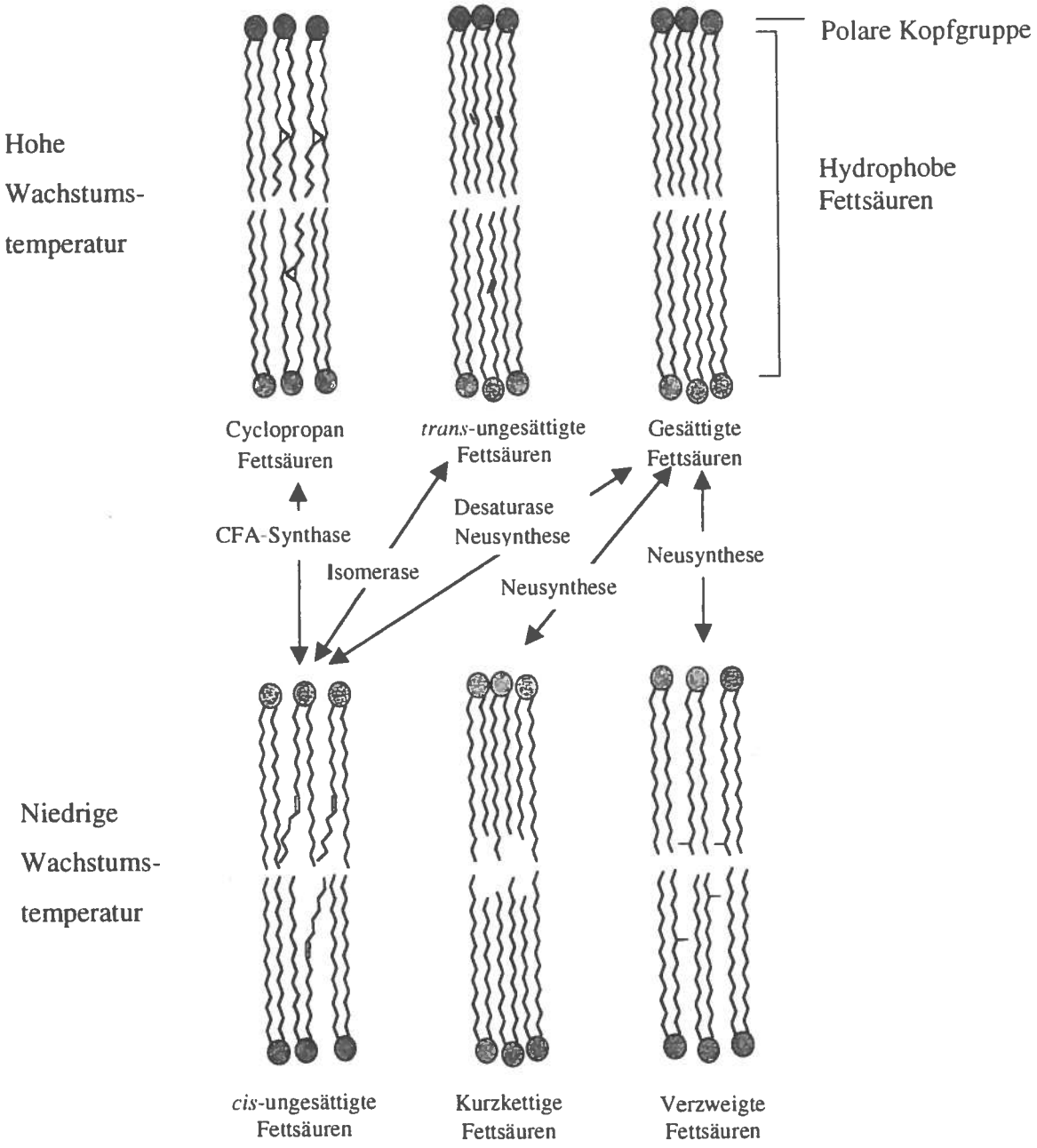


Abbildung 2: Beschriebene Veränderungen der Lipidfettsäurenzusammensetzung von Bakterienmembranen zur Regulation der Fluidität bei unterschiedlichen Wachstumstemperaturen. CFA-Synthase = Cyclopropanfettsäuren-Synthase.

Die meisten Bakterien bilden ihre Phospholipidfettsäuren jedoch über den sogenannten "Anaeroben Fettsäure Biosyntheseweg" (Schweizer, 1989). In diesem sauerstoffunabhängigen Syntheseweg verläuft die Bildung gesättigter und ungesättigter Fettsäuren bis zu einer Acylkette von 10 Kohlenstoffatomen gemeinsam, bevor der Enzymkomplex Hydroxydecanoyldehydratase den weiteren Verlauf zu einer gesättigten oder ungesättigten

Fettsäure determiniert. Die jeweilige Fettsäure wird in die Phospholipide eingebaut und anschließend in die Membran integriert, in der keine nachträgliche Umwandlung von einer gesättigten in die jeweilige ungesättigte Fettsäure (oder umgekehrt) stattfinden kann (Schweizer, 1989). Man kann daher diese häufig temperaturabhängige Veränderung als eine langsame Anpassungsreaktion bezeichnen, die an den Einbau von neusynthetisierten Phospholipiden in die Membran und somit an Wachstum gekoppelt ist. Gleiches gilt für die Veränderung des Anteils kurzkettiger oder verzweigter Fettsäuren (Gounot und Russel, 1999; Kaneda, 1991).

7. Zielsetzung der Arbeit

SRB spielen in marinen Sedimenten eine bedeutende ökologische Rolle. Obwohl im größten Teil der marinen Habitate permanent niedrige Temperaturen herrschen, wurden erst wenige SRB isoliert, die bei solch niedrigen Temperaturen wachsen. Küstennahe Sedimente der gemäßigten Breiten weisen dagegen jahreszeitliche Temperaturschwankungen auf, die dadurch wechselnde Anforderungen an die vorherrschende mikrobielle Lebensgemeinschaft stellen. Bisher ist jedoch noch nicht bekannt, welchen Einfluß die Temperatur auf zellulare Bestandteile und Reaktionen der SRB hat. Als essentielle Zellbestandteile sollten die zellularen Fettsäuren von marinen SRB sowohl in Reinkulturen als auch direkt in marinen Sedimenten verschiedener Klimabereiche untersucht werden.

1. Der Einfluß der Temperatur auf die zellulare Fettsäurenzusammensetzung von SRB sollte an psychrophilen, moderat psychrophilen und mesophilen SRB gezeigt werden, die von verschiedenen, überwiegend marinen Standorten isoliert wurden. Zum einen wurde die Fettsäurenzusammensetzunge nach der Kultivierung bei unterschiedlichen, konstanten Temperaturen untersucht, die in den jeweiligen Wachstumstemperaturbereichen lagen. Zum anderen sollte der Einfluß von wechselnden Temperaturen und mögliche Anpassungsmechanismen durch wachstumsbegleitende Fettsäure-Analysen verfolgt werden.

2. Zur Charakterisierung von neuartigen SRB sollte die Bestimmung der zellularen Fettsäuren als ein chemotaxonomisches Merkmal für die phylogenetische Einordnung eingesetzt werden. Gerade innerhalb der Familie *Desulfobacteriaceae* bilden die zellularen Fettsäuremuster ein Gattungs- oder sogar Art-spezifisches Muster, das sich häufig mit den phylogenetischen Einordnungen über die 16S rRNA Gensequenzen deckt.

3. Durch *in situ* Analysen der Phospholipidfettsäuren in marinen Sedimenten verschiedener Klimabereiche sollten spezifische Fettsäuren von SRB und anderen Organismengruppen nachgewiesen werden. Durch diese Untersuchung sollten Hinweise über die Verteilung der mikrobiellen Lebensgemeinschaften in den jeweiligen Sedimenten erhalten werden. Als ein weiterer Aspekt sollte untersucht werden, ob die permanent kalten, arktischen Sedimente Svalbards höhere Anteile fluiditätserhöhender Fettsäuren (wie beispielsweise in psychrophilen SRB) enthalten, als die Sedimente gemäßigter Breiten (Wattsediment, Jadebusen).

4. Durch klassische mikrobiologische Methoden sollte das Vorkommen aerober und sulfatreduzierender Bakterien in den marinen Sedimenten untersucht werden und mit den Ergebnissen der *in situ* Phospholipidfettsäuren-Analyse verglichen werden. Zur Abschätzung von Bakterienpopulationen verschiedener stoffwechselphysiologischer Gruppen wurde die most-probable number (MPN) Methode angewendet. Die hohen Verdünnungsstufen der MPN Reihen dienten der Isolierung und anschließenden Identifizierung von numerisch dominanten Organismen der jeweiligen Sedimente. Die selektive Isolierung von kälteangepaßten Bakterien aus kalten, arktischen Sedimenten (Svalbard) erfolgte dabei unter permanent niedrigen Wachstumstemperaturen.

5. Die Entwicklung von Gasblasen in gasdicht verschlossenen Sedimentproben aus Svalbard ließ auf eine mögliche Methanogenese unter kalten Bedingungen (4 °C) schließen. Durch eine selektive Kultivierung in anoxischem, sulfatfreiem Medium unter der Zugabe verschiedener Elektronendonatoren und Kohlenstoffquellen sollten erstmals kälteangepaßte methanogene Archaea aus permanent kaltem, marinem Sediment der Arktis angereichert und isoliert werden.

B Ergebnisse und Diskussion

1. Einfluß der Temperatur auf die zellulare Fettsäurenzusammensetzung von marinen sulfatreduzierenden Bakterien

Die Temperaturen, bei denen die dissimilatorische Sulfatreduktion stattfinden kann, reichen von unter 0 °C bis über 100 °C (Isaksen und Jørgensen, 1996; Jørgensen et al, 1992). Der Einfluß der Temperatur auf die Sulfatreduktionsraten konnte bereits in vorausgehenden Studien in den natürlichen Habitaten und in Reinkulturen gezeigt werden (Jørgensen, 1977; Abdollahi et al., 1979; Isaksen und Jørgensen, 1996; Knoblauch und Jørgensen, 1999). Weiterhin zeigten Studien die Temperaturabhängigkeit der Wachstumsraten und des Wachstumertrags in SRB (Isaksen und Jørgensen, 1996; Sass et al., 1998; Knoblauch und Jørgensen, 1999). In der vorliegenden Arbeit wurde erstmals der Einfluß der Temperatur auf die zellulare Fettsäurezusammensetzung von SRB untersucht (**Publikation 1**). Die hierfür ausgewählten, überwiegend marinen SRB können aufgrund ihrer unterschiedlichen Wachstumstemperaturbereiche in psychrophile, moderat psychrophile, psychrotolerante und mesophile SRB unterteilt werden.

1.1. Zellulare Fettsäurenzusammensetzung von psychrophilen, psychrotoleranten und mesophilen SRB bei verschiedenen Wachstumstemperaturen

Mit Ausnahme von *Desulfofaba gelida* besaßen alle psychrophilen (*Desulfofrigus oceanense* und *Desulfotalea psychrophila*) und moderat psychrophilen (*Desulfotalea arctica* und *Desulforhopalus vacuolatus*) SRB extrem hohe Anteile (> 70 %) an *cis*-ungesättigten Fettsäuren mit Acylketten von überwiegend 16-18 Kohlenstoffatomen. Nach dem Wachstum bei unterschiedlichen Temperaturen innerhalb ihrer Wachstumstemperaturbereiche unterhalb des Optimums zeigten diese Arten keine signifikanten Veränderungen ihrer zellularen Fettsäuren-zusammensetzungen. Die hohen Anteile an *cis*-ungesättigter Fettsäuren, die niedrige Phasen-übergangstemperaturen besitzen (siehe Tabelle 4), können als besondere Anpassung an ihre permanent kalten Lebensräume angesehen werden. Dagegen besitzt *D. gelida* mit nur 40 % ungesättigten Fettsäuren einen hohen Anteil (> 70 %) an Fettsäuren, mit weniger als 16 Kohlenstoffatomen in den Acylketten. Der vermehrte Einbau von kürzerkettigen Fettsäuren, der die hydrophoben Wechselwirkungen innerhalb der Lipiddoppelschicht verringert, kann ebenfalls als besondere Anpassung an den permanent kalten natürlichen Lebensraum angesehen werden.

Die mesophilen SRB der Gattungen *Desulfovibrio*, *Desulfococcus* und *Desulfosarcina*, die die seltenen Fettsäuren i17:1 und a17:1 enthalten, zeigten ebenfalls nach dem Wachstum bei verschiedenen Temperaturen nur geringe Veränderungen innerhalb ihrer zellularen Fettsäurenzusammensetzungen. Die relativen Anteile ungesättigter Fettsäuren sind mit 50 % in *Desulfovibrio desulfuricans* und 23-25 % in *Desulfococcus multivorans* und *Desulfosarcina variabilis* deutlich unterhalb der Anteile, die in den psychrophilen SRB enthalten sind. Im Gegensatz dazu konnte in den mesophilen Arten der Gattung *Desulfobacter* (*D. curvatus*, *D. hydrogenophilus*, *D. latus* und *D. postagatei*) mit Erniedrigung der Inkubationstemperatur eine Erhöhung der Anteile an *cis*-ungesättigten Fettsäuren gefunden werden (1-2 % pro °C). Die signifikantesten Veränderungen der zellularen Fettsäurenzusammensetzung bei unterschiedlichen Temperaturen wurden in *D. hydrogenophilus* nachgewiesen, das als einziges der untersuchten mesophilen SRB in der Lage ist, noch bei 0 °C zu wachsen. Es kann daher als psychrotolerant eingestuft werden.

1.2. Wachstumsabhängige Fettsäuremuster von *Desulfobacter hydrogenophilus* bei wechselnden Temperaturen

Weil bei *D. hydrogenophilus* offensichtlich die Fähigkeit zur Regulation der Fettsäurenzusammensetzung besonders ausgeprägt ist, wurden deren mögliche Änderungen während des Wachstumsverlaufs bei mehreren konstanten Temperaturen untersucht. Die zellularen Fettsäuremuster von *D. hydrogenophilus* in unterschiedlichen Phasen des Wachstums in batch-Kulturen zeigten, daß sich bei mesophilen Temperaturen beim Übergang von der Wachstumsphase in die stationäre Phase die Anteile von cyc17:0 und 10Me16:0 auf Kosten von *cis*9 16:1 erhöhen. Es handelt sich vermutlich um dieselbe postsynthetische Modifikation durch Methylierung mit S-Adenosylmethionin, die bereits in anderen Bakterien gefunden und aufgeklärt wurde (Grogan und Cronan Jr., 1997). Bei Herabsetzung der Wachstumstemperatur verringerte sich der Grad der Methylierung. Bei 4 °C nahmen die Anteile von cyc17:0 und 10Me16:0 bis unter die Nachweisgrenze ab. Die zellulare Fettsäurenzusammensetzung von *D. hydrogenophilus* war also nicht nur abhängig von der Temperatur, sondern auch von der Wachstumsphase. Infolge der Inhibierung der Synthese von 10Me16:0 bei niedrigen Temperaturen ist die Anwendung dieser häufig als spezifischer Biomarker beschriebenen Fettsäure (Findlay und Dobbs, 1993) in kalten, marinen Sedimenten fragwürdig.

Mittels Zugabe von ^{13}C-markiertem Acetat konnte gezeigt werden, daß die Erhöhung des Gehalts an *cis*9 16:1 in *D. hydrogenophilus* bei Erniedrigung der Wachstumstemperatur durch

eine *de novo* Synthese erfolgt. Dieser Befund ist im Einklang mit dem strikt anaeroben Metabolismus von *D. hydrogenophilus*. Bei niedrigen Temperaturen führen also eine Inhibierung der Synthese von 10Me16:0 und cyc17:0 sowie die Neusynthese von *cis*9 16:1 zu einer Erhöhung des Gehalts an fluiditätserhöhenden Fettsäuren.

2. Neueinordnung von *Desulfobacterium phenolicum* als *Desulfobacula phenolica* comb. nov. und Beschreibung von *Desulfotignum balticum* gen. nov., sp. nov.

Vergleichende Untersuchungen der 16S rRNA Gensequenzen von SRB der δ-Proteobakterien machten es notwendig, den ursprünglich als *Desulfoarculus* spec. eingeordneten Stamm Sax (Drzyzga et al., 1993) und *Desulfobacterium phenolicum* (Bak und Widdel, 1986) phylogenetisch neu einzuordnen. Als weitere Charakteristika von Stamm Sax, *D. phenolicum* und verwandten SRB wurden die jeweiligen physiologischen und morphologischen Merkmale sowie die zellularen Fettsäuren als chemotaxonomisches Merkmal bestimmt (**Publikation 2**).

2.1. Phylogenetische Charakterisierung mittels 16S rRNA Gensequenz-Analyse

Aufgrund der 16S rRNA Gensequenz-Analyse stellt Stamm Sax eine neue Gattung innerhalb der δ-Unterklasse der Proteobakterien dar. Stamm Sax teilt maximal 94,1 % 16S rDNA Sequenzähnlichkeit mit dem nächsten Verwandten *Desulfospira joergensenii* und 93.9 % Sequenzähnlichkeit mit *Desulfobacterium phenolicum*. Die 16S rRNA Gensequenz von *Desulfobacterium phenolicum* weist 98,8 % Ähnlichkeit mit der vom nächsten verwandten sulfatreduzierendem Bakterium, *Desulfobacula toluolica*, auf.

2.2. Physiologische und morphologische Merkmale

Stamm Sax und *D. phenolicum* sind in der Lage, eine Vielzahl von organischen Substraten, wie Fettsäuren und aromatische Verbindungen vollständig zu Kohlendioxid abzubauen. Im Gegensatz zu *D. phenolicum* kann Stamm Sax mit Wasserstoff oder Formiat autotroph wachsen. Eine Kohlendioxidfixierung über den Kohlenmonoxid-Dehydrogenase-Weg konnte in Stamm Sax nachgewiesen werden. Neben Sulfat konnte Stamm Sax auch Thiosulfat oder Sulfit als Elektronenakzeptor nutzen. Stamm Sax war in der Lage mit Pyruvat, jedoch nicht mit Fumarat oder Malat durch Gärung zu wachsen. Durch die gerade Stäbchenform unterscheidet sich Stamm Sax morphologisch von den ovalen Zellen bzw. vibrioiden Zellen der phylogenetisch verwandten SRB *Desulfobacterium phenolicum* und *Desulfospira joergensenii* .

2.3. Chemotaxonomische Einordnung mittels zellularer Fettsäureanalyse

Das zellulare Fettsäuremuster von Stamm Sax mit den markanten Fettsäuren 10Me16:0 und cyc17:0 zeigte große Ähnlichkeit mit Fettsäuremustern von Angehörigen der Gattung *Desulfobacula* und *Desulfobacter* und unterstreicht damit die phylogenetische Verwandschaft zu diesen. Das Fettsäuremuster des phylogenetisch nächsten Verwandten, *Desulfospira joergensenii,* unterschied sich vor allem durch das Fehlen von 10Me16:0 und das Vorhandensein von 3-Hydroxyfettsäuren vom Muster von Stamm Sax. Die große Ähnlichkeit der zellularen Fettsäurenzusammensetzung zwischen *D. phenolica* und *D. toluolica* stimmt mit ihrer engen phylogenetischen Beziehung überein und unterstützt die Einordnung in eine gemeinsame Gattung. Basierend auf den 16S rRNA Gensequenz-Analysen und unter Einbeziehung der morphologischen, physiologischen und chemotaxonomischen Merkmale stellt Stamm Sax eine neue Art innerhalb einer neuen Gattung dar. Es wurde der Name *Desulfotignum balticum* vorgeschlagen. Unter den gleichen Aspekten muß *Desulfobacterium phenolicum* in die Gattung *Desulfobacula* eingeordnet werden. Es wurde die neue Artbezeichnung *Desulfobacula phenolica* vorgeschlagen.

Durch den Nachweis von 10Me16:0 im marinen SRB *Desulfotignum balticum* und der Gattung *Desulfobacula* ist die Spezifität dieser Fettsäure als Biomarker für Angehörige der Gattung *Desulfobacter* in marinen Sedimenten nicht mehr gegeben.

3. Charakterisierung der Mikroorganismenzusammensetzung von marinen Sedimenten mittels Phospholipidanalysen und Bestimmung der Lebendzellzahlen

Die Zusammensetzungen der aeroben und sulfatreduzierenden Mikroorganismengemeinschaften von vier Fjorden Svalbards (Arktischer Ozean) wurden mittels der kultivierungsunabhängigen Analyse der Phospholipide und der Bestimmung der Lebendzellzahl untersucht (**Publikation 3**). Um mögliche Einflüsse der im arktischen Ozean vorherrschenden permanent niedrigen Temperaturen auf die dort vorkommende Mikroorganismengemeinschaft darzustellen, wurden die Ergebnisse mit denen aus Untersuchungen von Wattsediment des Jadebusen (Deutschland) verglichen (**Publikation 4**).

3.1. Tiefenprofile der Phospholipidfettsäuren

Die Analyse der Phospholipide und der in ihnen enthaltenden Fettsäuren bietet die Möglichkeit, ohne Kultivierung und ohne Selektion von Bakteriengruppen Aussagen über das

Vorkommen von lebenden Organismen in ihren natürlichen Habitaten zu treffen (Findlay und Dobbs, 1993).

Zur Abschätzung der lebenden Biomasse in den beiden marinen Habitaten wurde das in Phospholipiden gebundene Phosphat analysiert. Sowohl in den Sedimenten Svalbards als auch im Wattsediment wurde die größte Biomasse pro g Trockengewicht Sediment in den obersten Schichten (0-1 cm). Dabei lagen die Werte des Wattsediments etwa um das Zehnfache höher als die der kalten Sedimente Svalbards. In beiden Habitaten nahmen die Phosphatkonzentrationen mit der Tiefe ab. Die Sedimente aus vier verschiedenen Fjorden Svalbards zeigten dabei unterschiedliche Profile. Die Ursachen liegen vermutlich am unterschiedlich starken terrestrischen Eintrag aus den angrenzenden Gletschern.

Große Ähnlichkeiten zwischen beiden Habitaten zeigten auch die Tiefenprofile von spezifischen Phospholipidfettsäuren (PLFA), die verschiedenen physiologischen Organismengruppen zugeordnet werden können. So konnten die größten Anteile von mehrfach-ungesättigten PLFA, die charakteristisch für eukaryotische Organismen sind, und typische PLFA aerober Organismen in den oberen Sedimentschichten (0-1 cm) festgestellt werden. Diese Dominanz der eukaryotischen und prokaryotischen Aerobier resultiert vermutlich aus der Verfügbarkeit von Sauerstoff, organischem Material und von Licht in diesen Bereichen des Sediments. Mit zunehmender Tiefe erhöhte sich der relative Anteil von verzweigten und anderen für fakultativ und obligat anaerobe Bakterien spezifische PLFA. So zeigte sich in beiden Habitaten mit zunehmender Tiefe ein Wechsel von einer aeroben Mikroorganismenpopulation zu einer von anaeroben Bakterien dominierten Gemeinschaft.

Die Verteilungen und Häufigkeiten der für SRB spezifischen PLFA i17:1 und 10Me16:0 zeigten Unterschiede zwischen den Sedimenten aus Svalbard und denen des Wattenmeers. Die beiden Biomarker konnten in den kalten Sedimenten nur in geringen Konzentrationen mit Maxima in den obersten Sedimentschichten nachgewiesen werden. Die niedrigen Konzentrationen i17:1, deren Anteil in Reinkulturen nicht von der Temperatur beeinflußt wird (siehe Publikation 1), weisen auf eine geringe Anzahl SRB der Gattung *Desulfovibrio* hin. Dieser Befund bestätigt die in molekularbiologischen Untersuchungen ermittelte, geringe numerische Häufigkeit dieser Gattung in den Sedimenten Svalbards (Ravenschlag, 2000). Durch die niedrigen Konzentrationen 10Me16:0, deren Synthese in SRB der Gattung *Desulfobacter* durch niedrige Temperaturen inhibiert wird (siehe Publikation 1), kann man nicht unbedingt auf eine geringe Anzahl von SRB der Familie *Desulfobacteriaceae* schließen. Dagegen wurden deutlich höhere Konzentrationen dieser Biomarker im Wattsediment

gefunden. Die Tiefenprofile der relativen Anteile von i17:1 und 10Me16:0 zeigten eine weitgehend konstante Verteilung der Biomarker unterhalb der oxischen Sedimentschicht von 0,5 cm.

Die unterschiedlichen Temperaturen zur Zeit der Probenentnahme von etwa 0 °C in den Sedimenten Svalbards und 17 °C des Wattsediments hatten offensichtlich auf die PLFA Zusammensetzung nur geringfügigen Einfluß. Die relativen Anteile an ungesättigten PLFA waren in beiden Habitaten in der obersten, oxischen Schicht mit etwa 60 % am häufigsten und nahmen mit der Tiefe ab. Unterschiede hinsichtlich der Länge oder der Verzweigung der Acylketten der PLFA konnten ebenfalls nicht festgestellt werden.

3.2. Abschätzung der Bakterienzahlen mittels der MPN-Methode

In guter Übereinstimmung mit den PLFA-Profilen wurden in den kalten Sedimenten Svalbards die höchsten MPN-Zahlen von aeroben heterotrophen Bakterien und von SRB (mit Formiat oder Wasserstoff als Elektronendonator) in der obersten Sedimentschicht gefunden. Während die Zahl der aeroben Bakterien von $4{,}6 \cdot 10^7$ Zellen $\cdot$ cm^{-3} in der obersten Schicht auf $2{,}4 \cdot 10^4$ Zellen $\cdot$ cm^{-3} in 5-6 cm Tiefe abnahm, zeigten die SRB nur eine geringe Abnahme mit zunehmender Tiefe. Die ermittelten SRB Zahlen bei 4 °C aus Svalbard mit einem Maximum von $2{,}4 \cdot 10^5$ Zellen $\cdot$ cm^{-3} nach 18 Monaten Inkubation waren um etwa zwei Zehnerpotenzen geringer als die maximalen Werte aus dem Watt (20 °C, 10 Monate Inkubation). Die MPN Zahlen der SRB aus Svalbard liegen im gleichen Größenbereich wie vorhergehende MPN Bestimmungen in verschiedenen Sedimenten Svalbards, die mit den Substraten Lactat, Propionat oder Acetat durchgeführt wurden (Knoblauch et al., 1999b). Die Abschätzungen der Bakterienzahlen mittels der MPN Methode liegen jedoch unter denen, die mittels molekularer Methoden ermittelt wurden und wohl eher die natürliche Populationsgröße widerspiegeln. Die deutlich geringeren MPN Zahlen sind zum einen in den selektiven Kulturbedingungen und zum anderen in der Tendenz der marinen SRB zur Aggregatbildung zu sehen.

3.3. Phylogenetische Einordnung der neuisolierten Reinkulturen aus Svalbard

Aliquots aus den höchsten MPN Verdünnungsröhrchen, in denen Wachstum festgestellt wurde, dienten als Inoculum für Isolierungsansätze. Dadurch sollten die am Standort häufigsten vorkommenden Bakterien der jeweiligen stoffwechselphysiologischen Gruppe isoliert werden. Aus dem permanent kalten Sedimenten Svalbards konnten durch wiederholtes

Ausstreichen auf Agarplatten 21 Reinkulturen aerober, heterotropher Bakterien isoliert werden. Die Isolate wurden in einem komplexen Medium bei 4 °C kultiviert. Durch partielle 16S rRNA Gensequenz-Analysen konnten diese den Gattungen *Moritella*, *Psychromonas* und *Pseudomonas* zugeordenet werden. Dabei handelt es sich um γ-Proteobakterien, die auch schon von anderen Arbeitsgruppen aus permanent kalten, marinen Habitaten isoliert wurden (Morita, 2000). Die γ-Proteobakterien wurden ebenfalls in großer Häufigkeit in Sediment des Smeerenburgfjord mittels Fluoreszenz *in situ* Hybridisierung und rRNA slot blot Hybridisierung nachgewiesen (Ravenschlag, 2000). Aus den anaeroben Ansätzen mit Formiat als Elektronendonator wurden ausschließlich SRB der Gattung *Desulfotalea* isoliert. Die beiden bereits bekannten Arten dieser Gattung, *Desulfotalea arctica* und *Desulfotalea psychrophila*, stammen ebenfalls aus marinem Sediment der Küste Svalbards und sind als psychrophil eingeordnet (Knoblauch et al., 1999a). Der erneute Nachweis dieser auf verschiedenen organischen Substraten wachsenden SRB bestätigt ihre vermutete Häufigkeit in diesem Habitat (Knoblauch et al., 1999b). Aus den MPN-Verdünnungsröhrchen, die mit Wasserstoff als Elektronendonator inkubiert wurden, konnten bisher drei Isolate von Sedimenten aus Isfjorden (Stamm DHA1), Krossfjorden (Stamm G1HA) und Smeerenburgfjorden (JHA1) isoliert werden. Aufgrund ihrer 16S rRNA Gensequenzen bilden sie neue Taxa innerhalb der Familien *Desulfobulbaceae* bzw. *Desulfobacteraceae*. Der Stamm DHA1 teilt dabei 99,9 % Sequenzähnlichkeit mit einem Isolat (Stamm LSv53) aus Svalbard (Sahm et al., 1999) und 95,9 % Sequenzähnlichkeit mit *Desulforhopalus singaporensis*. Zusammen bilden diese einen Cluster, der sich als neue Gattung von *Desulforhopalus vacuolatus* abtrennen läßt. Die 16S rRNA Gensequenz von Stamm G1HA zeigt eine enge Verwandtschaft mit der von *Desulfobacterium catecholicum* (97,2 %) und einer Klon-Sequenz aus Svalbard-Sediment (Sva0999; 97,3 %) (Ravenschlag, 2000). *D. catecholicum* ist bisher der einzige vollständig-oxidierende Vertreter innerhalb der *Desulfobulbaceae* und könnte gemeinsam mit Stamm G1HA in eine neue Gattung eingeordnet werden. Der Stamm JHA1 ist aufgrund seiner 16S rRNA Gensequenz eindeutig ein Angehöriger der *Desulfobacteriaceae* und teilt 94,8 % Sequenzähnlichkeit mit dem nächsten Verwandten *Desulfobacula phenolica*. Die 16S rDNA Sequenz von Stamm JHA1 zeigt weiterhin 98,6 % Ähnlichkeit mit einer partiellen 16S rDNA Sequenz einer DGGE Bande, die aus der Wassersäule des Mariager Fjords (Dänemark) isoliert wurde (Teske et al., 1996). Stamm JHA1 stellt aufgrund der phylogenetischen Untersuchungen ebenfalls einen

Kandidaten für die Etablierung einer neuen Gattung dar. Für eine vollständige Einordnung der neuen Isolate sind jedoch weitere, detailiertere Charakterisierungen notwendig.

4. Anreicherung und Isolierung von methanogenen Archaea aus permanent kalten, marinen Sedimenten (Svalbard)

Diese Arbeit liegt bislang nicht in Form eines Manuskriptes vor. Einige Versuche wurden von Christine Selz im Rahmen eines Studienprojekts durchgeführt. Die phylogenetische Charakterisierung wurde gemeinsam mit Enrique Llobet-Brossa (MPI für marine Mikrobiologie) durchgeführt.

Methanogene Archaea sind vorwiegend in anoxischen Süßwasserhabitaten von ökologischer Relevanz (Cavicchioli et al., 2000). Jedoch haben Studien in den letzten Jahren mittels biochemischer und molekularbiologischer Methoden gezeigt, daß die Archaea auch in kalten marinen Standorten abundant und an verschiedenen geochemischen Prozessen, wie beispielsweise der anaeroben Oxidation von Methan, beteiligt sein könnten (DeLong et al., 1994, 1999; Sahm and Berninger, 1998; Boetius et al, 2000). Bisher ist es einzig Franzmann und seinen Mitarbeitern gelungen, eine moderat psychrophile und eine psychrophile methanogene Reinkultur aus der Antarktis zu isolieren. Dabei handelt es sich um *Methanococcoides burtonii* (T_{min} = -2,5 °C, T_{opt} = 23 °C) und *Methanogenium frigidum* (T_{min} = -10 °C, T_{opt} = 15 °C). Sie wurden aus methanhaltigem, anoxischem Wasser des Ace Lake isoliert, das eine konstant niedrige Temperatur von 1-2 °C besitzt (Franzmann et al., 1992, 1997). Weitere Standorte, in denen die Methanogenese bei niedrigen Bedingungen nachgewiesen wurde, sind kalte, sumpfige Böden in Russland und der Bodensee (Zhilina und Zavazin, 1991; Schulz und Conrad, 1996). Reinkulturen methanogener Archaea, die noch bei niedrigen Temperaturen wachsen, konnten aus letzteren Habitaten noch nicht isoliert werden.

4.1. Anreicherung und Isolierung

Mit einer Sedimentprobe aus dem Hafenbereich von Ny-Ålesund (Svalbard) wurden Kulturen für die Anreicherung von Methanogenen beimpft. Das selektive Medium entsprach hinsichtlich der Zusammensetzung der gelösten Salze weitgehend dem Meerwasser. Zur Unterdrückung des Wachstums von SRB wurde lediglich das üblicherweise zugesetzte Magnesiumsulfat durch eine aquimolare Menge Magnesiumchlorid ersetzt. Als Energie- und Kohlenstoffquelle wurden Verbindungen gewählt, die von Methanogenen verwertet werden können (Wasserstoff + Kohlendioxid, Formiat, Acetat und Methanol). Nach vier Monaten Inkubation bei einer Temperatur von 4 °C konnte in den Ansätzen mit Methanol eine

Methanbildung mittels gaschromatographischer Methoden nachgewiesen werden. Durch die Verwendung der Epi-Autofluoreszenzmikroskopie (Doddema und Vogels, 1978) zur Anregung des für Methanogene spezifischen Co-Faktors F_{420} waren fluoreszierende Zellen erkennbar, die überwiegend in Aggregaten vorlagen (Abb. 3).

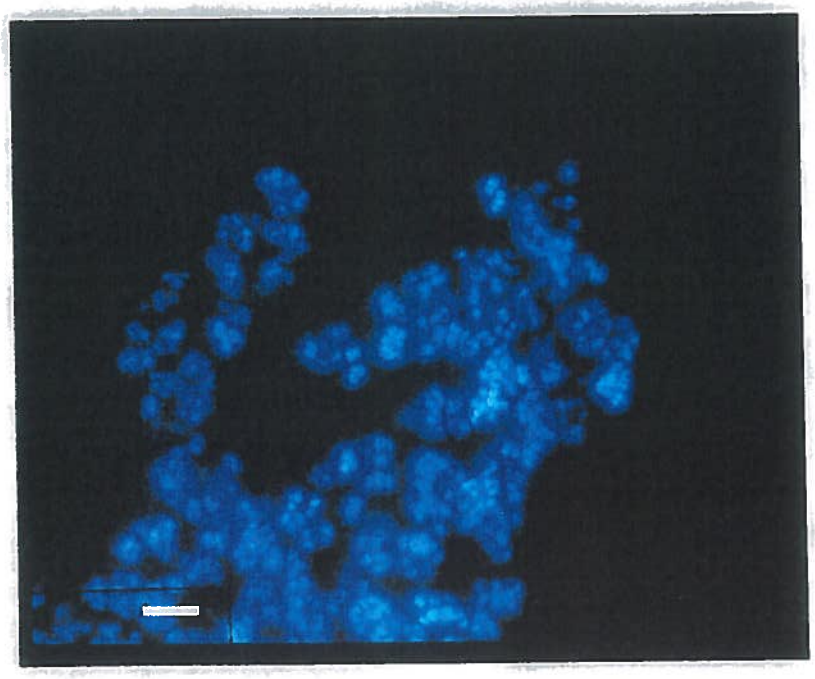


Abbildung 3: Autofluoreszenzmikroskopische Aufnahme der Anreicherungskultur von methanogenen Archaea mit Methanol bei 4 °C. Der eingezeichnete Maßstab entspricht 12 µm.

Versuche zur Isolierung dieser Methanogenen über Agarverdünnungsreihen waren nicht erfolgreich. Mittels aufeinander folgender Flüssigverdünnungsreihen, denen Antibiotika zur Hemmung des Wachstums von Bakterien zugesetzt wurde, konnten aus den Anreicherungen bisher sieben Reinkulturen isoliert werden. Weitere Anreicherungsexperimente, in denen neben den bereits untersuchten Substraten verschiedene organische Fettsäuren (Butyrat, Propionat, Capronat, Lactat, Palmitat, Benzoat) oder Trimethylamin als mögliche Substrate zugesetzt wurden, zeigten, daß zwischen 4 und 12 °C keine Unterschiede hinsichtlich der Substratverwertung bestanden. Während Benzoat als einziges der zugesetzten Verbindungen nicht verwertet wurde, wurden Wasserstoff, Trimethylamin und Methanol innerhalb des untersuchten Zeitraums von 9 Monaten bei beiden Temperaturen vollständig umgesetzt. Nach 2 Monaten Inkubation des Ansatzes mit Methanol bei 4 °C entsprach die Menge des gebildeten Methans 1,2 % der Menge, die nach 9 Monaten gebildet wurde. Während der Inkubation bei 12 °C entsprach die gebildete Menge Methan nach 2 Monaten bereits 53 % der Menge, die nach 9 Monaten gebildet wurde. In den Ansätzen mit Trimethylamin entsprachen

die Mengen Methan nach 2 Monaten bei 4 °C 16 % und bei 12 °C 56 % der Menge nach vollständigem Substratumsatz. Die methanogene Umsetzung der eingesetzten Substrate verläuft offensichtlich bei 4 °C langsamer als 12 °C, obwohl die *in situ* Temperatur der Probenentnahmestelle -0,5 °C betrug. Die optimale Temperatur der Methanogenese wurde in diesem Sediment nicht bestimmt.

4.2. Morphologische und physiologische Charakterisierung

Die neu isolierten methanogenen Archaea wiesen alle eine coccoide Zellform mit einem Durchmesser zwischen 0,8 und 1,7 µm auf. Zwei der sieben Isolate (16SvalB und 16Sval1) wuchsen als einzelne Zellen, während die Zellen der anderen Stämme (4Sval1, 4Sval2, 16Sval2, 16SvalA und 12TMA1) Aggregate bildeten. Bei Anregung des für Methanogenen spezifischen Co-Faktors F_{420} zeigten alle Isolate eine deutlich sichtbare Autofluoreszenz.

Die Untersuchung des Substratspektrums ergab, daß die Stämme 16Sval(A), 16Sval(B) und 12TMA1 lediglich Methanol und Trimethylamin verwerten können. Dimethylsulfid wurde von keinem der Stämme zu Methan umgesetzt. Die Umsetzung verläuft vermutlich, wie in anderen methylotrophen Methanogenen beschrieben, durch eine Disproportionierung gemäß folgender Gleichungen (Zehnder, 1993):

$$4\ CH_3OH \rightarrow 3\ CH_4 + CO_2 + 2\ H_2O$$

$$4\ (CH_3)_3NH^+ + 6\ H_2O \rightarrow 9\ CH_4 + 3\ CO_2 + 4\ NH_4^+$$

Die Zugabe von Biotin war für das Wachstum von 16Sval(A), 16Sval(B) und 12TMA1 essentiell, während Thiamin das Wachstum beschleunigte, jedoch nicht absolut notwendig war.

4.3. Phylogenetische Einordnung

Durch Klonierung und Sequenzierung von 16S rRNA Genen konnten die Isolate 16Sval(A) und 16Sval(B) zwei Verwandtschaftsgruppen innerhalb der *Methanosarcinaceae* zugeordnet werden (Abb. 4). Zu den anderen Isolaten lagen zur Zeit der Fertigstellung der Dissertation noch keine Ergebnisse vor. Der Stamm 16Sval(A) zeigt sehr große Ähnlichkeit (> 99 %) mit dem mesophilen Archaeon *Methanolobus taylorii*. Alle bisher bekannten Angehörigen der Gattung *Methanolobus* stammen aus marinen Sedimenten und verwerten ebenfalls nur Methylgruppen-enthaltende Verbindungen. Sie sind mesophil (T_{opt} = 28–40 °C) und im Gegensatz zu Stamm 16Sval(A) nicht in der Lage, bei Temperaturen unterhalb von 7 °C zu

wachsen (Kadam und Boone, 1995). Der nächste phylogenetisch Verwandte Organismus zu Stamm 16Sval(B) ist *Methanococcoides burtonii* (Ähnlichkeit > 99 %). *M. burtonii* stammt aus einem permanent kalten, terrestrischen Gewässer der Antarktis und wächst optimal bei 23 °C (Franzmann, 1992). Die geringste Temperatur, bei der Wachstum beobachtet wurde ist 5,6 °C, jedoch wurde mittels des Quadratwurzel-Modells (Ratkowsky et al., 1983) eine theoretische T_{min} von – 2,5 °C errechnet. Das Substratspektrum von *M. burtonii* ist ebenfalls auf Methanol und Methylamine begrenzt. Wachstum auf Wasserstoff wurde nicht beobachtet (Franzmann et al., 1992). Das einzige bisher bekannte psychrophile Archaeon ist *Methanogenium frigidum* (Franzmann et al., 1997). *M. frigidum* wurde bei 8 °C aus dem sulfidreichen (8 mM) Ace Lake (Antarktis) isoliert und wächst mit Wasserstoff und Kohlendioxid oder mit Formiat optimal bei 15 °C. *M. frigidum* ist das bisher einzige Archaeon in Reinkultur, das noch bei 0 °C wachsen kann.

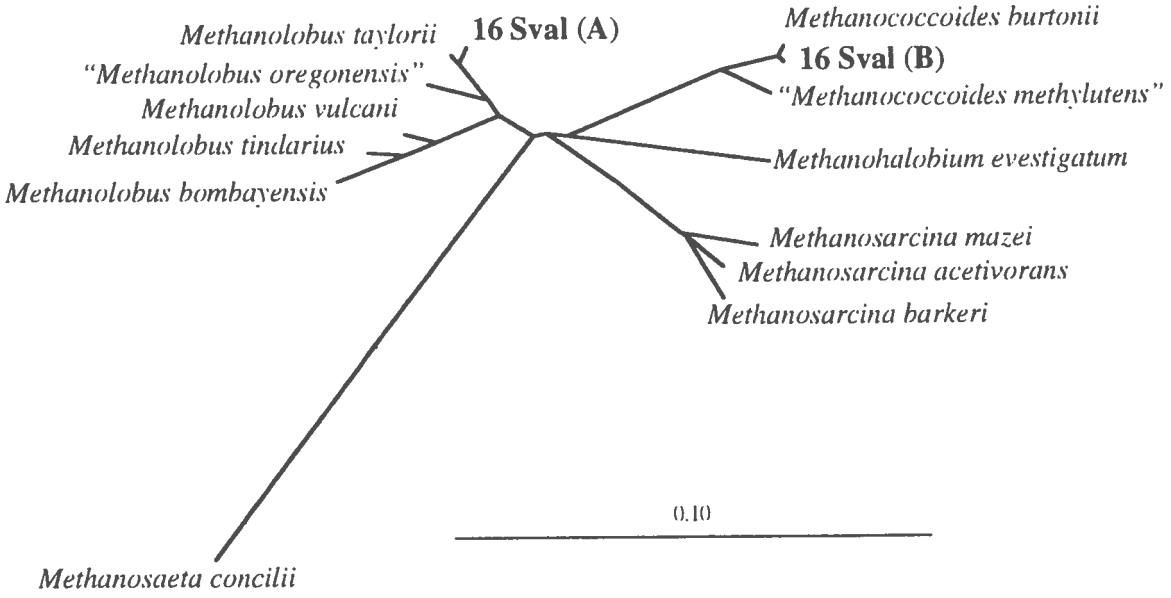


Abbildung 4: Phylogenetische Einordnung der Stämme 16Sval(A) und 16Sval(B) aufgrund ihrer 16S rRNA Gensequenzen.

Methylamine sind unter anderem Produkte des anaeorben Abbaus von methylierten Aminoverbindungen (z.B. Cholin und Betain), sowie der mikrobiellen Reduktion von Methylamin-Oxiden und in marinen Sedimenten weit verbreitet. Durch die Nutzung dieser Substrate stehen die Methanogenen nicht in Substratkonkurrenz mit den energetisch begünstigten SRB und können mit diesen in sulfatreichen Lebensräumen co-existieren (Oremland et al., 1982; Zinder, 1993).

Im zeitlichen Rahmen dieser Arbeit konnten keine weiteren Versuche zur Charakterisierung oder hinsichtlich möglicher Kälteanpassungen der neuisolierten Methanogenen durchgeführt werden. Die neuisolierten Stämme sind die ersten marinen methanogenen Archaea, die aus permanent kaltem, arktischem Sediment in Reinkultur isoliert wurden und die Fähigkeit besitzen, noch bei 4 °C zu wachsen.

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Teil II: Publikationen

A Publikationsliste mit Erläuterungen

Die vorliegende Dissertation beruht zum größten Teil auf den folgenden Publikationen. Die angefügten Erläuterungen stellen den eigenen Beitrag an der jeweiligen Arbeit dar.

1. **Effect of temperature on the composition of cellular fatty acids in sulphate-reducing bacteria.**

   Martin Könneke and Friedrich Widdel
   In preparation

Entwicklung des Konzepts und Durchführung aller mikrobiologischen und analytischen Arbeiten. Gemeinsame Erstellung des Manuskripts mit F. Widdel.

2. **Reclassification of *Desulfobacterium phenolicum* as "*Desulfobacula phenolica*" comb. nov. and description of strain Sax^T as *Desulfotignum balticum* gen. nov., sp. nov..**

   Jan Kuever, Martin Könneke, Alexander Galushko and Oliver Drzyzga (2001)
   Int. J. Syst. Evol. Microbiol. ***51****, 171-177*

Durchführung der mikrobiologischen und analytischen Arbeiten zur Bestimmung der zellularen Fettsäuren. Redaktionelle Mitarbeit bei der Erstellung des Manuskripts.

3. **Aerobic and sulfate-reducing bacterial communities of Arctic sediments characterized by phospholipid analysis and cultivation methods.**

   Martin Könneke, Jan Kuever and Bo Barker Jørgensen
   In preparation

Initiierung des Projekts durch B.B. Jørgensen. Entwicklung des Konzepts und Durchführung der mikrobiologischen und analytischen Arbeiten. Erstellung des Manuskripts unter redaktionellen Mitwirken von J. Kuever und B.B. Jørgensen.

4. Community structure and activity of sulfate-reducing bacteria in an intertidal surface-sediment: A multi-methods approach

Enrique Llobet-Brossa, Ralf Rabus, Michael E. Böttcher, Martin Könneke, Andreas Schramm, Rikke L. Meyer, Niko Finke, Stefan Götzschel, Ramon Rosselló-Mora and Rudolf Amann

Limnology and Oceanography, submitted

Beteiligung an der Entwicklung des Konzepts und Durchführung der Phospholipidfettsäuren-Analyse. Redaktionelle Mitarbeit bei der Erstellung des Manuskripts.

1

Effect of temperature on the composition of cellular fatty acids in sulphate-reducing bacteria

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Abstract

The effect of the growth temperature on the cellular fatty acid composition was studied in twelve species (of eight genera) of sulphate-reducing bacteria (SRB); they included psychrophilic and mesophilic species mostly of marine origin. With the exception of four *Desulfobacter* species, the SRB (including all psychrophiles) exhibited only minor increases (by <3% per 10°C) of *cis*-unsaturated fatty acids if the temperature was decreased. In contrast, *Desulfobacter* species increased the amount of *cis*-unsaturated (measured in late growth phase) significantly (by >14% per 10°C) if the temperature was decreased. The by far highest content of *cis*-unsaturated fatty acids was always observed in psychrophilic species (>70%). Time courses of changes of the fatty acid composition were measured with *D. hydrogenophilus*, the most psychrotolerant mesophile (growth range 0–35°C) among the examined species. The ratio between unsaturated and saturated fatty acids in this species varied not only with temperature, but also during growth in batch cultures at constant temperature; this was not observed with psychrophiles. Isotope (^{13}C) labelling of *D. hydrogenophilus* confirmed that that increases in the relative amount of *cis*-9-hexadecenoic acid are not due to a postsynthetic modification but to a de novo synthesis, as generally observed in an anaerobic metabolism. Furthermore, D. hydrogenophilus formed *cis*-9,10-methylenehexadecanoic acid (a cyclopropane fatty acid) and 10-methylhexadecanoic acid, presumably from *cis*-9-hexadecenoic acid, toward the end of growth at moderate temperature, but not at low temperature.

Introduction

By the use of sulphate as an abundant electron acceptor (28 mM in ocean water), sulphate-reducing bacteria (SRB) play a key role in the anaerobic mineralisation of natural organic compounds in marine sediments (Jørgensen 1982; Widdel 1988). There has been significant progress during the past few decades in our knowledge of the ecology, molecular (rRNA-based) systematics, growth physiology, metabolism, protein structures, and genes of sulfate-reducing bacteria (for overview see Widdel, 1988; Odom and Singleton, 1993; Rabus *et al.*, 2000). The genome sequence of *Desulfovibrio vugaris* has been recently elaborated (www.tigr.org). However, only very few studies have been dealing with adaptative responses and regulatory mechanisms in SRB if these encounter changes of physical and chemical parameters, e.g. in oxygen gradients (Johnson *et al.*, 1997; Cypionka, 2000), or changes in the availability of electron donors (Rabus *et al.*,1993) or electron acceptors.

An important environmental change which often triggers adaptative responses in organisms is the frequent decrease or increase of temperature in moderate climate regions. Effects of temperature on SRB have been examined so far with respect to the sulphate reduction rate, the growth rate and the growth yield, viz. on the level of "macroscopic" physiological parameters. The temperature dependence of sulphate reduction rates in sediments or cultures (Jørgensen 1977; Abdollahi and Nedwell, 1979; Jørgensen *et al.*, 1992; Isaksen and Jørgensen, 1996; Sagemann *et al.*, 1998; Knoblauch and Jørgensen, 1999) or of growth rates in cultures (e.g. Isaksen and Jørgensen, 1996; Knoblauch and Jørgensen, 1999) exhibits typical asymmetric optimum curves and may follow an Arrhenius function over a certain suboptimal temperature range. In contrast, correlation curves between temperature and growth yields do not reveal a general principle (Isaksen and Jørgensen, 1996; Knoblauch and Jørgensen, 1999; Sass *et al.*, 1998) but rather indicates complex and diverse temperature effects on the efficiency of energy conservation and its regulation. The existence of obligately cold-adapted SRB has been recently revealed in a study of Arctic sediment (Knoblauch and Jørgensen, 1999).

Cellular mechanisms of cold-adaptation have been studied in various animals and plants, as well as in microorganisms (Margesin and Schinner, 1999), in particular in *E. coli* and *Bacillus subtilis* (Grau *et al.*, 1994; Graumann and Marahiel, 1996), but not so far in SRB. The cytoplasmic membrane has been given much attention in such studies. Lipid bilayers decrease their fluidity (mobility of lipid molecules within one layer or eventually between the

two layers) with decreasing temperature, and undergo a transition from the liquid crystalline state (the mobile in vivo state) to the gel ("solid") state at a certain temperature (phase transition temperature); membrane functioning is hampered below this temperature (Russel, 1989). Cold adaption in various organisms involves a decrease of the phase transition temperature; this can be achieved by an increased synthesis of *cis*-unsaturated, short-chain or alkyl-branched fatty acids (Gounot and Russel, 1999). To gain first insights into mechanistic principles of cold adaption in SRB, we examined how temperature influences the composition of lipid fatty acids in a number of psychrophilic and mesophilic species.

Results

Effect of growth temperature on cellular fatty acid composition of various SRB. Twelve species of SRB comprising strictly psychrophilic, moderately psychrophilic and mesophilic types (Table 1) were grown at different temperature and subsequently used for fatty acid analysis. Growth temperatures were never above the optimum to avoid damaging effects and responses to unusual stress. Hence, growth experiments with obligately psychrophilic species were only possible within a relatively narrow temperature range. Fatty acid analyses were carried out when cells were still in the course of growth and had reached 3/4 of the maximum (final) optical density. In this way, a certain standardisation was achieved that allowed comparison of analyses without too much possible influence of fresh inoculation or of ageing on fatty acids patterns (see next section). Measured fatty acids patterns were regarded to represent those of the cytoplasmic membrane, because storage fatty acids such as neutral fats have not been found in bacteria (Wilkinson, 1988). The outer membrane usually contributes around 20% to the fatty acids and exhibits nearly the same ratios between fatty acids as the cytoplasmic membrane (Wilkinson, 1988).

The most frequent change observed in the examined species was an increase of *cis*-unsaturated fatty acids with decreasing temperature. However, the change of the cellular fatty acid composition was relatively small in most SRB (Fig. 1). Only four *Desulfobacter* species (*D. curvatus*, *D. hydrogenophilus*, *D. latus*, and *D. postgatei*) exhibited significant variation of the cellular fatty acid composition over the examined temperature ranges (Fig. 1). In species analysed before (Taylor and Parkes, 1983; Vainshtein *et al.*, 1992; Kohring *et al.*, 1994; Knoblauch *et al.*, 1999), the presently observed fatty acid patterns were in agreement with the previous results obtained at a single growth temperature. We confirmed high portions (around 70%) of fatty acids with less than 16 carbon atoms in *Desulfofaba gelida*,

Table 1 Species of sulphate-reducing bacteria included in the study of temperature effects on cellular fatty acids.

Genus and species (DSMZ[a] collection number)	Origin	Temperature for growth[b] (°C)		Organic growth substrate, and medium[c]	Reference
		Range	Optimum		
Strictly psychrophilic					
Desulfofaba gelida (12344)	Arctic marine sediment	-1.8 - 10	7	Propionate, marine	Knoblauch *et al.*, 1999
Desulfofrigus oceanense (12341)	Arctic marine sediment	-1.8 - 16	10	Acetate, marine	Knoblauch *et al.*, 1999
Desulfotalea psychrophila (12343)	Arctic marine sediment	-1.8 - 19	10	Lactate, marine	Knoblauch et al. (1999)
Moderately psychrophilic					
Desulfotalea arctica (12342)	Arctic marine sediment	-1.8 - 26	18	Lactate, marine	Knoblauch *et al.*, 1999
Desulforhopalus vacuolatus (9700)	Temperate marine sediment	0 - 24	18-19	Lactate, marine	Isaksen and Teske, 1996
Mesophilic, psychrotolerant					
Desulfobacter hydrogenophilus (3380)	Temperate marine sediment	0 - 35	29-32	Acetate, salt	Widdel, 1987
Mesophilic					
Desulfobacter curvatus (3379)	Temperate marine sediment	nr	28-31	Acetate, salt	Widdel, 1987
Desulfobacter latus (3381)	Temperate marine sediment	nr	29-32	Acetate, salt	Widdel, 1987
Desulfobacter postgatei (2034)	Brackish sediment	10 - 37	32	Acetate, salt	Widdel and Pfennig, 1981
Desulfovibrio desulfuricans (642)	Wet soil	25 - 40	nr	Lactate, freshwater	Widdel and Pfennig, 1984
Desulfosarcina variabilis (2060)	Mediterranean sediment	15 - 38	28-33	Acetate, salt	Widdel and Pfennig, 1984
Desulfococcus multivorans (2059)	Sewage digestor	15 - 40	28-35	Acetate, salt	Widdel and Pfennig, 1984

a. DSMZ, Deutsche Sammlung von Mikroorganismen (German Collection of Microorganisms and Cell Cultures), Braunschweig.

b. Precise temperature ranges (especially lowest growth temperatures) or temperature optima have not been determined in all cases.

c. All cultures were grown in chemically defined media. The amounts of NaCl, $MgCl_2 \cdot 6\ H_2O$ and $CaCl_2 \cdot 2\ H_2O$ added per litre were as follows: 1 0, 0.4 and 0.1 in freshwater medium; 7, 1.2 and 0.1 in brackish medium; 20, 3 and 0.15 in salt medium; and 26, 5 and 1.4 in marine medium. For details see Widdel and Bak (1992).

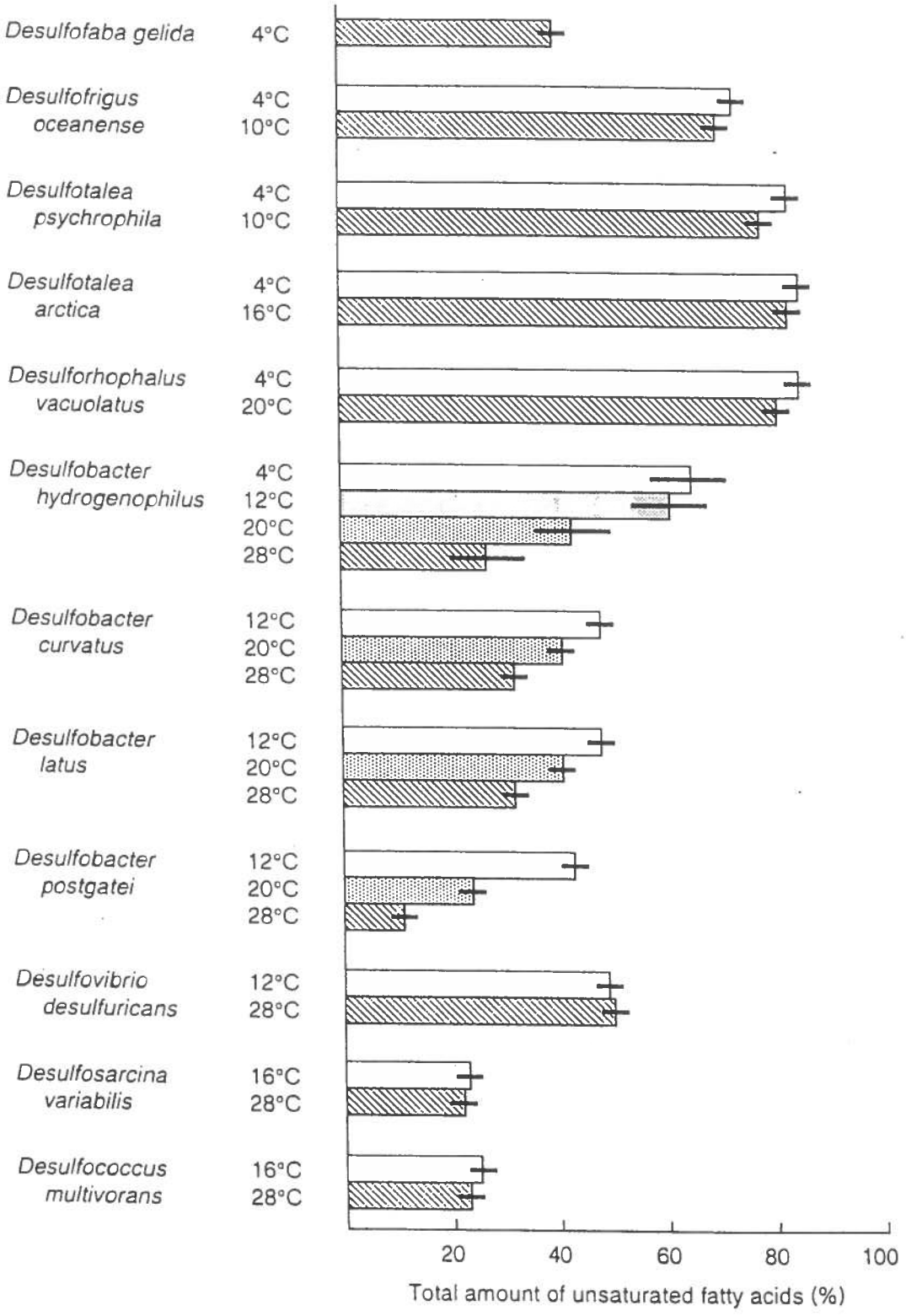

Fig. 1. Relative portions of unsaturated fatty acids among total fatty acids in psychrophilic and mesophilic species of sulphate-reducing bacteria at different growth temperatures. The bars indicate highest variations of fatty acids which were found in comparable analyses.

and high portions (>70%) of *cis*-monounsaturated fatty acids with 16 to 18 carbon atoms in the other psychrophiles. Special, characteristic fatty acids (besides fatty acids with unbranched chains) in mesophiles were iso- and anteiso-branched unsaturated fatty acids in *Desulfococcus multivorans*, *Desulfosarcina variabilis* and *Desulfovibrio desulfuricans*, and 9,10-methylenehexadecanoic acid (*cyc*9 17:0) and 10-methylhexadecanoic acid (10Me 16:0) in *Desulfobacter* species; however, significant portions of *cyc*9 17:0 and 10Me 16:0 were only formed under mesophilic conditions.

Detailed study of fatty acid changes in *Desulfobacter hydrogenophilus*. The fatty acid composition of *Desulfobacter hydrogenophilus* and *D. postgatei* depended not only on the growth temperature (Fig. 1), but also on the time point at which cells were harvested for analyses. Such a change of the fatty acid composition with the growth state of batch cultures was not observed in psychrophilic species (other strains so far not examined). The time course of the variation of the fatty acids at constant temperature was examined in *Desulfobacter hydrogenophilus* in a growth experiment at the optimum temperature of 28°C (Fig. 2). The inoculum was from a stationary phase culture that had been also grown at 28°C. An initial increase of *cis*-9-hexadecenoic acid (*cis*9 16:1) and decrease of hexadecanoic acid (16:0) occurred after inoculation. When growth began to cease due to acetate depletion, the content of *cis*9 16:1 decreased markedly while that of *cyc*9 17:0 and 10Me 16:0 increased.

In another time course experiment we examined the obvious effect of temperature on formation of *cyc*9 17:0 and 10Me 16:0 in *D. hydrogenophilus* (see preceding section) in more detail. The experiment was performed in one culture with subsequent incubation periods at different temperatures. In this way, the experiment could be combined with a ^{13}C-labelling study to prove simultaneously whether an increase of *cis*9 16:1 is due to a *de novo* synthesis (rather than to a postsynthetic modification), similar as in other anaerobes. An initial growth period at 28°C was used for isotope labelling of cellular fatty acids by providing a limiting amount ^{13}C-enriched acetate. After growth had ceased, the temperature was changed to 12°C and another limiting, but non-labelled amount acetate was added. After growth ceased again, the temperature was shifted back to 28°C and acetate was added a third time. Results revealed the following: (a) At 12°C, the fatty acids *cyc*9 17:0 and 10Me 16:0 remained at constant, low level after cessation of growth. Subsequent increase of the temperature to 28°C showed that cells synthesised these fatty acids (Fig. 3A) as in the experiment before (Fig. 2). (b) The ^{13}C-label was incorporated into all cellular fatty acids during the first growth phase at nearly the same extent. In the second growth phase, the amount of *cis*9 16:1 increased due to the

temperature downshift (and eventually partly due to the change caused by acetate addition). Simultaneously, the ^{13}C-content in this fatty acid decreased much more than in any other one (Fig. 3B) indicating a *de novo* synthesis of *cis*9 16:1 from unlabelled acetate. The portion of unsaturated fatty acids such as *cis*9 16:0 in this experiment was somewhat lower than in other cultures of *D. hydrogenophilus* (Fig. 1). This difference is explained by the fact that there was already a relatively high cell density and hence a relatively high pool of saturated fatty acids when the temperature was decreased in the present experiment.

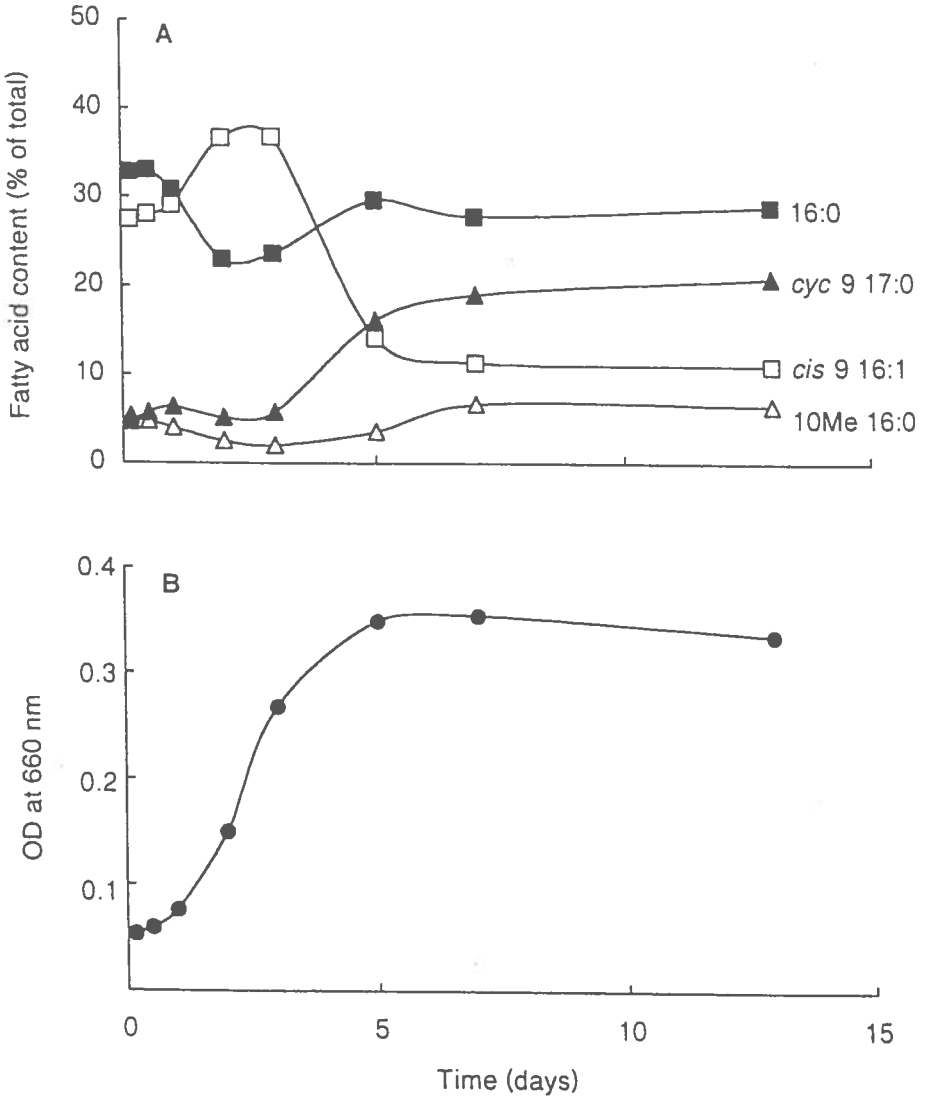

Fig. 2. Time course of temperature-independent changes of characteristic fatty acids *in Desulfobacter hydrogenophilus* after inoculation and incubation at 28°C. The preculture was also grown at 28°C and used for inoculation in the deceleration phase (the phase following the exponential growth phase). A, Results of fatty acid analysis. B, Growth recorded as optical density at 660 nm.

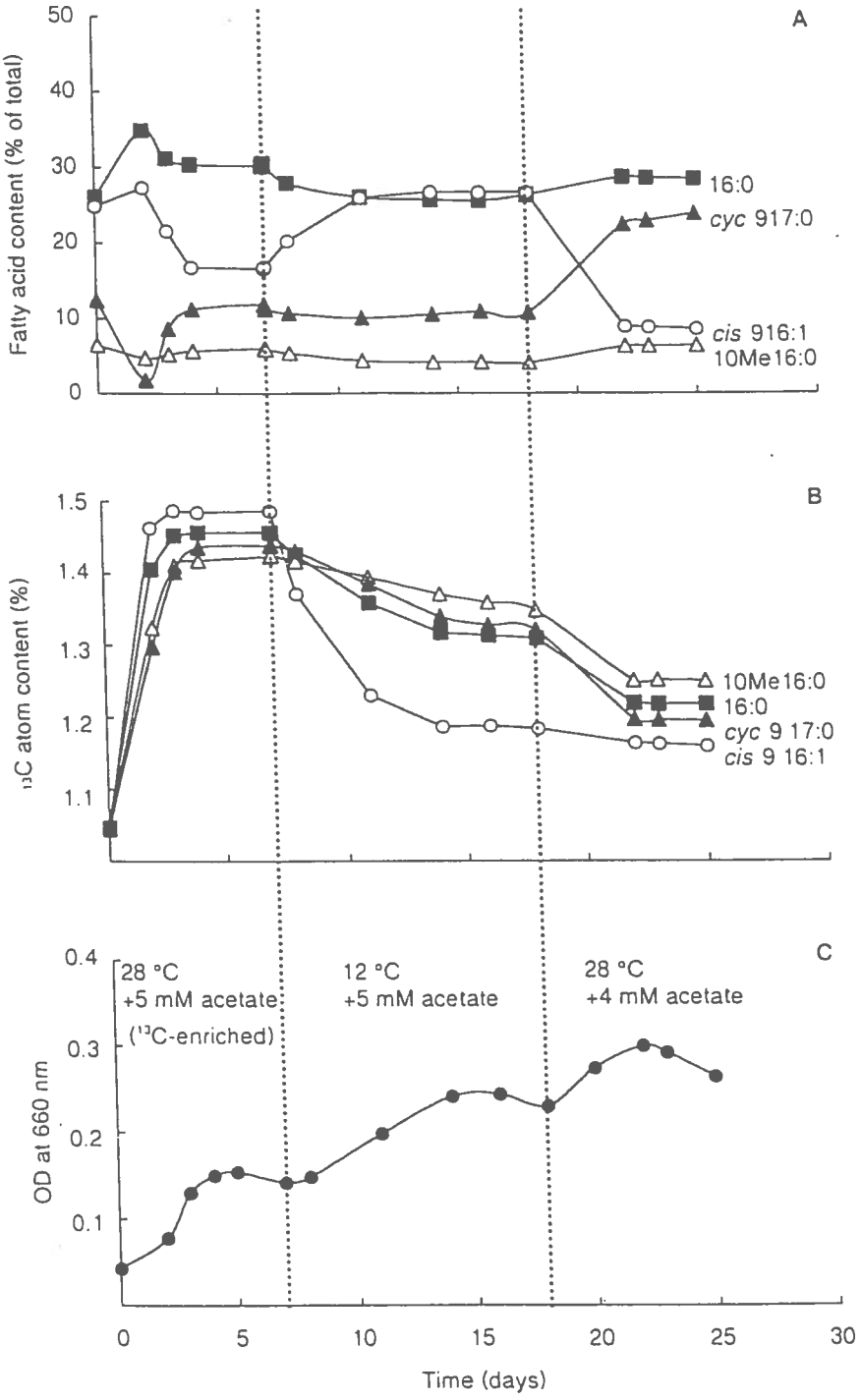

Fig. 3. Time course of the changes of characteristic fatty acids in *Desulfobacter hydrogenophilus* upon temperature shifts. In addition, synthesis of fatty acids was followed by stable isotope labelling. Medium with ^{13}C-enriched acetate was inoculated with a preculture grown at 28°C and first incubated at 28°C. Upon depletion of ^{13}C-enriched acetate (not shown) after 7 days, the culture was shifted to 12°C and non-enriched acetate was added. When acetate was agin depleted after 18 days, the temperature was shifted back to 28°C and non-enriched acetate was added again. A. Results of fatty acid analysis. B, 13C-content of fatty acids. C, Growth recorded as optical density at 660 nm.

Discussion

Cellular fatty acids in SRB have been examined mostly as biomarkers for chemotaxonomy and for the identification of these bacteria in natural habitats (Taylor and Parkes, 1985; Dowling *et al.*, 1986; Vainshtein *et al.*, 1992; Kohring *et al.*, 1994; Boschker *et al.* 1998). An influence of the growth substrate on cellular fatty acids has been demonstrated with some species (Taylor and Parkes, 1983; Dowling *et al.*, 1986). The present study is, to our knowledge, the first one that reveals effects of temperature and of the growth phase on fatty acid patterns in SRB.

The more or less pronounced increase of *cis*-unsaturated fatty acids in most of the investigated SRB with decreasing temperature matches, in principle, observations in other bacteria (for overview see Gounot and Russel, 1999). The by far highest portions of *cis*-unsaturated fatty acids were measured in the psychrophilic species. These bacteria are well-adapted to permanently cold environments, as suggested by relatively high sulfate reduction and growth rates in comparison to mesophilic species at the same low temperature (Knoblauch and Jørgensen, 1999). One may, therefore, assume that the fatty acids pattern in the psychrophiles represents a special, "optimised" adaptation to the permanently cold environment.

Several species revealed little or even no changes of the fatty acid composition with temperature. This may indicate that membrane functioning over a certain temperature range is possible with the same fatty acid composition. There are examples of other bacteria with little response of the fatty acid composition to temperature (e.g., Bhakoo and Herbert, 1980), and it is still a matter of discussion as to which extent membrane fatty acids have to change over a given temperature range to allow full membrane functioning (Jackson and Cronan, 1978; Gounot and Russel, 1999). On the other hand, there might be presently unidentified components or insufficiently understood mechanisms for temperature adaptation of the membrane. In contrast, the *Desulfobacter* species apparently employ a rather sensitive system for regulation of the lipid fatty acid composition; this system is not only triggered by temperature changes but also by other, presently unknown factors that may be endogenic (growth phase dependent) or caused by chemical changes in the medium during growth. Further research is needed to elucidate whether the pronounced ability of *Desulfobacter* to change and to regulate its membrane fatty acid composition offers a special competitive advantage in environments with fluctuation of temperature and other conditions.

A conversion of *cis*9 16:1 to *cyc*9 17:0 in the late growth phase as in *D. hydrogenophilus* has also been documented in other bacteria (Grogan and Cronan, 1997). The formation of cyclopropane fatty acids is a post-synthetic modification of *cis*-unsaturated fatty acids in the lipid bilayer by methyl transfer from *S*-adenosylmethionine and proton elimination (Grogan and Cronan, 1997). The simultaneous decrease of the *cis*9 16:1 with the formation of *cyc*9 17:0 is in agreement with such a mechanism also in *D. hydrogenophilus*. The physiological role of cyclopropane fatty acids and their influence on membrane fluidity is insufficiently understood (Grogan and Cronan, 1997). An assumed biochemical variant of the formation of *cyc*9 17:0 from *cis*9 16:1 is the formation of 10Me 16:0 which involves an additional reduction step (Grogan and Cronan, 1997). 10Me 16:0 has been previously suggested as a potential biomarker for *Desulfobacter* species in sediments (Taylor and Parkes, 1985; Dowling *et al.*, 1986). However, the finding of this fatty acid in other genera of sulfate-reducing bacteria (Vainshtein et al., 1992; Kohring et al., 1994; Kuever et al., 2001) and the presently observed very low content of 10Me16:0 in *Desulfobacter* species at low temperature questions the applicability of this fatty acid as a reliable biomarker. The obvious inhibition of the formation of *cyc*9 17:0 and 10Me 16:0 at low temperature is to the advantage of high levels of *cis*9 16:1 which is particularly suited to decrease the phase transition temperature.

The ^{13}C-labelling experiment with *D. hydrogenophilus* (Fig. 3B) is fully in agreement with a strictly anaerobic metabolism. In contrast to several aerobic bacteria, which can desaturate fatty acids after synthesis of the carbon chain by oxygen-dependent desaturases, anaerobic bacteria cannot convert a saturated to an unsaturated hydrocarbon chain. Anaerobic formation of unsaturated fatty acids always needs *de novo* synthesis. The anaerobic introduction of *cis*-double bonds is assumed to occur during chain elongation by a complex process resulting in 3,4-dehydration of 3-hydroxyacyl-ACP (ACP = acyl carrier protein) when the acyl chain has the appropriate length. The common 2,3-dehydration of 3-hydroxyacyl-ACP followed by reduction leads to saturated lipid fatty acids (Schweizer, 1989).

Experimental procedures

Cultivation. For analysis of the cellular fatty acid composition upon growth at different temperatures (Table 1), SRB were cultivated in chemically defined, bicarbonate-buffered, strictly anoxic media (Widdel and Bak, 1992) with appropriate salinity and organic substrates

(Table 1). Every strain was cultivated at a given growth temperature in parallel batches, each with 230 ml medium under 20 ml of anoxic head space (N_2 + CO_2 [90:10, v/v]); bottles were sealed with black rubber stoppers. Precultures for inoculation (12 ml for each bottles) were grown at the same temperature. Cells were harvested by centrifugation (20 min, 10,000 × g) during the late growth phase from each bottle and separately subjected to fatty acid analysis.

To monitor the time course of changes in the fatty acid composition of *Desulfobacter hydrogenophilus*, the strain was essentially grown as decribed above, however, the culture volume was 2000 ml and the inoculum size was 200 ml. Samples at different time points (Figs. 2 and 3) were taken from the closed, anoxic bottles by application of slight overpressure (10 kPa) of N_2 + CO_2 and the use of an inserted outlet tube. At the initial low cell density, the sample volume was 200 ml; it could be gradually decreased during growth to 50 ml. For stable isotope labelling of the cellular fatty acids, the acetate added as carbon source and electron donor was amended with 0.5 % of the pure ^{13}C-compound (sodium ^{13}C-acetate, >99%; Sigma-Aldrich).

Growth was recorded by measuring both the optical density at 660 nm and produced sulphide; the latter was determined photometrically as CuS (Cord-Ruwisch, 1985).

Fatty acid analysis. Cellular fatty acids in centrifuged cells were methylated by the method of Sasser (1997). Extracted fatty acid methyl esters (FAMEs) were separated by gas chromatography (GC) and quantified via a flame ionisation detector (FID). Identification was based on retention times and mass spectrometry (MS) using authentic FAME standards (BioTrends). GC/FID analysis was carried out with an Autosystem (Perkin Elmer) equipped with an Optima 5 fused silica capillary column (Machery und Nagel). The carrier gas was H_2 (2.8 ml min^{-1}). The oven temperature was run from 60 to 140°C (10°C min^{-1}) and then to 300°C (3°C min^{-1}, finally 15 min isotherm). The FID temperature was 350°C. GC/MS analysis was carried out using the same conditions for gas chromatography, and a GC/Q ion trap (Finnigan MAT). Mass spectra were collected in full scan mode (m/z 30-400) at a transfer line temperature of 280°C, a source temperature of 180°C, and an ionisation energy of 70 eV.

The ^{13}C-content the FAME was measured using gas chromatography (conditions as above) in combination with combustion interfaced isotope ratio mass spectrometry (GC-C-IRMS) on a Finnigan MAT DELTA plus instrument (Finnigan MAT) with an oxidation and reduction temperature of 940 and 600°C, respectively. CO_2 and a long-chain alkane (*n*-$C_{26}H_{54}$) with known isotope ratio were used as standards.

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2

Reclassification of *Desulfobacterium phenolicum* as *"Desulfobacula phenolica"*, comb. nov. and description of strain Sax as *Desulfotignum balticum* gen. nov., sp. nov.

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Abstract

A mesophilic, sulfate-reducing bacterium (strain Sax^T) was isolated from marine coastal sediment in the Baltic Sea and originally described as a *"Desulfoarculus"* sp. It used a large variety of substrates ranging from simple organic compounds and fatty acids to aromatic compounds as electron donors. Autotrophic growth was possible with H_2 and CO_2 and formate in the presence of sulfate. Sulfate, thiosulfate and sulfite were used as electron acceptors. Sulfur and nitrate were not reduced. Fermentative growth was obtained with pyruvate, but not with fumarate or malate. Substrate oxidation was usually complete leading to CO_2, but at high substrate concentrations acetate accumulated. Carbon monoxide dehydrogenase activity was observed indicating the operation of the carbon monoxide dehydrogenase pathway (reverse Wood-pathway) for CO_2 fixation and complete oxidation of acetyl-CoA. The rod-shaped cells were 0.8-1.0 μm in width and 1.5-2.5 μm in length. Spores were not produced and cells stained Gram negative. The temperature limits for growth were between 10 °C and 42 °C (optimum growth at 28-32 °C). Growth was observed at salinities ranging from 5 to 110 g of NaCl per liter, with an optimum at 10 to 25 g NaCl per liter. The G + C content of the DNA is 62.4 mol%. Vitamins were required for growth. Based on the 16S rRNA gene sequence strain Sax^T represents a new genus within the *delta* subdivision of the *Proteobacteria.* The name *Desulfotignum balticum* is proposed.

After the 16S rDNA sequences of all members of the genus *Desulfobacterium* were published (Stackebrandt, 1999), the need to reclassify most members of the genus *Desulfobacterium* became obvious due to their strong phylogenetic affiliation to other genera. Here, we propose to reclassify *Desulfobacterium phenolicum* as *"Desulfobacula phenolica"*.

***Desulfotignum balticum*, *Desulfobacterium phenolicum*, and *"Desulfobacula toluolica"* contain fatty acids which were so far only found in members of the genus *Desulfobacter*.**

Introduction

Among the sulfate-reducing bacteria of the δ-subdivision of the Proteobacteria several marine isolates are known to grow on a large variety of aromatic compounds including phenolic compounds and toluene. They were originally classified as members of the genera *Desulfobacterium* and *"Desulfobacula"* (Bak & Widdel, 1986; 1986a; Brysch *et al.*, 1987; Rabus *et al.*, 1993). Beside the use of various aromatic compounds, members of the genus *"Desulfobacula"* are characteristically restricted to the utilization of short chain fatty acids and simple organic compounds as electron donors; whereas members of the genus *Desulfobacterium* can also grow chemoautotrophically on H_2 and CO_2 (Bak & Widdel, 1986; Bak & Widdel, 1986; 1986a; Brysch *et al.*, 1987; Rabus *et al.*, 1993; Schnell *et al.*, 1989). In contrast, members of the genera *Desulfococcus*, *Desulfonema*, and *Desulfosarcina* only use a limited number of aromatic compounds, mainly benzoic acid derivatives, but also long chain fatty acids as their sole electron donor and carbon source (Fukui *et al.*, 1999; Widdel, 1980; Widdel, 1988; Widdel & Bak, 1992; Widdel & Hansen, 1992; Widdel *et al.*, 1983). Recently obtained isolates with interesting degradation capacities might indicate that in general marine sulfate-reducing bacteria are more versatile than isolates obtained from freshwater habitats (Galushko *et al.*, 1999; Harms *et al.*, 1999; Rueter *et al.*, 1994). However, there are many other sulfate-reducing bacteria (including spore-forming ones) isolated from freshwater habitats which use a large variety of organic compounds as electron donor and are able to grow in marine media (Kuever *et al.*, 1999). Another marine isolate which was isolated with benzoate and tentatively classified as a *"Desulfoarculus"* (the correct spelling would *"Desulfarculus"*) sp. could grow slowly on fatty acids with a chain length higher than butyrate (Drzyzga *et al.*, 1993). Therefore, it would resemble the physiological properties of *Desulfobacterium* and *"Desulfobacula"* spp. In the present paper we describe this species as a new genus within the δ-subdivision of the *Proteobacteria*.

The genus *Desulfobacterium* was established by using mainly physiological properties (Brysch *et al.*, 1987). After the 16S rDNA sequences of all members of this genus were published (Stackebrandt, 1999), the need to reclassify most members of the genus *Desulfobacterium* became obvious due to their strong phylogenetic affiliation to other genera. A comparative analysis using the sequences published by Stackebrandt (1999) indicates that the genus comprises only the type species of the genus *Desulfobacterium autotrophicum* and

two other species; the not validly published *"Desulfobacterium vacuolatum"* (so far not described, only listed in Widdel (1988)) and the originally as *Desulfococcus niacini* (Imhoff and Pfennig, 1983) described *"Desulfobacterium niacini"*. That both species are members of the genus *Desulfobacterium* was already demonstrated by several phylogenetic analyses (Rabus et al., 1993) and by use of the genus-specific oligonucleotide probe 221 (Devereux et al., 1992; Manz et al., 1998). All other members of the genus have to be reclassified, because they belong to other genera or represent new genera. Here we suggest that the former *"Desulfobacterium phenolicum"* be incorporated within the genus *"Desulfobacula"* (Rabus *et al.*, 1993).

Methods

Source of organism. Strain Sax was isolated from a benzoate enrichment culture inoculated with anoxic marine mud from Saxild, Denmark (Drzyzga, et al., 1993). Strain Sax was deposited in the DSMZ under the accession number DSM 7044. *"Desulfobacula toluolica"* (DSM 7467), *Desulfobacterium phenolicum* (DSM 3384), *Desulfobacterium autotrophicum* (DSM 3382), and *Desulfobacter postgatei* (DSM 2034) were obtained from the DSMZ.

Media and culture conditions. For enrichment and cultivation the medium was prepared as described previously using benzoate (2.5 mM) as electron donor (Drzyzga, et al., 1993). Pure cultures were obtained by repeated use of deep agar dilution series (Widdel and Bak, 1992). Substrate utilization was determined by adding the carbon and energy sources from sterile stock solutions; the cultures were incubated for about 3 weeks. In order to avoid possible toxic effects of the substances, toluene and xylene isomers were supplied to cultures following adsorption in the deaerated organic solvent 2,2,4,4,6,8,8-heptamethylnonane (HMN; 2 %, v/v) (Rabus, et al., 1993). A check was made to ensure that HMN did not inhibit growth of strain Sax on benzoate.

To test the capability of autotrophic growth, cultures were grown under an headspace of 80% H_2-20% CO_2 at an overpressure of 101.3 kPa. The temperature range for growth was determined by incubation in a temperature gradient block from 35 to 80 °C in increments of 2-4 °C. The pH range of growth was determined in mineral medium with pH from 5 to 9. The dependence of growth on the concentration of NaCl was determined in mineral medium with NaCl concentrations from 0 to 130 g NaCl per liter. Strain Sax was routinely cultivated in a carbonate-buffered, sulfide-reduced medium for marine sulfate-reducing bacteria supplied

with 2.5 mM of benzoate as a growth substrate. Salt composition of the medium and incubation conditions were as described before (Widdel and Bak, 1992).

Chemical and biochemical characterization. The presence of desulfoviridin was tested as described by (Postgate, 1959). The mol% G + C content was determined by thermal denaturation. Analysis of aromatic compounds, identification of cytochromes, and measurement of sulfide production was described previously (Drzyzga, et al., 1993).

For fatty acid methyl ester (FAME) analysis Strain Sax, *"Desulfobacula toluolica"*, and *Desulfobacterium phenolicum* were grown in marine mineral medium with benzoate (2.5 mM) as only electron donor and carbon source, and sulfate (28 mM) as electron acceptor. *Desulfobacterium autotrophicum* and *Desulfobacter postgatei* were grown with butyrate (10 mM) and acetate (20 mM), respectively. Cells from the late exponential growth phase were centrifuged and used for a comparative FAME analysis. The whole-cell fatty acid composition was determined by means of capillary GC and mass spectral analysis. The preparation of the fatty acid methyl ester was carried out using a method as described previously (Sasser, 1997).

Preparation of cell extract. Strain Sax was grown in 1200 ml bottles containing 1000 ml mineral medium supplied with 2.5 mM benzoate under a gas phase of N_2/CO_2 (80/20 vol. %). Cells from the late exponential growth phase were harvested by centrifugation and washed once in anoxic saline buffer (potassium phosphate 50 mM; pH 7.3; containing NaCl, 26 $g*l^{-1}$; $MgCl_2 * 6H_2O$, 11.2 $g*l^{-1}$). Collected cells were resuspended in anoxic potassium phosphate buffer (50 mM; pH 7.3) supplied with 2.5 mM $MgCl_2 * 6H_2O$ and 2.5 mM dithiothreitol and were passed 2-3 times through an anoxic French press cell at 137 MPa. Cell debris and intact cells were removed from the homogenate by centrifugation (30 000 x g, 20 min). The extract was placed in a small glass vial under N_2 gas phase and stored on ice. Measurements of enzyme activities were done at the day of preparation.

Enzyme assays. All enzyme assays were done under strictly anoxic conditions at 30 °C in 1.5 ml glass cuvettes sealed with butyl rubber stoppers. Assays were performed in 1 ml potassium phosphate buffer (50 mM; pH 7.3) which was slightly reduced by addition of several µl of 0.05 % dithionite solution. All additions were done from anoxic stock solutions with microliter syringes. One unit of enzyme activity was $1\mu mol*min^{-1}$ and mg $protein^{-1}$.

The presence of 2-oxoglutarate: electron acceptor oxidoreductase was checked by following the reduction of benzyl viologen (2 mM) at 578 nm in the presence of 2-oxoglutarate (3 mM) and CoA (0.2 mM).

Activity of CO dehydrogenase was determined by following the reduction of benzyl viologen (5 mM) in the presence of CO. To perform the assay, cuvettes were flushed with CO until the assay buffer was saturated with CO.

Protein content in cell free extract was determined with bichinchoninic acid as a reagent by following standard protocol assay (BCA protein assay kit; Pierce, Germany) and with bovine serum albumin fraction V (Pierce, Germany) as a standard for calibration. Gases were purchased from Messer-Griesheim (Darmstadt, Germany) and Sauerstoffwerke Friedrichshafen (Friedrichshafen, Germany).

PCR amplification and sequencing of the 16S rRNA gene. To amplify the almost complete 16S rRNA encoding gene (1,500 bp) of strain Sax, primers GM3F and GM4R were used in a 35-cycle PCR with an annealing temperature of 40 °C (Muyzer *et al.*, 1995). PCR products were purified by using the QIAquick Spin PCR purification kit (Qiagen, Inc., Chatsworth, Calif.) as described by the manufacturer. The *Taq* Dyedeoxy Terminator Cycle Sequencing kit (Applied Biosystems, Foster Cit, Calif.) was used to directly sequence the PCR products according to the protocol provided by the manufacturer. The sequencing primers have been described previously (Buchholz-Cleven *et al.*, 1997). The sequence reaction mixtures were electrophoresed on an Applied Biosystems 373S DNA sequencer.

Phylogenetic analyses of 16S rRNA gene sequence data. The sequences were loaded into the 16S rRNA sequence data base of the Technical University of Munich using the program package ARB (Strunk et al. 1999). The tool ARB_ALIGN was used for sequence alignment. The alignment was visually inspected and corrected manually. Tree topologies were evaluated by performing maximum parsimony, neighbor joining, and maximum likelihood analysis with different sets of filters. Only sequences with at least 1200 nucleotides were used for the calculation of different trees. The partial sequence of strain Sax (1462 nucleotides) was added to the reconstructed tree by applying parsimony criteria without allowing changes in the overall tree topology. The strain designations and nucleotide sequence accession numbers which were not included in the ARB database are as follows: "*Desulfobacula toluolica*^{T}" DSM 7467, X70593; *Desulfobacterium phenolicum*^{T} DSM 3384,

AJ237606 (submitted by E. Stackebrandt); *Desulfospira joergensenii*[T], DSM 10085; X99637; *Desulfotignum balticum* (strain Sax), DSM 7044, AF 233370; Clone SB-9, AF029042; Clone Sva0605, AF230098.

Nucleotide sequence accession number. The nearly complete 16S rRNA sequences of strain Sax (DSM 7044) is AF233370.

Results

FAME analysis. The fatty acid composition of strain Sax and its phylogenetically closest relatives are listed in Table 1. Significant amounts of unidentified fatty acids (ECL16.07, ECL 18.09) were found in *"Desulfobacula toluolica"* (7.5 %) and *"Desulfobacula phenolica"* (16.6 %) and also in traces in strain Sax. The FAME analysis for *"Desulfobacula toluolica"* grown on ethanol was very similar to our results (van der Maarel et al., 1996).

Enzyme activities. In cell free extracts of strain Sax active CO dehydrogenase was found (2.6 U * mg protein^{-1}), whereas 2-oxoglutarate dehydrogenase (a key enzyme of the citric acid cycle) was not detected. This finding indicated the presence of the CO dehydrogenase pathway for the oxidation of acetyl-CoA (Thauer, 1988).

Table 1 Major cellular fatty acids in % of strain Sax and some phylogenetically related species.

Fatty acid	strain Sax	*"Desulfobacula phenolica"*	*"Desulfobacula toluolica"*	*Desulfospira joergensenii*[a]	*Desulfobacterium autotrophicum*	*Desulfobacter postgatei*
14:1	-	1.4	-	-	-	-
14:0	3.7	8.7	8.3	13.9	2.8	15.4
i15:0	-	-	-	1.8	-	0.7
15:1 *c*9	0.8	-	-	-	6.5	-
15:0	0.2	2.0	1.6	1.3	5.1	3.1
3OH14:0	-	1.3	-	1.6	-	-
16:1 *c*9	8.5	16.5[b]	19.7	38.9	30.7	10.0
16:0	24.2	20.4	31.2	28.4	13.2	20.9
10Me16:0	14.0	17.2	17.6	-	7.4	11.2
17:1 *c*11	-	-	-	-	10.5	-
17:0 cyc	19.5	3.9	2.6	2.4	-	31.5
17:0	0.4	0.7	0.7	0.6	2.9	2.3
3OH16:0	-	-	-	2.3	0.5	-
18:1 *c*9	3.7	-	-	-	-	-
18:1 *c*11	7.4	3.9	6.0	5.3	2.7	1.2
18:0	9.8	2.1	3.1	0.7	1.1	0.5
i19:0	1.7	-	2.5	-	-	-
19:0 cyc	1.1	-	-	-	-	-

Percentage of total fatty acids are shown. Fatty acids present in all strains at less than 1 % are not listed. *c*, *cis*; i, iso; cyc, cyclopropane; OH, hydroxy; Me, methyl.

Physiological and morphological properties. All physiological and morphological properties of strain Sax, *Desulfobacterium phenolicum* and *"Desulfobacula toluolica"* are listed in Table 2 and compared to the closely related species *Desulfospira joergensenii*. For a more detailed description the original publications should be used (Bak & Widdel, 1986; Drzyzga, et al., 1993; Finster *et al.*, 1997; Rabus, et al., 1993).

Strain Sax did not grow on toluene, or o-, m-, p- xylenes supplied adsorbed in HMN, nor did this organic solvent inhibit growth of the bacterium on benzoate.

Phylogenetic analyses. The 16S rRNA gene sequence of strain Sax shares less than 94.1 % identity with the 16S rRNA gene sequences of other sulfate reducers of the delta subdivision of the *Proteobacteria* (data not shown). The phylogenetic position of strain Sax and its closest relatives is shown in Figure 1. The closest affiliation was found with a similarity value of 96.6 % to clone SB-9 (Phelps *et al.*, 1998), followed by *Desulfospira joergensenii* (94.1 %) (Finster, et al., 1997) and *Desulfobacterium phenolicum* (93.9). As can be seen in Figure 1 the sequence of clone Sva 0605 obtained from permanently cold sediments at Svalbard falls in close proximity to these organisms (Ravenschlag *et al.*, 1999). Clearly, they are all members of the delta subdivision of the *Proteobacteria*. Furthermore, the comparative phylogenetic analyses indicated *Desulfobacterium phenolicum* to be a member of the genus *"Desulfobacula"*. It was closely related *to "Desulfobacula toluolica"* (98.8 % similarity of their 16S rRNA sequence), which is the only species of this genus so far (Figure 1). Based on the sequence of the 16S rRNA, strain Sax could not be affiliated with any of the other genera and was therefore proposed as a new genus in its own right.

Table 2 Comparison of selected characteristics of *"Desulfobacula toluolica"*, *"Desulfobacula phenolica"* (formerly *Desulfobacterium phenolicum*), *Desulfospira joergensenii*, and strain Sax^T.

Characteristic	*"Desulfobacula toluolica"*	*"Desulfobacula phenolica"*	*Desulfospira joergensenii*	Strain Sax^T
Morphology	Oval	Oval to curved rod	Vibrio	Rod
Width x length (µm)	1.2-1.4 x 1.2-2.0	1-1.5 x 2-3	0.7-0.8 x 1-2	0.5-0.7 x 1.5-3.0
Motility	+*	+(sp)	-	+(sp)
G + C content (mol%)	42	41	50	62
Desulfoviridin	-	-	-	-
Major menaquinone	NR	MK-7(H_2)	MK7 and MK-7(H_2)	NR
Salinity optimum (g/l)	20	20	12-20	20
Optimal temperature (°C)	28	28	26-30	28-32
Oxidation	C	C	C	C
Electron donors:				
H_2	-	-	+	(+[a])
Formate	-	(+)	+[a]	(+[a])
Acetate	-	(+)	-	(+)
Fatty acid : C atoms	4	(4)	4, 8, 12, 14	4-(10, 12, 16, 18)
Isobutyrate	-	-	-	-
2-Methylbutyrate	-	-	-	-
3-Methylbutyrate	-	-	-	-
Ethanol	+	(+)	-	-
Lactate	-	-	+	+
Pyruvate	+	+	+	+
Fumarate	+	(+)	+	+
Succinate	+	(+)	+	(+)
Malate	+	(+)	-	+
Benzoate	+	+	-	+
4-Hydroxybenzoate	+	+	-	+
Phenol	-	+	-	(+)
Phenylacetate	-	+	-	(+)
toluene	+	(+)	-	-
Others	Propanol, butanol, p-cresol, glutarate	Propanol, butanol, 2-hydroxybenzoate, p-cresol, glutarate	Crotonate, glutarate, maleinate, glycolate, glycerol, betaine, proline, yeast extract	Crotonate, maleinate
Fermentative growth on:	NR	NR	-	Pyruvate
Electron acceptors:				
Sulfate	+	+	+	+
Sulfite	NR	-	+	+
Sulfur	NR	-	+	NR
Thiosulfate	NR	+	+	+
Nitrate	NR	-	-	-
Growth factor requirement	Vitamins	-	Biotin	Vitamins

*Cells are motile during exponential growth phase, but motility can rapidly decline during growth. NR, Not reported; +, good growth; (+), poor growth; -, no growth; [a], autotrophic growth; sp, single polar flagellum. Data obtained from Bak and Widdel (1996a), Drzyzga et al. (1993), Finster et al. (1997) and Rabus et al. (1993). All strains were negative for desulfoviridin and for growth on isobutyrate, 2-methylbutyrate and 3-methylbutyrate and could use sulfate as electron acceptor. Substrate oxidation was complete for all strains.

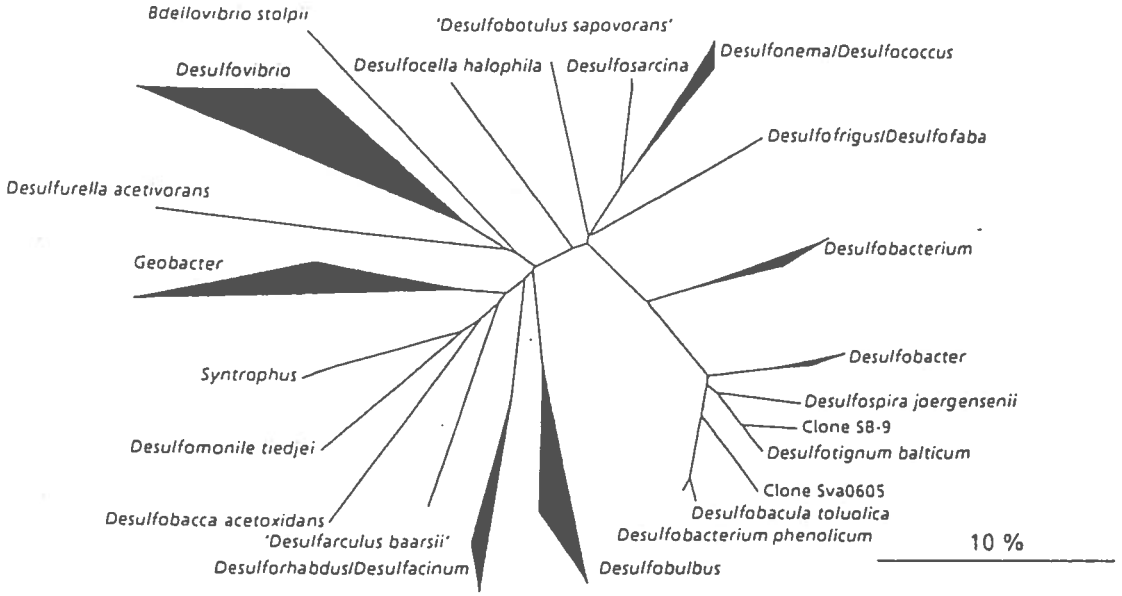

Figure 1 Phylogenetic tree showing the affiliations of 16S rDNA sequences from *Desulfobacula phenolica* and *Desulfotignum balticum* to selected reference sequences of the delta subdivision of the *Proteobacteria*. The tree was calculated by neighbor-joining analysis and corrected with filters which considered only 50% conserved regions of the 16S rRNA of _-Proteobacteria. The sequence of *Desulfurella acetivorans* was used as out group. The bar represents 10% estimated sequence divergence.

Discussion

The physiological and morphological properties (Table 2) in combination with the comparative 16S rDNA and FAME analysis (Figure 1 and Table 1) clearly demonstrate that *Desulfobacterium phenolicum* is a member of the genus "*Desulfobacula*". It can be clearly distinguished from "*Desulfobacula toluolica*" by its motility, morphology, missing vitamin requirement and use of different electron donors for sulfate-reduction. Therefore, we propose to rename it as *Desulfobacula phenolica*.

In view of the 16S rRNA gene sequence analyses presented in this study strain Sax should be regarded as belonging to a new genus, since it was not closely related to other genera (a maximum of 94.1% 16S rRNA sequence identity). The distinct morphological and physiological properties (Table 2) in accordance with the FAME analysis (Table 1) argue in the same direction. Both "*Desulfobacula*" species and strain Sax contain relative high amounts of the fatty acid 10Me16:0, whereas this fatty acid is completely absent in *Desulfospira joergensenii* (Finster, et al., 1997). The finding of this fatty acid in

"Desulfobacula toluolica" is consistent to the results obtained by van der Maarel and coworkers (1996). Strain Sax can be distinguished from both *"Desulfobacula"* species by the high amount of the cyclopropane fatty 17:0, which is also a dominant fatty acid in *Desulfobacter* spp. Therefore, the designation *Desulfotignum balticum* is proposed for strain Sax^T.

Ecological relevance of members of the genera *"Desulfobacula"* and *Desulfotignum*. The cellular fatty acid composition of strain Sax shows the highest similarity to that of *Desulfobacter postgatei*. It is distinguished from the composition of *"Desulfobacula toluolica"* and *"Desulfobacula phenolica"* by the presence of cyc17:0. Nevertheless strain Sax, *"Desulfobacula phenolica"* and *"Desulfobacula toluolica"* contain significant amounts of 10Me16:0 which was previously described as a biomarker for *Desulfobacter* spp. (Dowling *et al.*, 1986) and was also found in lower amounts (< 10 %) in *Desulfobacterium autotrophicum* (Vainshtein *et al.*, 1992). Therefore, the use of this fatty acid as a specific biomarker for microorganisms belonging to the genus *Desulfobacter* should no longer be considered reliable. The presence of this fatty acid in marine sediment could also account for sulfate-reducing bacteria belonging to the aforementioned genera.

Emended description of the genus *Desulfobacula* (Rabus, et al., 1993). The sentence about the motility should be changed into: Oval cells which may be motile or nonmotile.

Description of *Desulfotignum* gen. nov.

Desulfotignum (De.sul.fo.tig´num. L pref. de from, L. n. sulfur sulfur; M.L. n. tignum stick; M.L. neut. n. Desulfotignum sulfate-reducing stick). Cells are straight sometimes slightly curved rods that are motile. They are strict anaerobes, using sulfate as the terminal electron acceptor that is reduced to sulfide. Aromatic compounds, fatty acids and a number of low-molecular-weight aliphatic acids may be utilized as electron donor. Autotrophic growth on H_2 plus CO_2 and formate. Electron donors are completely oxidized to CO_2 via the carbon monoxide dehydrogenase pathway. *Desulfotignum* belongs to the delta subdivision of the Proteobacteria; the closest relative are *Desulfobacula* and *Desulfospira* spp. The type strain of the genus is *Desulfotignum balticum* (= DSM 7044).

Description of *Desulfotignum balticum* sp. nov.

Desulfotignum balticum (bal.ti´cum. M.L. neut. adj *balticum* from the Baltic Sea, pertaining to location of the sampling site). Cells are short rods, 0.5-0.7 by 1.5-3.0 µm. Spore formation is absent. Cells are motile. Gram stain reaction of cells is negative. Strict anaerobe. Growth on H_2/CO_2, formate, acetate, butyrate, crotonate, straight long chain fatty acids up to C_{18}, lactate, pyruvate, fumarate, succinate, maleinate, malate, benzoate, 4-hydroxybenzoate, phenol, phenylalanine, and phenylacetate. Substrate oxidation is usually complete leading to CO_2, but at high substrate concentrations acetate can accumulate. Electron acceptors used are sulfate, sulfite and thiosulfate. Not used: nitrate and nitrite. Slow fermentative growth on pyruvate. Addition of at least 10 g/l of NaCl is necessary. Optimum NaCl concentration for growth is 20 g/l. NaCl is tolerated up to 110 g/l. Vitamins are required for growth. Temperature requirements: T_{min}, 10 °C; T_{opt}, 28-32 °C; T_{max}, 42 °C. The pH range for growth is 6.5 to 8.2; pH optimum at 7.3. The G + C content of the DNA is 62.4 mol% (T_m). The GenBank accession number for the 16S rRNA gene sequence is AF233370. The type strain is Sax^T (=DSM 7044).

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3

Aerobic and sulfate-reducing bacterial communities of Arctic sediments characterized by phospholipid analysis and cultivation methods

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Abstract

Phospholipid fatty acid (PLFA) analysis in permanently cold sediments from fjords of Svalbard (Arctic Ozean) showed that the total microbial biomass determined as lipid-bound phosphate decreased with depth. Polyunsaturated PLFAs of eukaryotes and PLFAs generally characteristic of aerobes had highest concentrations in the top 5 cm of the sediments. Branched-chain PLFAs indicative of facultatively and obligately anaerobic bacteria were most abundant below that depth. Two specific biomarkers for sulfate-reducing bacteria (i17:1 and 10Me16:0) were present at low concentration with an absolute maximum in the top 0-1 cm but with the highest relative amounts in a depth of 10 cm. High amounts of unsaturated PLFA (>60%) typical for cold-adapted organisms were found in the surface sediment and decreased with depth in the anoxic sediment. A reduced diversity of PLFAs with depth reflects a decrease of the microbial diversity in the deeper sediment, probably associated with the limited quality and quantity of electron donors and electron acceptors. The abundance of bacteria as estimated by most probable number (MPN) counts over different horizons correlated well with the PLFA profiles. The cell numbers of aerobic heterotrophic bacteria and of sulfate-reducing bacteria (SRB) were highest in the top layer. Isolates obtained from the highest dilutions representing the most abundant culturable organisms among these physiological types were identified as members of the genera *Moritella*, *Pseudomonas*, *Psychromonas* and *Desulfotalea*. Pure cultures isolated with hydrogen as electron donor represented new taxa of the families *Desulfobacteraceae* and *Desulfobulbaceae* within the δ-subclass of *Proteobacteria*. All isolates showed fastest growth below 20 °C which indicated the dominance of cold-adapted bacteria in this permanently cold environment.

Introduction

Measurements of the sulfate reduction rate in permanently cold sediments of the Arctic Sea have been found to be comparable with those of temperate sediments [1]. From the same location, new psychrophilic sulfate-reducing bacteria (SRB) were selectively isolated at low temperatures with acetate, lactate and propionate [2, 3] which are typical products of the anaerobic food chain and common substrates for SRB [4]. Further studies in the cold sediments, using molecular methods have shown a high abundance of SRB; most of them are unknown species, which have not been isolated before [5-7]. These findings indicate the importance of SRB for the remineralization of organic matter in the cold sea floor, and that the SRB community of the Arctic is dominated by psychrophiles [2]. Members of the genera *Desulfovibrio* and *Desulfobacter* , that are often found in marine sediments were only detected at low numbers [8] and were not among the isolates obtained.

Phospholipid analysis of environmental samples has often been used as an alternative approach to molecular methods for the quantification of the microbial community structure that does not depend on the cultivation of organisms and that is free of potential selections of the biochemical compound to be analysed [9]. Specific signature phopholipid fatty acids (PLFA) have been reported for a number of eukaryotic and prokaryotic organisms and can be used as biomarkers in different habitats [10-16]. Genus-specific fatty acid patterns of SRB have been found, containing 2-methyl-hexadecenoic acid (i17:1) for *Desulfovibrio* species and the 10-methyl-hexadecanoic acid (10 me16:0) for members of the genera *Desulfobacter*, *Desulfobacterium* and *Desulfobacula* [17-20]. These specific fatty acids were often described as SRB biomarkers in marine habitats [21-23], but the practical application is limited because the cellular fatty acid compositions of SRB in pure cultures can be influenced by the type of carbon source [17, 24], by the growth phase, and by temperature [Könneke and Widdel, unpublished data].

In addition to molecular community structure analyses, PLFA profiles can elucidate physiological features of microbial communities *in situ*, such as the adaptation of the lipid membrane composition to changing temperature. A well described adaptation of eukaryotes and prokaryotes is the regulation of the fluidity of their membrane to low temperatures by an increase in the amount of unsaturated fatty acids or an incorporation of short-chain fatty acids into the lipid membrane [25, 26].

In the present study, we investigated the microbial communities of four permanently cold marine sediments of north-western Svalbard by the use of cultivation-independent PLFA analyses. The total viable biomass was estimated from the total amount of lipid bound phosphate for different sediment layers.

The PLFA profiles were compared with MPN-counts of SRB and aerobic heterotrophic bacteria, two of the most abundant bacterial groups. For the cultivation and isolation of SRB, hydrogen and formate were used as electron donors. Both are common end-products of

fermentative bacteria and can be utilized by methanogens, homoacetogens, or especially in marine environments due to the high sulfate concentration, by SRB [27]. Hydrogen together with a low concentration (1-2 mM) of acetate as carbon source should be selective for the isolation of *Desulfovibrio* species [28]. Aliquots of the highest positive dilution of the MPN series were used for the isolation of the most abundant bacteria at low temperatures. The physiological properties and phylogenetic position were determined.

Material and Methods

Sampling site. Four permanently cold sediments located at the north-western coast of Svalbard, were sampled during a cruise in July of 1998: Isfjorden (78°10´907N, 14°34´124E), Kongsfjorden (78°55´259N, 12°17´222E), Krossfjorden (79°16´535N, 11°58´065E), and Smeerenburgfjorden (79°42`815N, 11°05`189E). Water depths of the sampling sites were between 100 and 246 m, with bottom water temperatures around 0 °C. The sediment was collected with a Haps corer, subsampled with acrylic cores and plugged with rubber stoppers. The subcores were transported at 1-4°C over 1-3 days, until further processing.

PLFA analysis. The analytical procedure involved one-phase extraction, fractionation on silicic acid, derivatization to methyl-esters, and analysis by capillary gas chromatography.

Total lipids from the sediment were extracted as described by Bligh and Dyer [29] with some modifications. The total lipids were seperated on a silicic acid column (Isolute SPE column, IST Mid Glamorgan, UK). The eluents, having different polarity, were dichlormethane, acetone, and methanol. The methanol fraction contained the polar phospholipids, which were used for further analysis.

A mild alkaline methanolysis was used to transmethylate the ester linked fatty acids of phospholipids to methyl esters [30]. Nonadecanoic acid methyl ester was used as an internal standard.

A 2.0 µl sample volume was injected splittless onto a fused silica capillary column (Optima-5-MS, Macherey und Nagel, Düren, Germany). The fatty acid methyl esters were identified by a mass spectrometer (GC-Q, Finnigan, Bremen, Germany) and quantified by a flame-ionisation-detector (GC-Autosystem, Perkin Elmer, Überlingen, Germany). The GC-temperature-program was described previously [30]. The mass spectra and retention times of 68 fatty acid methyl esters were determined by injection of authentic standards (Supelco, Deisenhofen, Germany and BioTrend, Köln, Germany). We used a fatty acid nomenclature in following form: x A:B, where A designates the total number of carbon atoms, B the number of double bonds, and x the distance of the closest double bond to the carboxyl group. The suffixes *c* for *cis* and *t* for *trans* refer to the geometric isomers of the double bond, whereas i (iso) and a (anteiso) indicate the position of a methyl group in branched fatty acids.

Determination of biomass by phosphate analysis of PLFA. After extraction, aliquots of all samples were used for calorimetric phosphate analysis to determine the viable biomass [31].

Cultivation and isolation of bacteria. For liquid master dilution series, 27 ml anoxic artificial seawater medium with no organic substrates was inoculated with 3 ml sediment of each sampling site, mixed for several minutes and transferred into the next dilution flask by a volume ratio of 1:9 (inoculum:media). For most probable number (MPN) estimates, we used these master dilution series to inoculate triplicate MPN-series of aerobic heterotrophic and sulfate-reducing bacteria. All cultures were incubated at 4 °C over different time periods, depending on the physiology of the organisms.

The SRB were cultured in a defined saltwater medium as described by Widdel and Bak [28], with hydrogen (0,75 bar overpressure of a mixture of 90% (v/v) H_2 and 10% (v/v) CO_2) or formate (20 mM) as electron donor. Acetate at a final concentration of 1 mM was added as an alternative carbon source for growth. Growth of SRB was determined by microscopy and via sulfide formation measured by the method described by Cord-Ruwisch [32]. Pure cultures were isolated from the highest dilution that showed growth using the deep agar dilution technique [28].

A complex organic medium was used for MPN counts of aerobic heterotrophic bacteria (Marine Broth 2216, Difco). During growth the cultures were shaken two times a day. Growth was determined by measuring turbidity and by microscopy. For isolation of the most abundant organisms, an aliquot of the highest diluted positive tube was transferred onto agar plates and streaked out. Colonies were transferred at least three times before considered pure.

The MPN calculations were based on the method published by the American Public Health Association [33].

Isolation of nucleic acid and sequencing. Nucleic acids were isolated from liquid cultures after centrifugation (5min, 10000 x g) as described by Isaksen and Teske [34]. The 16S rRNA gene was amplified by PCR using primers GM3F (5´-AGAGTTTGATC(A/C)TGGC_3´) and GM4R (5´-TACCTTGTTACGACTT-3´) as described previously [34].

PCR products were sequenced by AMODIA Bioservice GmbH (Braunschweig, Germany) by the use of an automatic sequencer (Li-Chor 4200, MWG-Biotech AG, Ebersberg).

Phylogenetic analysis of 16S rRNA gene sequence data. The sequences were loaded into the 16S rRNA sequence data base of the Technical University of Munich using the program package ARB [35]. The tool ARB_ALIGN was used for sequence alignment. The alignment was inspected and manually corrected. Tree topologies were evaluated by performing maximum parsimony, neighbor joining, and maximum likelihood analysis with different sets of filters. Only sequences with at least 1300 nucleotides were used for the calculation of different trees. The nucleotide sequence accession numbers which were not included in the ARB database are as follows: *Desulfobacula phenolica*^{T}, AJ237606; *Desulfospira joergensenii*^{T}; X99637; *Desulfotignum balticum*^{T}, AF 233370; *Desulforhopalus vacuolatum*^{T}, L42613; *Desulfobacterium catecholicum*^{T},

AJ237602; *Desulforhopalus singaporensis*, xxxx; *Desulfocella halophila*^{T}, AF022936; *Desulfofaba gelida*^{T}; AF099063; *Desulfofustis glycolicus*^{T}, X99707; *Desulfocapsa sulfoexigens*^{T}, Y13672; *Desulfotalea arctica*^{T}, AF099061; Strain LSv53, AJ241014; Strain LSv23, AF099059; Strain LSv24, AF099060; Strain DHA1, xxxxx; Strain G1HA, xxxxx; Strain JHA1, xxxxxx; Clone SVA0632, AJ241014; Clone Sva0113, AJ240982; Clone Sva0999, AJ241013; DGGE band h, L40787.

Results and Discussion

Estimation of the viable biomass. For all four fjords the highest content of lipid-bound phosphate, as a measure of the total microbial biomass, was found in the top layer of the sediments (Figure 1). The phospholipid phosphate decreased with depth and changed from 70 to 10 nmol/g sediment dry weight, which is in the range observed also from other sedimentary environments [36]. Based on the assumption that 1 nmol phosphate is equivalent to 3.4 x 10^7 cells [31], a total cell number for eukaryotic and prokaryotic microorganisms of 1.5 x 10^9 cells per gram dry weight sediment in the top layer can be calculated, which is in the range of cell numbers determined by molecular methods [8]. The total biomass for different depth intervals varied between the four sampling sites. A very pronounced decrease with depth was found for samples from Isfjorden and Krossfjorden, which are both located near the open sea, whereas sediment samples from Kongfjorden and Smeerenburgfjorden showed less decrease with depth and are more strongly influenced by terrestrial input of organic and inorganic matter from the neighboring glaciers.

Vertical distribution of signature PLFA for functional microbial groups. The more detailed PLFA profiles of the sediment from Krossfjorden is shown in Figures 2-4, however, similar tendencies were found at all other sampling sites. Specific signature PLFA, previously described for aerobic organisms and for anaerobic bacteria were grouped together into different plots (Figure 2). Polyunsaturated fatty acids, c5 20:4 and c5 20:5 which have often been found in significant amounts in diatoms and other eukaryotes [37], were abundant in the upper sediment layers. The c4 22:6 , a typical fatty acid of eukaryotes [9] decreased with depth. The same fatty acid has been detected in psychrophilic/barophilic bacteria that have been isolated from different cold marine environments [38]. The polyunsaturated PLFAs were extremely low in the deepest horizon. In contrast to the polyunsaturated PLFAs, the relative amounts of iso and anteiso branched PLFAs, specific for facultative or strict anaerobic bacteria, increased with depth and reached a maximum at 10-11 cm. The bacterial specific fatty acid a15:0 [39], was the most abundant branched PLFA with a relative amount of up to 10%. All together, the data imply that a large fraction of the biomass of the upper sediment layer originated from eukaryotes and aerobic bacteria, either actively growing in the sediment or deposited from the overlaying water column, and

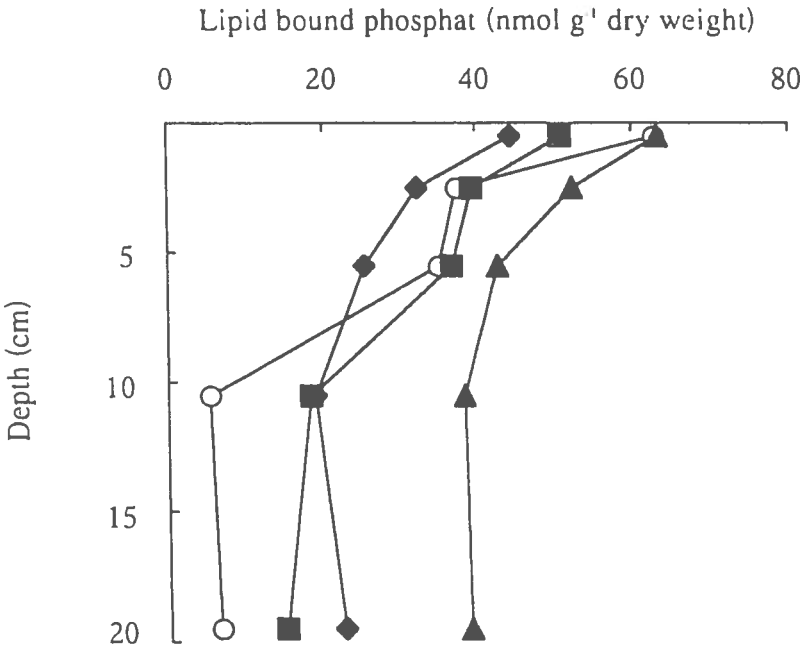

Figure 1 Depth-profiles of lipid-bound phosphate as indication for viable biomass in Isfjorden (○), Kongsfjorden (◆), Krossfjorden (■) and Smeerenburgfjorden (▲), permanently cold Arctic fjords of north-western Svalbard.

aerobic bacteria feeding on them. Below a depth of 6 cm the microbial community seemed to be dominated by facultative or obligate anaerobic bacteria.

SRB specific biomarker. The absolute and relative abundances of 10Me16:0 and i17:1, specific signature PLFAs for *Desulfobacter* and *Desulfovibrio* spp., are shown in Figure 3. In comparison to PLFA profiles of temperate estuarine sediments from different locations [9, 40], the low amounts of both biomarkers show that these SRB play a minor role in cold marine environments. The highest absolute abundance was detected in the top sediment and a five fold lower amount at a depth of 20 cm, whereas the maximum relative abundance was found in the deep horizon. These data agree with previous molecular studies from the same environment [8].

A quantification of SRB by specific biomarkers is, however, not as reliable since pure culture studies have shown that the synthesis of 10Me16:0 in *Desulfobacter* species inhibited by low temperatures [Könneke and Widdel, unpublished data]. The absolute amount of 10Me16:0 declined with depth in association with the total biomass and MPN-counts, whereas i17:1 was found in same concentrations at depths from 2 to 10 cm, yet with a relative maximum at 10 cm.

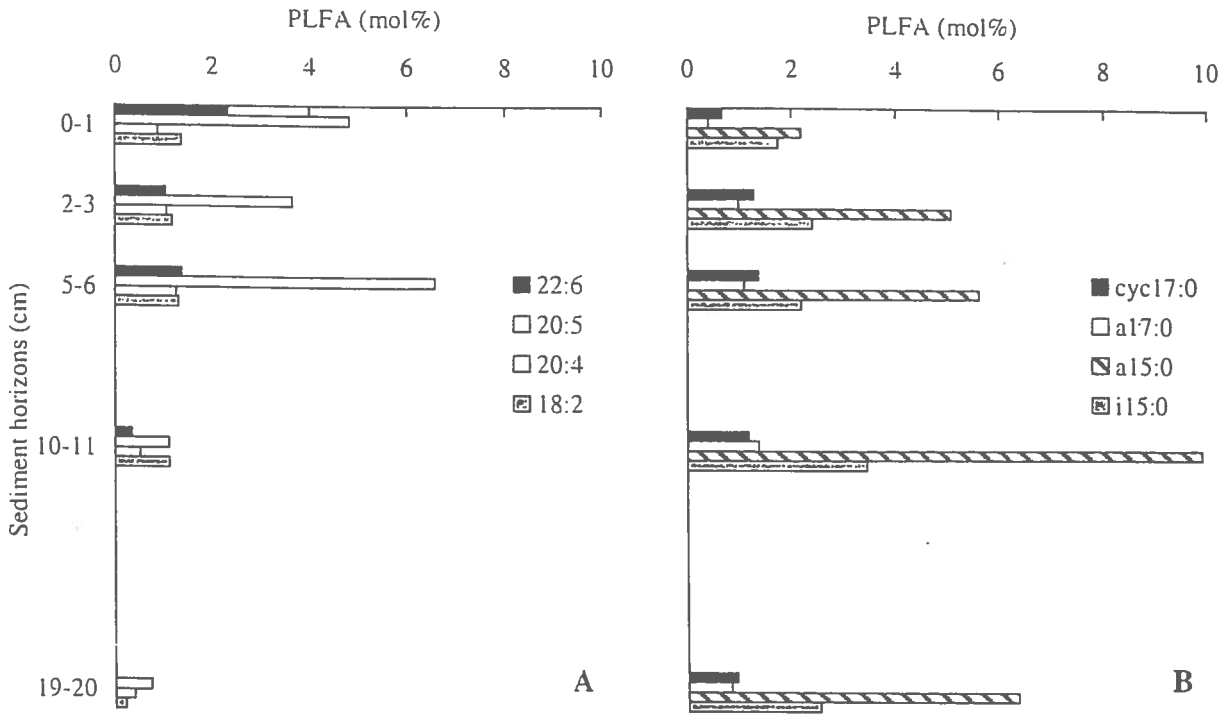

Figure 2 PLFA profile of selected biomarkers for (A) aerobic eukaryotes and prokaryotes, and (B) facultative and obligate anaerobic bacteria. The relative amounts of PLFA are shown in different horizons of sediment sampled from Krossfjorden.

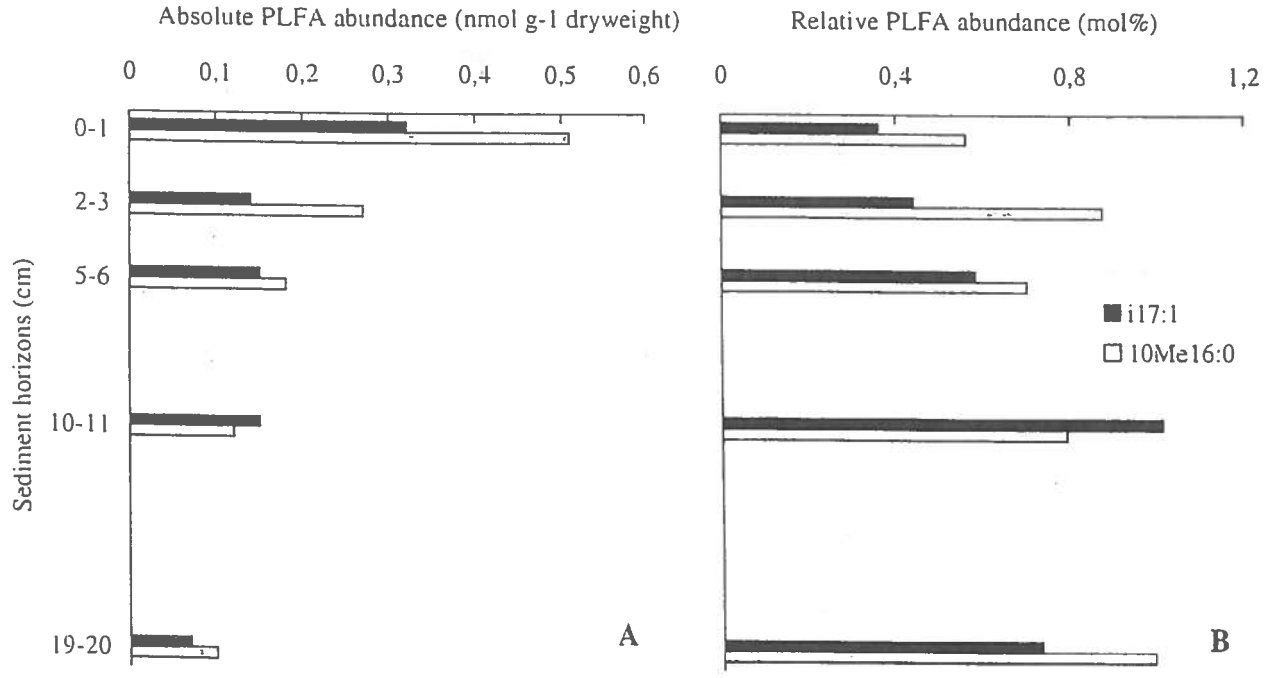

Figure 3 PLFA profiles of 10-methyl-pentadecanoate (10Me16:0) and 2-methyl -hexadecenoate (i17:1) as specific biomarkers of SRB of the Genera *Desulfobacter*/*Desulfobacterium*/*Desulfobacula* and *Desulfovibrio*, respectively. The signature PLFA are shown in (A) absolute abundance and (B) relative abundance in different horizons of sediment sampled from Krossfjorden.

The largest amount of total unsaturated PLFAs (mono-unsaturated 50%, poly-unsaturated 12%) was detected in the top layer of the sediment (Fig 4A). The relative amounts of total unsaturated PLFA decrease with depth to 55 % between 2 to 6 cm, and to about 40% below a depth of 10 cm. The total amount of branched PLFAs, originate from bacteria, increase with depth from 7% in the top to 16% in the depth of 20 cm (Fig. 4A). The most of the PLFA consist of even-numbered acyl chains (> 80%). A slight increase of acyl chains with 14 carbon atoms were found with increasing depth.

The fatty acid composition is an important factor that determine the membrane fluidity. Eukaryotic and prokaryotic microorganisms generally respond to a decrease in growth temperature by increasing acyl chain unsaturation or branching, or by decreasing the acyl chain length. These changes decrease the liquid-cristalline to gel phase transition temperature of the phospholipids. Therefore the organisms regulate their membrane fluidity to changing temperatures. The portions of unsaturated, branched and short chain PLFAs from Svalbard sediments match with those reported previously for shelf sediments of temperate regions.

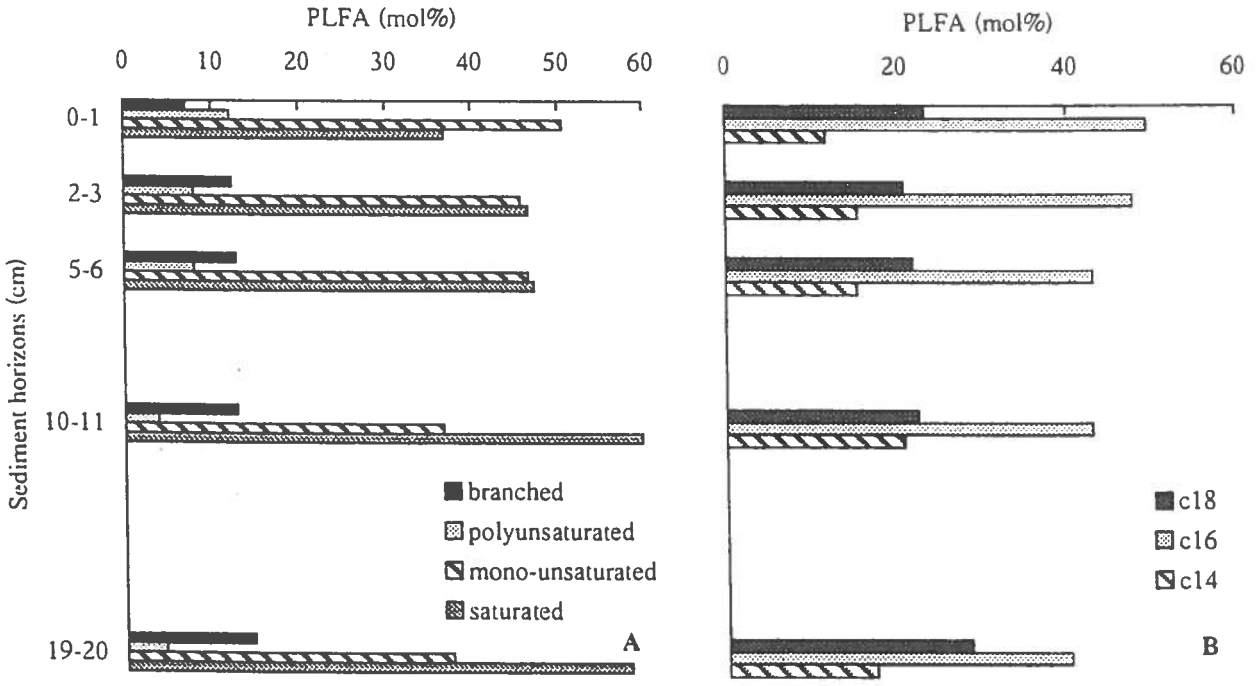

Figure 4 PLFA profiles of sediment from Krossfjorden of (A) total relative amounts saturated, mono-and poly-unsaturated PLFA. Additionally the total relative amount s of branched-chain PLFA are presented. The vertical distribution of PLFA with even-numbered acyl-chains (14 to 18 carbon atoms) is shown in (B).

In contrast, psychrophilic SRB isolated from Svalbard sediments exhibited high portions of unsaturated (70-90%) or short chain fatty acids (Knoblauch et al, 1999). High amounts of unsaturated and short-chain fatty acids have also been found in psychrophilic and psychrotolerant bacteria originate from other permanently cold habitats (Gounot and Russel, 1999).

One can speculate that membrane functioning is possible over a certain temperature range with the same fatty acid composition or about other cold adaptations to regulate the membrane fluidity. It´s also possible that only a minor part of the recent microbial community exhibited cold adapted PLFA compositions and therefore are active under these cold conditions. A high density of active cells in the upper layer of the sediment might also be the reason for the changes of unsaturation with depth. In bacteria the changes are usually achieved by *de novo* synthesis or by postsynthetic modifications of the lipids (Gounot and Russel, 1999).

MPN-counts of aerobic bacteria. The highest MPN-counts (4.6 x 10^7 per ml) of aerobic heterotrophic bacteria were found in the oxic surface of sediment from Krossfjorden (Table 1). The cell numbers in the depth of 2-3 cm were two and in a depth of 5-6 cm three orders of magnitude lower. The abundance of aerobes in the deeper sediment layers is probably limited by the availability of oxygen as electron acceptor. Pure cultures obtained from the highest positive MPN tubes were identified by 16S rRNA genesequenze-analyses as members of the genera *Moritella*, *Psychromonas* or *Pseudomas*, which have often been isolated from cold marine environments. These bacteria belong to the γ- subclass of Proteobacteria, which have been previously detected in high adundance in Svalbard sediments with a maximum in the upper first cm by using molecular methods. All identified strains were previously described as psychrophiles, that indicate the important role of cold-adapted aerobic bacteria in this permanently cold sediment.

Table 1 Vertical MPN counts of aerobic heterotrophic and sulfate-reducing bacteria from Krossfjorden. The triplicate MPN series were cultivated at 4 °C.

	Sediment horizons		
	0-1 cm	2-3 cm	5-6 cm
Aerobic heterotrophic bacteria (cells cm^{-3})			
After 6 weeks	4.6 (0.71-24)*·10^7	4.6 (0.71-24) ·10^5	2.4 (0.36-13) ·10^4
Sulfate-reducing bacteria (cells cm^{-3})			
After 18 months with hydrogen	2.1 (0.35-4.7) ·10^4	4.6 (0.71-24) ·10^3	2.4 (0.36-13) ·10^3
After 18 months with formate	4.6 (0.71-24) ·10^4	4.6 (0.71-24) ·10^3	7.5 (1.4-23) ·10^3

* 95% confidence limits.

MPN-counts of SRB. In agreement with the absolute abundance of the SRB specific biomarkers the highest cell numbers of SRB grown with hydrogen or formate were found in the top layer of the sediment (Table 1). The estimates for SRB were in the range of those reported previously for permanently cold marine sediments at low temperatures with other substrates like acetate, propionate or lactate [2]. Ten fold lower numbers were found at a depth of 2-3 cm and 5-6 cm depth. In contrast to Isfjorden (Table 2), no substrate-depending differences in the cell numbers were found in Krossfjorden and Smerenburgfjorden, but we observed faster growth of SRB with formate than with hydrogen.

In Smeerenburgfjorden, the estimated numbers of SRB were significant lower than those detected by molecular approches (> $5 \cdot 10^8$ SRB per cm^{-3}). Differences between cultivation and molecular methods have often been described. In the present study the selective media and the low growth temperature, combined with the microscopic observation that all obtained pure cultures form aggregates might enhance the differences between both approches.

The formation of cell aggregates could be a strategy of the SRB to escape oxygen stress in the oxic-anoxic sediment surface where the highest MPN numbers of SRB were found.

Table 2 MPN counts of sulfate-reducing bacteria from different fjords of Svalbard. The triplicate MPN series were incubated at 4 °C.

	Isfjorden (5-6 cm)	Kongsfjorden (8-9 cm)	Smeerenburgfjorden (5-6 cm)
Sulfate-reducing bacteria (cells cm^{-3})			
After 18 months with hydrogen	2.4 (0.36-13)*·10^3	2.4 (0.36-13) ·10^5	2.4 (0.36-13) ·10^3
After 18 month with formate	2.4 (0.36-13) ·10^4	2.4 (0.36-13) ·10^5	2.4 (0.36-13) ·10^3

* 95% confidence limits.

SRB isolates and phylogenetic characterization. From the MPN series with formate as electron donor, seven isolates were obtained that could be identified by 16S rRNA gene sequence analysis as *Desulfotalea psychrophila* or *D. arctica*. These are psychrophilic SRB from permanently cold Arctic sediment which are able to grow on a variety of organic substrates [3]. The repeated isolation of these strains indicates the abundance of *Desulfotalea* species in the sediment around Svalbard.

Until now, three SRB were isolated in pure culture with hydrogen as electron donor from Isfjorden (strain DHA1), Krossfjorden (strain G1HA1) and Smeerenburgfjorden (strain JHA1). According to its 16S rRNA gene sequence strain DHA1 forms a cluster together with clones and isolates from Svalbard and the previously described *Desulforhopalus singaporensis* [53] within the

"*Desulfobulbaceae*" (Fig. 5). According to 16S rRNA sequences, strain DHA1 is identical to strain LSv53, an isolate obtained with lactate as electron donor and carbon source from the same habitat [2]. It shares between 95.9 and 99.9 % identity with the 16S rRNA gene sequences of other members of this cluster, whereas *Desulforhopalus vacuolatus* is only distantly related (93.6% identity). This might indicate that the aforementioned cluster forms a new genus separate from the genus *Desulforhopalus* represented by its type strain.

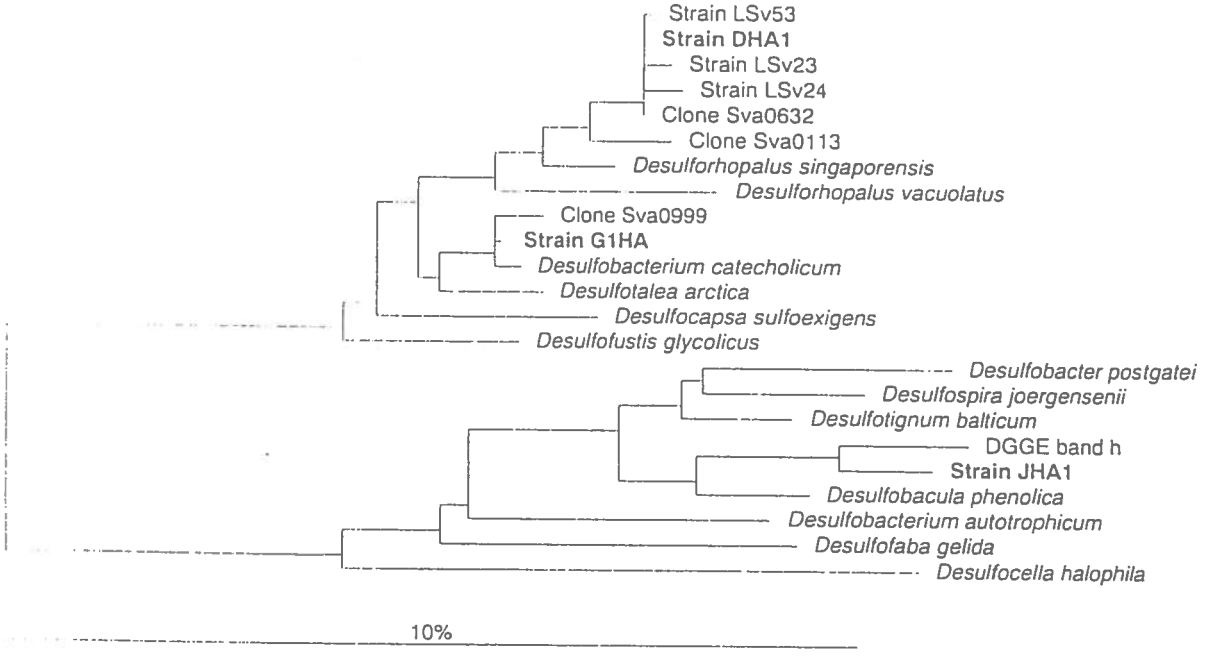

Fig. 5. Phylogenetic tree showing the affiliations of 16S rDNA sequences from strain DHA1, strain G1HA, and strain JHA1 to selected reference sequences of the delta subdivision of the Proteobacteria. The tree was calculated by using neighbor-joining analysis corrected with a position variability and a termini filter which considered only 1255 bases of the 16S rRNA gene sequences. The shorter sequence for the DGGE band h was added to the existing tree by using parsimony criteria. The bar represents 10% estimated sequence divergence.

The closest relative of strain G1HA is *Desulfobacterium catecholicum* (97.2% identity of the 16S rRNA gene), an organism that has to be reclassified, and a clone sequence Sva0999 from Svalbard (97.3% identity); all other sequences showed lower identities (< 95%). So far, *Desulfobacterium catecholicum* is unique within the "*Desulfobulbaceae*", because it is the only species that shows complete substrate oxidation. The cluster containing strain G1HA probably presents another new genus.

Strain JHA1 is a member of the *Desulfobacteraceae* with *Desulfobacula phenolica* being its closest described relative (94.8% identity). As can be seen in Figure 5, the 16S rRNA gene sequence of DGGE band h obtained from a stratified marine water column of Mariager Fjord, Denmark [49] falls in close proximity to this organisms (98.6% identity). Based only on the sequence of the 16S rRNA gene, strain JHA1 probably represents also a new genus within the family *Desulfobacteraceae*. A detailed characterization has to reveal whether these strains indeed represent new genera.

The utilization of hydrogen as electron donor combined with acetate as carbon source were often described as selective substrate for the isolation of members of the genus *Desulfovibrio* from marine habitats. The absence of *Desulfovibrio* species in high dilutet MPN tubes and the low concentration of the *Desulfovibrio* specific biomarker i17:1 confirm the low abundance of the genus in Svalbard sediment which have been previously reported [8].

In conclusion, the microbial population of the permanently cold marine environment along the coast of Svalbard might be dominated by psychrophilic bacteria as indicated by newly isolated bacteria of different physiological groups, which grow optimally below 20 °C. Most of them are highly related to known psychrophiles from other cold marine habitats. A significant indication for a cold adaptation of the PLFA profiles was not observed.

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4

Community structure and activity of sulfate-reducing bacteria in an intertidal surface-sediment: A multi-methods approach

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Abstract

The community structure of sulfate-reducing bacteria (SRB) in an intertidal mud flat of the German Wadden Sea (Site Dangast) was studied and related to sedimentary biogeochemical gradients and processes. Below the penetration depths of oxygen (~3 mm) and nitrate (~ 4 mm), the presence of dissolved Fe and Mn and the absence of dissolved sulfide indicate suboxic conditions within the top 10 cm of the sediment. Moderate to high bacterial sulfate reduction rates were measured with radiotracers throughout the sediment and dissimilatory sulfate reduction was also demonstrated by the presence of acid-volatile sulfides (AVS, essentially iron monosulfide). Stable sulfur isotope ($^{34}S/^{32}S$) discrimination between dissolved sulfate and AVS was dominated by sulfate reduction, but a contribution from anaerobic metabolism of sulfur intermediates is likely. The diversity of SRB was studied using denaturant gradient gel electrophoresis (DGGE) of 16S rDNA and counting viable cells with the most probable number (MPN) technique. Phylogenetic groups of SRB identified with these two techniques were evenly distributed throughout the vertical profile (0-20 cm) of the studied sediment. However, application of fluorescence in situ hybridization (FISH) demonstrated a maximum of the *Desulfovibrio* and *Desulfosarcina-Desulfococcus-Desulfofrigus* groups between 2 and 3 cm depth. These two groups encompass acetate and lactate utilizing SRB. The coincidence of this SRB maximum with a local maximum of sulfate reduction rates and the depletion of acetate and lactate reflects the biogeochemical processes related to sulfate reduction.

Introduction

Continental margin sediments include shelf sediments and intertidal mud flats of the coastal ranges. These sediments are generally characterized by a high input of organic matter. Up to 30% of the oceanic primary production takes place in the shelf areas. From this 25-50% sinks to the sediment (Wollast 1991). In addition, the deltaic areas of the shelf regions are receiving up to 90% of the global flux of particles and organic matter transported by rivers to the oceans (Gibbs 1981). Since most of the deposited matter is remineralized (Berner 1982), continental margin sediments play a prominent role in the marine carbon cycle, even though they encompass only 10% of the total ocean area. The oxidation of organic carbon is performed by microorganisms that employ an array of electron acceptors, each allowing a different yield of free energy. In accordance with the decreasing yields, a zonation of the oxidants is usually observed in the vertical sediment profiles: O_2, NO_3^-, Fe(III) and Mn(IV), and finally SO_4^{2-} (Froelich et al. 1979). Using the $^{35}SO_4^{2-}$ radiotracer technique it was shown that up to about 50% of the organic matter in marine sediments is mineralized via microbial sulfate reduction (Jørgensen 1982b). Since sulfate concentration in ocean water is about 28 mM, it may still be abundant in deeper layers of the sediment, and consequently the dominant electron acceptor (Fenchel et al. 1998). The areal rates of dissimilatory sulfate reduction in intertidal sediments may vary considerably between about 0.2 and 104 mmol $\cdot$ m^{-2} $\cdot$ d^{-1} (Trudinger 1992). SRR are generally controlled by temperature (Vosjan 1974), the availability of reactive organic compounds (Schubert et al. 2000), and correlated to the abundance of sulfate-reducing bacteria (Sahm et al. 1999; Böttcher et al. 2000).

The importance of sulfate reduction for the remineralization of organic carbon in marine sediments is reflected also by the variety of metabolic capacities of sulfate-reducing bacteria (SRB) isolated from this environment. Pure cultures of SRB have been shown to utilize organic substrates such as short chain fatty acids, alcohols and aromatic compounds, and to completely oxidize acetate to CO_2 (Widdel 1988; Rabus et al. 2000); all important substrates in the anoxic zones of marine sediments. Phylogenetic analysis of isolates (Devereux et al. 1990) and the introduction of 16S rRNA based molecular tools (Amann et al. 1995) allowed to identify and quantify SRBs in their natural habitat. Only recently this type of investigation was applied to marine sediments (Devereux et al. 1996; Llobet-Brossa et al. 1998; Ravenschlag et al. 1999; Sahm et al. 1999; Bowman et al. 2000; Ravenschlag et al. 2000; Wieringa et al. 2000).

A key question in microbial ecology is the influence of sulfate-reducing bacteria with different activity and nutritional properties on the biogeochemical gradients in marine

sediments. The present study focuses on the diversity, abundance and activity of SRB and their relation to the biogeochemical processes in a tidal sediment of the southern North Sea, (Wadden Sea, Site Dangast). To advance our understanding on the relationship between the community of sulfate-reducers and biogeochemical gradients, we combined cultivation dependent and independent microbiological approaches with biogeochemical and stable isotope analyses.

Materials and Methods

Study site. The river Weser is one of four major rivers draining into the German Bight of the southern North Sea. The Jade Bay, a meso- to macrotidal embayment, is situated in the coastal area to the west of the Weser estuary in the northern part of Lower Saxony (Germany). The Jade Bay is under influence of the fluvial input of the river Weser, and the mean tidal range in the southern part reaches 3.75 m (Irion 1994). The sampling station, "Site Dangast", is located 2 km west to the small village Dangast, about 25 m west of a tidal creek ("Dangast Tief"), connected to a freshwater outlet ("Dangast Siel") and about 15 m north from the shore-line (Fig. 1). Thus the site represents a highly dynamic system.

The sediment experienced tides which exposed it to the air for about 5 h and left it inundated for about 7 h, with some variability due to the wind velocity and direction (Llobet-Brossa et al. 1998).

Processing of sediment samples. Sediment cores were obtained on June 28th, 1999 at low tide between 7 and 9 am with polycarbonate tubes (diameter 8-10 cm; length 50 cm). The sediment cores were closed with air-tight rubber stoppers on both ends and transported cool (approx. 4°C) and dark to the laboratory for further processing within about 3 hours.

Sediment cores were sliced by extruding them from the polycarbonate tube and cutting with a thin aluminum plate into the following layers: 0-0.5, 0.5-1, 1-2, 2-3, 3-4, 4-5, 5-10, 10-15 and 15-20 cm (top-down). Sediment samples for enumeration of viable cells (MPN) and molecular analysis (DGGE and FISH) were taken from the same sediment layers of a single core. For geochemical measurements of pore water and sediments, parallel cores were used and sliced in 1 cm layers under inert gas (N_2) in a temperature controlled room (4 °C). All cores were taken from an area of 1 by 2 m.

In September 1999 additional samples of Wadden Sea surface sediments (2-5 cm depth) were taken from the same site in Dangast and from Horumersiel, located approx. 20 km north of Dangast.

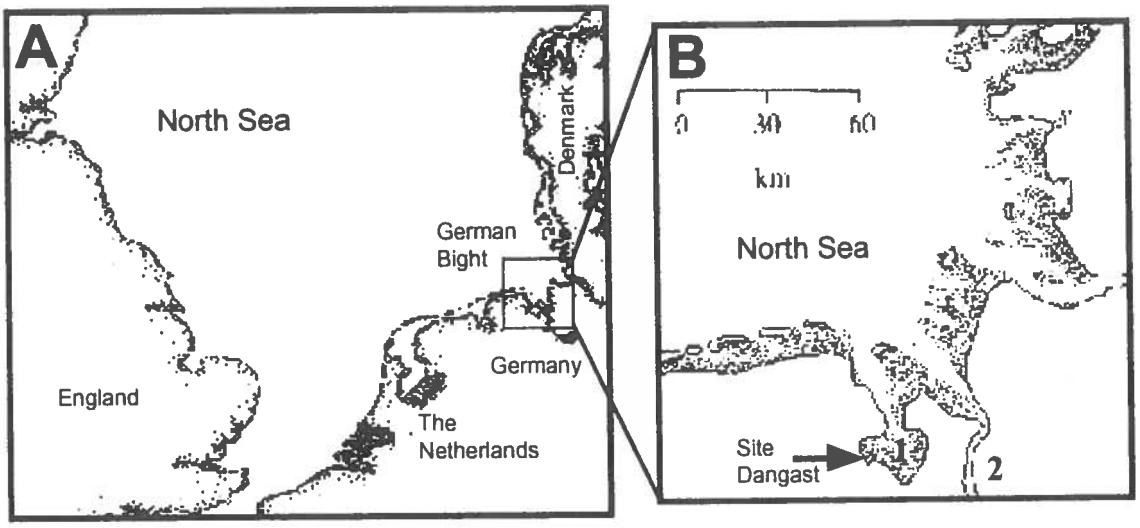

Figure 1: Map of the sampling site, Site Dangast. (A) Position of the German Bight in the North Sea. The black dot (●) marks the location of the city Bremen. (B) Position of the sampling site, Site Dangast in the Jade Bay (1) is indicated by the arrow. The river Weser is indicated by (2).

Sediment characterization. A variety of methods were applied to study physicochemical properties and biological activities related to sulfate-reducing bacteria of the sediment under investigation.

*(i) Characterization of organic material and sulfur speciation*_Pore water contents in the sediments were determined gravimetrically by drying sediment sections at 70°C until weight constance. Total carbon was measured using a Leco® induction furnace and total inorganic carbon on a CM 5012 coulomat with a CM 5130 acidification modul (UIC). Total organic carbon (TOC) contents were obtained from the difference of total carbon and total inorganic carbon. Phospholipid fatty acids (PLFA) were extracted essentially as described by Bligh and Dyer (1959). The procedure involves a one-phase extraction, fractionation on silicic acid, derivatization to methyl-ester, and analysis by capillary gas chromatography. The polar lipids were fractionated by the use of silicic acid columns (Isolute SPE columns, IST Mid Glamorgan, UK) with chloroform, acetone and methanol as eluents. The mild alkaline methanolysis procedure was used to transmethylate the ester linked fatty acids of phospholipids to methyl ester (Palojärvi and Albers 1998). Nonadecanoic acid methyl ester was utilized as an internal standard. 2.0 µl sample volume was injected splittless onto a fused silica capillary column (Optima-5-MS, Macherey und Nagel, Düren, Germany). The fatty acid methyl esters were identified by the use of a mass spectrometer (GC-Q, Finnigan, Bremen, Germany) and quantified by the use of a flame-ionization-detector (GC-Autosystem, Perkin Elmer, Überlingen, Germany). The GC-temperature-program is described elsewhere

(Palojärvi and Albers 1998). The mass spectra and retention times of 68 fatty acid methyl esters were determined by injection of authentic standards (Supelco, Deisenhofen, Germany and BioTrend, Köln, Germany). Unsaturated *trans* -isomers were not determined. Biomass was determined by phosphate analysis of PLFA. After the Bligh and Dyer (1959) extraction, aliquots of all samples were used for calorimetric phosphate analysis to determine the viable biomass (Findlay et al. 1985; Findlay et al. 1989).

The fraction of acid volatile sulfide (AVS) was separated from the wet, Zn-acetate preserved sediment by the reaction with cold 6N HCl containing $SnCl_2$ (Duan et al. 1997) in a stream of nitrogen. The addition of $SnCl_2$ increased the recovery of the AVS fraction within the first 5 cm, but no further influence was observed at greater depths (Böttcher, unpublished data). Sulfur isotope ratios (see below) of the AVS fraction recovered by both methods agreed within 1‰ (Böttcher, unpublished data). The sum of (essentially) pyrite and (minor) elemental sulfur (fraction 'Cr-II') was obtained by the distillation with hot acidic Cr(II)chloride solution (Fossing and Jørgensen 1989). H_2S was trapped as Ag_2S in a $AgNO_3$ solution and quantified gravimetrically.

For stable sulfur isotope analysis ($^{34}S/^{32}S$), pore water sulfate was precipitated from filtered Zn-acetate preserved samples as $BaSO_4$, carefully washed and dried. Sulfur isotope ratios of the AVS, Cr-II, and pore water sulfate fractions were measured by C-irmMS (Pichlmayer and Blochberger 1988). Samples were converted to SO_2 using a Eurovector elemental analyser which was connected to a Finnigan MAT Delta$^+$ gas mass spectrometer via a Finnigan Conflo II split interface. Isotope ratios are given in the δ-notation versus the SO_2-based Vienna-Canyon Diablo Troilite (V-CDT) standard. International standards IAEA-S-1, IAEA-S-2, IAEA-S-3, and NBS 127 were used to calibrate the mass spectrometer.

*(ii) Pore waters*_Air and pore water temperatures were measured with a digital sensor (GTH 1150 digital thermometer) at the beginning and the end of the sampling session. After transport to the laboratory, subcores (3.6 cm diameter) for pore water analyses were taken in a temperature-controlled room. Pore waters were separated from the sediment by centrifugation in closed centrifugation vessels under inert gas. Prior to analyses, pore waters were filtered through membrane filters (0.45 µm; Sartorius) and acidified with nitric acid (reagent grade quality) into pre-cleaned PE bottles. Concentrations of dissolved iron, manganese and sulfate were analyzed after appropriate dilution by means of ICP-OES (Perkin Elmer Optima 3000 XL). It is assumed that dissolved iron essentially consisted of Fe(II), although a contribution from complexed Fe(III) can not be completely ruled out (Luther III et al. 1996). Dissolved sulfate was additionally quantified gravimetrically as $BaSO_4$ from the pore water of a parallel

sediment core which was immediately cut into sections and preserved in 20% zinc acetate solutions. The results of the two methods agreed very well. H_2S was measured in selected samples preserved with 2% $ZnCl_2$ solution according to Cline (Cline 1969). Salinity of filtered samples was measured with a hand refractometer.

Pore waters for the analysis of volatile fatty acids (VFA) analysis was extracted by centrifugation of the sediment in precombusted glas centrifuge tubes. 2 ml of the supernatant were sampled with a glas syringe into precombusted borosilicate vials (4 ml) with teflone lined caps and frozen (-20°C) until analysis. The VFA were measured by HPLC as 2-nitrophenyl hydrazin derivats as described by Albert and Martens (1997). A Sykam S1211 HPLC pump combined with a linear UV/VIS detector and a Gilson 232XL autosampler were used for separation and measurement of the acids. The detected signal was recorded and integrated by a Knauer Eurochrom 2000 integration software. For the separation of the acids a 25 cm LiChrosphere RP8 column with a 1.5 cm LiChrosphere RP8 guard column from Knauer was used. 2.5 cm polymeric reversed phase (PRP-1) cartridge (Hamilton) installed in the sample loop was used as a sample concentrator. Differing from the original method, the flow rate was reduced to 1 ml/min, the concentration of tetrabutylammonium hydroxide of 'solvent A' was reduced to 1 mM and the concentration of tetradecyltrimethylammonium bromids in 'solvent B' to 25 mM. Additionally, the pH of the solvent was adjusted with HCl instead of phosphoric acid. The detection limit for glycolate and lactate was 0.2 µM, for acetate and propionate 0.3 µM and formate 0.6 µM.

(iii) Microsensor measurements- Profiles of oxygen, hydrogen sulfide, and nitrate concentrations at the sediment-water interface were measured in the laboratory with microelectrodes. The determination of oxygen profiles was done with Clark-type O_2 sensors with guard cathodes (Revsbech 1989). The O_2 sensors had tip diameters between 10 µm and 20 µm, a stirring sensitivity <2 %, and a 90 % response time t_{90}< 1 s. To calibrate the sensors, readings in the overlying air saturated water and in the anoxic zone of the sediment were taken. A two point calibration curve was calculated with oxygen solubility values for different temperatures and salinities based on equations from Garcia and Gordon (1992). Amperometric H_2S microsensors were constructed, calibrated and applied as described by Kühl et al (1998). Nitrate was measured with a microbiosensor in which nitrate and nitrite are reduced to N_2O by bacteria and N_2O is detected electrochemically (Larsen et al. 1997). The nitrate sensor showed no detectable stirring sensitivity and a 90 % response time t_{90}< 45 s with a tip diameter of 70 µm. Calibration was done by measuring sensor readings in nitrate solutions with salinity and temperature identically to the sample.

From steady-state oxygen profiles areal fluxes of oxygen were calculated based on Fick´s first law of one dimensional diffusion $J = D_e * dC(z)/dz$ (Kühl et al. 1996) with D_e as effective diffusion coefficient and dC/dz as concentration gradient for oxygen. Volumetric production and consumption rates were calculated based on Fick´s second law of diffusion using the second derivative of the measured concentration profile. Nitrate values were calculated correspondingly. Furthermore, it was also determined if consumed nitrate originated from the water phase (Dw) or was produced by nitrification (Dn) by calculating back a concentration profile from an activity profile with no nitrate production (Meyer et al. 2000).

Sulfate reduction rates (SRR). Bacterial sulfate reduction rates (SRR) were measured using the whole-core incubation technique with the injection of a carrier-free $^{35}SO_4^{2-}$ tracer (Jørgensen 1978; Fossing and Jørgensen 1989). Although the sediment temperature during sampling varied only slightly and was close to 17°C, much higher changes in pore water temperatures were found on a daily base between 26th and 30th of June, 1999 (Böttcher, unpublished data). Therefore, sediment cores were equilibrated after sampling in the laboratory at 10 and 20°C for several hours and subsequently incubated with the radiotracer (~ 200 kBq per injection along 1 cm intervals) for 4.5 h in the dark. Activities counted with a Packard liquid scintillation counter were corrected for blank contributions derived from the counting and the distillation procedures. The apparent activation energy calculated from an Arrhenius equation using the depth-integrated SRR for the 10 and 20°C incubations gives a value of 66 kJ · mol^{-1} which is similar to observations on a seasonal base in tidal mudflats (Kristensen et al. 2000) and general findings on microbial sulfate reduction (Westrich and Berner 1988).

Nucleic acid extraction and DGGE amplification. In addition to the sediment core used for MPN counts and VFA analysis, a second core was processed for molecular analysis. Replicate sediment cores were analysed to avoid changes in the community composition caused by handling and containment as described before (Rochelle et al. 1994). DNA and RNA of each sediment horizon were extracted from 1.5 ml wet sediment by bead-beating, phenol extraction and isopropanol precipitation as described previously (Sahm and Berninger 1998).

PCR amplification specific for SRB of the δ-subclass of *Proteobacteria* was carried out with the forward primer SRB385 carrying a GC-clamp and the reverse primer 907 (Sass et al. 1998). DGGE, excision of bands, reamplification, and sequencing were performed as previously described (Muyzer et al. 1996). DGGE partial sequences were added to an alignment of about 15,000 homologous bacterial 16S rRNA genes (Maidak et al. 2000) by

Table 1. Oligonucleotide probes used in this study.

Probe	Target	Sequence (5'-3') of probe	[%] FA[a] in situ	Reference
EUB338	Bacteria 16S rRNA, position[b] 338-355	GCT GCC TCC CGT AGG AGT	0-35	27
SRB385	_-Subdivision of Proteobacteria 16S rRNA, position 385-402	CGG CGT CGC TGC GTC AGG	35	27
DNMA657	*Desulfonema sp.* 16S rRNA, position 657-676	TTC CGY TTC CCT CTC CCA TA	35	27
DSV698	*Desulfovibrio* (16 species) 16S rRNA, position 698-717	GTT CCT CCA GAT ATC TAC GG	35	29
DSV1292	*Desulfovibrio* (13 species) 16S rRNA, position 1292-1310	CAA TCC GGA CTG GGA CGC	35	29
DSV407	*Desulfovibrio* (3 species) 16S rRNA, position 407-424	CCG AAG GCC TTC TTC CCT	50	29
DSD131	*Desulfovibrio* (1 species) 16S rRNA, position 131-148	CCC GAT CGT CTG GGC AGG	20	29
DSV214	*Desulfovibrio/D'microbium* 16S rRNA, position 214-230	CAT CCT CGG ACG AA TGC	10	29
DSS658	*Desulfosarcina/D'coccus/D'frigus* 16S rRNA, position 658-675	TCC ACT TCC CTC TCC CAT	60	29
DSB985	*Desulfobacter/D'bacula* 16S rRNA, position 985-1003	CAC AGG ATG TCA AAC CCA G	20	29
221	*Desulfobacterium* 16S rRNA, position 221-238	TGC GCG GAC TCA TTC AAA	35	29
660	*Desulfobulbus* 16S rRNA, position 660-679	GAA TTC CAC TTT CCC CTC TG	60	29
DSBO224	*Desulfobotulus* 16S rRNA, position 224-242	GGG ACG CGG ACT CAT CCT C	60	29
DSMA488	*Desulfovibrio/D'monile* 16S rRNA, position 488-507	GCC GGT GCT TCC TTT GGC GG	60	29
DSR651	*Desulforhopalus* 16S rRNA, position 651-668	CCC CCT CCA GTA CTC AAG	35	29
DTM229	*Desulfotomaculum* 16S rRNA, position 229-246	AAT GGG ACG CGG AXC CAT	15	29
SVAL428	*Desulfotalea/D'fustis* 16S rRNA, position 428-446	CCA TCT GAC AGG ATT TTA C	25	36
NON338	none (negative control)	ACT CCT ACG GGA GGC AGC	0-35	27

[a] Percentage of formamide (FA) in hybridization buffer.

[b] *Escherichia coli* numbering.

using the aligning tool of the ARB program package (Strunk et al. 1998-2000). Aligned sequences were inserted within a stable tree by using the ARB parsimony tool (Ludwig et al. 1998). Accession numbers of the partial sequences are XXX and YYY, and deposited at ZZZ.

In situ hybridization and cell counts. Hybridizations, microscopic examination and counting of hybridized cells and total cell counts were performed as previously described (Snaidr et al. 1997; Llobet-Brossa et al. 1998). For each sample between 700 and 1000 DAPI stained cells were analyzed and counted; duplicates were carried out for each sample. The oligonucleotide probes used in this study were purchased from Interactiva (Ulm, Germany) with Cy3 fluorochrome at the 5' end. Probes used are listed in Table 1. For fluorescence in situ hybridization (FISH) of sediment samples two replicate sediment cores were cut and processed as described before (Llobet-Brossa et al. 1998). From MPN-tubes that showed

growth, aliquots of 1-2 ml were withdrawn with N_2-flushed syringes. Cells were sedimentated by centrifugation and washed once with 1x PBS. Subsequent fixation and hybridization were performed as previously described (Snaidr et al. 1997).

Media and enumeration of viable cells. A defined, bicarbonate-buffered, sulfide-reduced (1 mM) mineral medium, essentially having the same salt composition as natural seawater was used for cultivation experiments (Widdel and Bak 1992). Na-dithionate at a final concentration of 10 µg/ml was applied as an additional reductant. Organic substrates were added from concentrated stock solutions. Gaseous substrates (H_2/CO_2) were supplied by applying an overpressure of 1 atm to the headspace of the culture tubes.

Viable sulfate-reducing bacteria were enumerated using an MPN technique with liquid media and with agar shakes. Samples from each layer of the sediment core were transferred via a funnel to glas bottles (250 ml volume) and mixed 1:1 with substrate-free, anoxic media under a steady stream of N_2/CO_2 (90:10 [v/v]), yielding approximately 100 ml of homogenate. The bottles were then anoxically sealed with butyl-rubber stoppers and screw caps. These sediment slurries were diluted in steps of 1:10 by transferring aliquots to substrate-free, anoxic media under N_2/CO_2 (90:10 [v/v]) in butyl-rubber sealed glass-bottles (250 ml volume of bottle, 100 ml total volume of dilution). The transfer of sediment suspensions between the glass-bottles was carried out with N_2-flushed syringes. Prior to transfer, the sediment suspensions were shaken vigorously to achieve optimal mixing. These "master"-dilutions were used to inoculate culture tubes for MPN-counts in liquid media. Similarily, "master"-dilutions were prepared for the inoculation of MPN-counts in agar-shakes. However, in this case not every single sediment layer was diluted but rather a homogenous mix of the top 5 cm of the sediment core.

*(i) MPN-counts with liquid media*_These MPN-counts were carried out in glass tubes (160 × 16 mm) sealed with butyl-rubber stoppers and screw caps that contained anoxic media with or without substrate under an N_2/CO_2-atmosphere (90:10 [v/v]). Substrates used were as follows: H_2/CO_2 (90:10, [v/v], applied with 1 atm to the gas head space); H_2/CO_2 + 2 mM acetate; 15 mM formate; 7.5 mM acetate; 5 mM propionate; 5 mM lactate. Each tube contained 9 ml medium and was inoculated with 1 ml from the corresponding "master" dilution. Three replicates were prepared for each substrate condition. To sustain reduced conditions, 0.5 mM Na_2S were added after about four weeks of incubation. The tubes were incubated at 23 °C for 10 months. Growth of sulfate-reducing bacteria was determined by measurement of the optical density (660 nm) and sulfide using the semiquantitative method described by Cord-Ruwisch (1985) and by macroscopic and microscopic examination.

*(ii) MPN-counts in agar-shakes and isolations*_MPN-counts in agar shakes were conducted to obtain single colonies. Butyl-rubber stopper sealed glass tubes containing approximately 9 ml of anoxic media with molten agar and substrates as described above were prepared as described previously for the isolation of sulfate-reducing bacteria (Widdel and Bak 1992). The tubes were inoculated with 1 ml from the corresponding master dilution. Replicates and incubation conditions were as described above for MPN-counts in liquid media. Agar shakes were incubated at 23 °C. Growth was determined by microscopically observing formation of brownish colonies which are typical for sulfate-reducing bacteria. Such colonies were picked by means of finely drawn Pasteur pipettes (Widdel and Bak 1992) and transferred to liquid medium containing the corresponding substrate.

Results

Sediment. Downcore temperatures in the sediments varied between about 16.0 and 17.5 °C during sampling with corresponding air temperatures between 16.0 °C (in the wind) and 20.2 °C (without wind). The sediment cores displayed 2 distinct color changes: the oxic upper part was brown which turned within the first 1-2 cm into a darker olive green color with some greyish diffuse streaks. At about 9-12 cmbsf (cm below surface), the coupling of increasing accumulation of iron monosulfide (acid-volatile sulfide, AVS) with decreasing porosity was indicated by a diffuse blackening of the sediment, remaining black until the bottom of the investigated sediment section. These vertical changes were also reflected by biological and geochemical parameters. The sediment was characterized by bioturbation, and living polychaetes were found down to about 16 cmbsf. An analysis of the grain size distribution of surface sediments was carried out on sediments recovered in May 1998 (Böttcher & Hespenheide, unpublished data) and showed that the first 10 cm almost completely consisted of mud (clay and silt grain size fraction < 63µm; essentially phyllosilicates (Böttcher et al. 2000). An increase of the sand grain fraction was observed below that depth (Böttcher et al. 2000). Correspondingly, the pore water contents decreased continuously with depth. During sampling in June 1999, for instance, maximum water contents of 67% were observed near the surface (0.5 cm depth) which decreased to 52% and 29% at 10 and 29 cm depth, respectively. The TOC (Fig. 2E) decreased with depth in parallel with the water content.

Acid volatile sulfides (AVS; essentially iron monosulfide) were found in all sections and showed a maximum at around 8 cm depth (Fig. 2C). The stable sulfur isotopic signature of AVS should essentially mirror that of H_2S which is derived from the overall metabolic processes in the sulfur cycle (Böttcher et al. 1998b). The AVS fraction ($\delta^{34}S$ values between -

21.1 and -26.2‰) was significantly enriched in the lighter sulfur isotope compared to coexisting pore water sulfate ($\delta^{34}S$ values between +20.4 and +25.4‰; Fig. 2D). Apparent sulfur isotope enrichment factors between -41 and -52‰ are calculated from the data given in Fig. 2D which are at the upper end or even exceed the results obtained in experiments with pure cultures of sulfate-reducing bacteria (Kaplan and Rittenberg 1964; Chambers et al. 1975). The isotopic composition of the Cr-II fraction which consists essentially of pyrite was more or less constant in the top 11 cm ($\delta^{34}S$ of -16 ± 1 ‰) but decreased at greater depths (Fig.2D).

Pore waters. The salinities of the pore waters during sampling in June 1999 were rather constant with 28‰. This corresponds well to earlier data obtained during sampling on a seasonal base over a two year period, where it has been found that salinities in the pore waters of the mud flat at Site Dangast varied between 22 and 30‰, averaging about 26‰ (Böttcher, unpublished data).

During sampling in June 1999 the microsensor measurements were impeded by high bioturbation activity in the upper 1.5 mm of the sediment and, therefore, no steady state O_2 profiles could be gained. During this measurement only the oxygen penetration depths (3.1 mm ± 0.17 mm) could be determined. Under similar environmental conditions but slightly less bioturbation a sediment core from the same site was investigated in June 1998 and the results are presented in Fig. 3. In the upper 0.7 mm the sediment was significantly supersaturated with respect to oxygen as it has been found previously for a situation in April 1998 (Böttcher et al. 2000). The maximum concentration occurred at 0.3 mm depth with 0.43 mM. Below 0.7 mm the oxygen concentration declined constantly and the oxygen penetration depth was 2.7 mm. The volumetric rate calculations also showed a distinct area of oxygen production in the upper 1.5 mm of the sediment sample (Fig. 3), with a maximum in the upper 0.5 mm. Below 1.5 mm oxygen was consumed.

Nitrate concentrations from measurements in June 1999 are plotted in Fig. 4, as a mean of two profiles. During the measurements the sediment was heavily bioturbated. Down from the water/sediment interface the nitrate concentration increased to a maximum value of more than 10 µM at 1 mm depth. At 2 mm the concentration was still higher than in the water phase but declined in deeper layers. Nitrate could be measured down to 3.6 mm. The calculated activity rates showed a clear separation between nitrate production in the upper 2.3 mm of the sediment and nitrate consumption below that layer (Fig. 4). Highest production rates occurred concomitant with the maximum nitrate concentration.

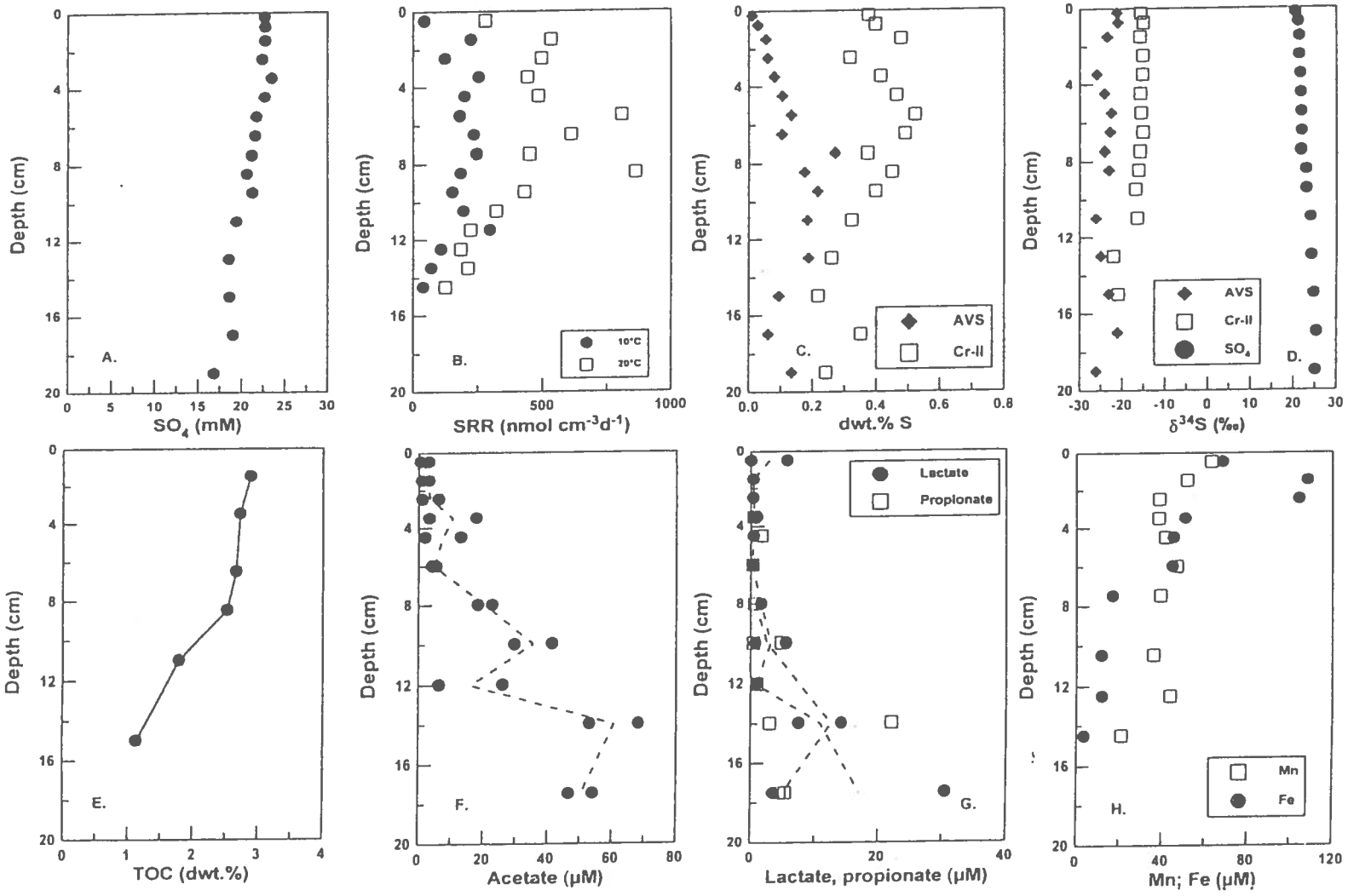

Fig. 2. Biogeochemical analysis of the vertical sediment profile. A, SO_4^{2-}; B, sulfate reduction rate (SRR); C, Cr(II)-reducible sulfur (Cr-II) and acide volatile sulfides (AVS). D, isotopic fractionation of sulfur species; E, total organic carbon (TOC); F, acetate; G, lactate and propionate; H, Mn(II) and Fe(II).

No hydrogen sulfide could be detected in the sediment cores within the first 20 mm, the maximum depth reached by our microsensor measurements. Sub-oxic, non-sulfidic conditions were indicated by the presence of dissolved Fe(II) and Mn(II) (Fig. 2H) and absence of sulfide at concentrations exceeding 5 µM between about 5 mm down to at least 12 cm depth (data not shown). Sulfate remained essentially constant within the first 4-5 cm and decreased further downcore (Fig. 2A), associated with an enrichment in ^{34}S. Despite of minor changes in dissolved sulfate, moderate to high microbial sulfate reduction rates were measured with radiotracers through the whole sediment core and showed a maximum with up to 485 nmol · cm^{-3} · d^{-1} in the sub-oxic zone at around 7 cmbsf (Fig. 2B). The apparent activation energy of 66 kJ mol^{-1} was used to relate the measured SRRs at 10 and 20°C to the mean temperature of 17°C measured during field sampling.

The concentrations of volatile fatty acids (VFA; acetate, propionate, and lactate; Fig. 2F and 2G) were determined in the pore waters of two sediment cores. In both cases concentrations of VFA except for acetate were below 5 µM in the top 5 cm of the sediment. Acetate concentration increased with depth to concentrations of about 50-60 µM. Concentrations of glycolate and formate on the other hand did not exceed 5 µM throughout the entire core. Elevated concentrations of lactate and propionate ranging between 10 and 30 µM were detected in the deepest horizon of 15-20 cm. This accumulation of organic acids occurred only below the maximum of the sulfate reduction rates (Fig. 2B, F and G).

PLFA analysis. Three different depth-depending PLFA patterns were observed in the studied sediment cores (Table 2). The first 0.5 cm are dominated by high amounts of polyunsaturated PLFA. In the depth between 0.5 and 10 cm, the PLFA patterns did not show any pronounced variations. The PLFA patterns included many branched fatty acids, cyclopropane fatty acids and a high amount of c11 18:1. The deepest layer is distinguished from the upper layers by the low amount of c9 16:1 and a higher amount of 18:0. The content of phospholipid phosphate decreased with depth (967 nmol · g^{-1} dry sediment at 1 cm depth to 228 nmol · g^{-1} dry sediment at 15-20 cm depth).

DGGE profiles of sediment samples. Changes in the diversity of the SRB population with depth were analyzed by DGGE of PCR-amplified 16S rDNA fragments and of the reversely transcribed 16S rRNA fragments. In the same sediment sample, the electrophoretic profiles of the 16S rDNA fragments were more complex than the ones observed after reverse transcription of the 16S rRNA (Fig. 5).

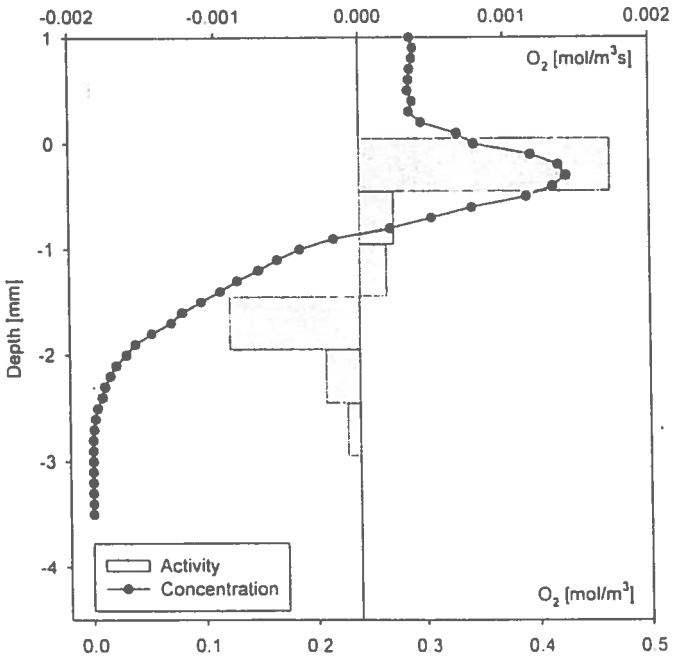

Fig. 3. Vertical sediment profile of oxygen concentrations determined with an O_2 microsensor and calculated oxygen production/consumption rates in a sediment core from Dangast (6/98). Profile and rates represent the mean of two data sets

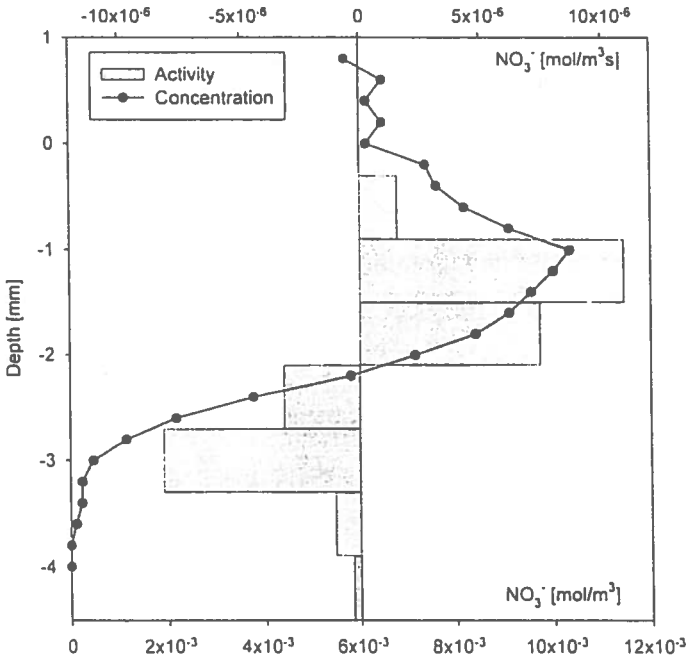

Fig. 4. Vertical sediment profile of nitrate concentrations determined with a NO_3^- microsensor and calculated nitrate production/consumption rates in a sediment core from Dangast (7/99). Profile and rates represent the mean of two data sets.

Table 2. PLFA profile of site Dangast. PLFA were analysed as fatty acid methyl esters and are shown in relative amounts (mol%). PLFA methyl esters present in all horizonts less than 0,5 % are not listed. Not detectable PLFA are abbreviated with nb.

	Sediment horizons (cm)								
PLFA methyl ester[a]	**0.0-0.5**	**0.5-1.0**	**1.0-2.0**	**2.0-3.0**	**3.0-4.0**	**4.0-5.0**	**5-10**	**10-15**	**15-20**
i14:0	1.4	0.9	1.0	1.1	1.0	0.8	1.0	0.4	0.3
c7 14:1	0.7	nd	0.1	0.3	nd	nd	0.3	0.2	0.6
14:0	6.3	3.6	3.5	0.4	3.1	0.9	3.3	1.6	1.6
i15:0	2.0	3.2	3.8	4.1	3.8	3.9	3.8	3.1	3.2
a15:0	2.8	5.5	6.6	7.3	6.9	7.3	7.6	7.3	8.0
15:0	3.6	2.8	2.1	2.2	2.0	2.0	2.1	1.8	1.4
16:3[b]	8.4	nd	nd	nd	nd	nd	nd	nd	nd
i16:0	nd	1.6	1.7	1.8	1.7	1.8	1.6	1.7	1.6
c7 16:1	1.2	1.8	1.6	1.8	1.7	1.8	1.5	1.5	1.3
c9 16:1	16.5	17.2	17.8	19.2	18.3	19.5	17.9	14.0	9.4
c11 16:1	4.1	1.6	2.1	2.4	2.3	2.7	2.6	2.2	1.4
c13 16:1	1.3	2.4	2.2	2.5	2.4	2.9	2.5	2.8	2.7
16:0	10.5	19.2	17.6	18.2	17.1	17.4	16.7	15.7	17.4
i17:1	0.3	1.2	1.2	1.1	1.4	1.1	0.8	1.2	2.9
10me16:0	0.4	1.3	1.7	1.9	1.9	2.3	2.4	2.3	2.1
a17:1	0.5	0.2	0.7	0.7	0.7	0.5	0.5	0.6	nd
i17:0	0.5	0.8	0.9	1.0	1.0	1.0	0.9	1.1	1.2
a17:0	0.4	1.1	1.2	1.3	1.3	1.4	1.4	1.8	2.0
c9 17:1	3.2	1.5	1.3	1.4	1.5	1.4	1.4	1.2	0.5
cyc 17:0	0.7	1.3	1.5	1.6	1.6	1.6	1.6	0.3	0.2
17:0	1.0	1.6	1.6	1.6	1.6	1.5	1.5	1.5	1.2
c6 18:2	1.9	0.8	0.7	0.6	0.5	0.5	0.5	0.4	0.1
c6 18:3	nd	0.6	0.6	0.5	0.5	0.5	0.6	0.5	nd
c9 18:2	1.4	0.4	0.7	0.7	0.8	0.7	0.4	0.6	0.7
c9 18:1	3.4	4.4	3.6	4.1	3.8	3.5	4.0	4.3	4.1
c11 18:1	3.9	10.7	11.6	13.3	13.3	13.3	12.7	15.6	14.6
c13 18:1	0.5	4.2	3.0	0.7	2.2	2.4	3.3	4.1	8.0
18:0	2.4	3.2	2.8	3.3	2.6	2.4	2.7	3.8	8.2
cyc19:0	nd	0.4	0.6	0.3	0.5	0.1	nd	0.5	0.7
c5 20:4	1.6	0.9	0.8	1.0	0.9	0.8	0.7	1.4	0.5
c5 20:5	13.7	2.1	1.4	1.2	1.0	0.8	0.8	2.3	0.4
c9 20:2	nd	0.8	0.2	nd	0.8	0.2	0.2	1.6	nd
c4 22:6	5.7	0.7	0.7	0.5	0.2	0.2	0.2	0.2	nd
24:0	nd	0.2	0.2	nd	0.1	1.6	0.3	0.2	0.1
branched[c]	8.8	16.7	20.2	22.1	20.6	20.7	21.2	20.3	22.9
saturated[d]	32.5	47.1	47.7	46.6	47.0	46.6	47.8	43.9	49.9
unsaturated[e]	67.5	53.0	52.3	53.4	53.0	53.5	52.2	56.1	50.1

[a]used short-hand nomenclature: i=iso-branched; a=anteiso-branched; c=*cis*; cyc=cyclopropane; me=methylgroup; x:y x= number of carbon atoms; y=number of double bonds
[b]position of double bond not determined
[c]include all iso, anteiso and 10Me branched PLFA-methyl esters
[d]all PLFA methyl esters without a double bond in the carbon chain
[e]all PUFA methyl esters with one or more double bonds in the carbon chain

DGGE of 16S rDNA showed between 6 and 9 bands of different intensities. There were no major changes between different sediment layers, except for the deepest layer at 15-20 cm depth. DGGE of 16S rRNA showed only two to four bands in all the samples. In contrast to the 16S rDNA-based DGGE, these were not evenly distributed but appeared as distinct patterns. It was not possible to get any PCR product from the deepest layer. As rRNA-based DGGE is influenced by the "activity-regulated" ribosome contents, this pattern might better indicate the identity and distribution of the active SRBs in the sample.

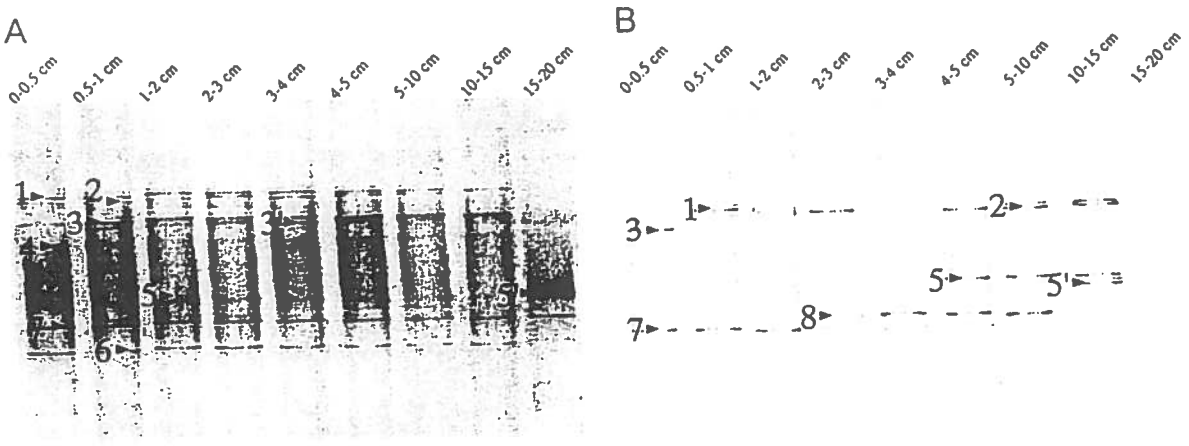

Fig. 5. DGGE profiles of 16S rDNA (A) and reversely transcribed 16S rRNA (B) by using the SRB specific forward primer SRB385 which carries a GC-clamp and the reverse primer 907 (Sass et al. 1998). DGGE bands 6 and 7 affiliate with the *Desulfonema* group. It has to be noted, that probe DSS658 does not target this group.

Identification of the most prominent DGGE bands by sequencing. All the bands analyzed by sequencing were shown to originate from members of the _-subclass of *Proteobacteria*, the taxonomic group that encompasses most gram-negative SRB (Fig. 6). Bands 1 and 2 were affiliated with a sequence similarity of 99% to *Desulfobulbus* sp. (accession numbers L40786 and L40785, respectively). DGGE bands 3 and 4 were found to be closely related to *Desulfovibrio* species. Band 3 was related to *Desulfovibrio caledoniensis* (accession number U53465) sharing similarities of 97%, and band 4 was related to *Desulfovibrio* sp. Ac5 (accession number AF228117) with 99% similarity. Band number 5 was identified as *Desulfobacter* sp. with 96% similarity (accession number L40787). Bands 6 and 7 were related with 99% similarity to *Desulfonema limicola* (accession number U45990),

and to *Desulfonema ishimotoei* (accession number U45991), respectively. The band number 8 had 98% sequence similarity with *Desulfuromonas palmitatis* (accession number U28172).

MPN counts of SRB. A variety of defined substrates was used to meet specifc substrate preferences of different SRBs possibly occurring in the studied sediment. For example, chemolithoautotrophic SRBs, such as *Desulfobacterium autotrophicum,* can grow with H_2 and CO_2 as the sole sources of energy and carbon, respectively. The bacterial numbers from MPN counts with liquid media are shown in Table 3.

Table 3. Total cell counts of SRB from different sediment horizons from Site Dangast after 1.5 and 10 months of incubation at 23 °C[a].

	Horizon (cm)								
	0-0.5	0.5-1	1-2	2-3	3-4	4-5	5-10	10-15	15-20
Hydrogen (CO_2)*									
1.5 months	1.1×10^3	4.6×10^3	4.6×10^3	1.1×10^3	1.1×10^3	1.1×10^3	nd	1.1×10^3	nd
10 months	4.6×10^6	1.1×10^7	1.1×10^6	4.6×10^6	1.1×10^6	4.6×10^6	4.6×10^6	4.6×10^6	1.1×10^6
Hydrogen (CO_2+Acetate)*									
1.5 months	4.6×10^4	1.1×10^5	1.1×10^5	1.1×10^4	1.1×10^5	4.6×10^5	4.6×10^5	1.1×10^5	1.1×10^3
10 months	1.1×10^7	1.1×10^7	1.1×10^7	1.1×10^6	1.1×10^6	1.1×10^6	4.6×10^6	4.6×10^6	4.6×10^5
Formate									
1.5 months	nd	nd	1.1×10^4	1.1×10^4	4.6×10^4	4.6×10^3	1.1×10^4	1.1×10^4	4.6×10^3
10 months	1.1×10^6	nd	4.6×10^5	1.1×10^6	4.6×10^6	4.6×10^6	1.1×10^7	1.1×10^6	4.6×10^5
Acetate									
1.5 months	4.6×10^3	1.1×10^4	1.1×10^4	1.1×10^4	1.1×10^4	1.1×10^4	4.6×10^4	1.1×10^4	1.1×10^3
10 months	1.1×10^5	4.6×10^5	1.1×10^4	1.1×10^6	1.1×10^4	4.6×10^5	1.1×10^7	3.9×10^6	4.6×10^3
Lactate									
1.5 months	1.1×10^4	1.1×10^4	4.6×10^5	4.6×10^3	4.6×10^4	4.6×10^4	1.1×10^4	4.6×10^4	4.6×10^3
10 months	1.1×10^4	3.9×10^5	4.6×10^5	4.6×10^6	4.6×10^6	4.6×10^6	1.1×10^4	4.6×10^6	4.6×10^5
Propionate									
1.5 months	1.1×10^4	1.1×10^4	1.1×10^4	1.1×10^4	1.1×10^4	1.1×10^4	1.1×10^4	1.1×10^4	4.6×10^3
10 months	1.1×10^5	1.1×10^5	4.6×10^5	1.1×10^6	1.1×10^6	4.6×10^6	4.6×10^5	1.1×10^6	4.6×10^3

nd = not detected; * carbon source

[a] To confirm the low numbers of bacterial cells observed after the short incubation period a control experiment was performed. A comparative MPN study was carried out with sediment (2-5 cm depth) from the same location at Dangast and from sediment at Horumersiel, north of Jadebusen in September 1999. The latter site is characterized by a locally high organic input and a high sulfidogenic activity. The MPN tubes were incubated for 2.5 months at 23 °C. MPN counts with all substrates tested (H_2 + CO_2, H_2 + CO_2 + 2mM acetate, 7.5 mM acetate, 5 mM lactate and 5 mM propionate) were between 1.1×10^4 an 1.1×10^5 per ml for the Dangast sediment. In contrast, MPN counts for the Horumersiel site were between 1.1×10^6 and 1.1×10^8 cells per ml.

During incubation of MPN tubes first a fast growing population and then after prolonged incubation a slow growing population developed. After about six weeks of incubation only

low cell numbers ranging between 1.1×10^3 and 4.6×10^4 cells per ml could be observed. The only exception were MPN cultures with H_2/CO_2/acetate as substrates yielding counts of up to 4.6×10^5 cells per ml. In general, cells grew homogeneously in the medium. With all substrates tested, rather similar cell numbers were obtained for all horizons analyzed. Thus this first population of fast growing SRBs appears to be evenly distributed across a depth profile of 0 to 15 cm. Cell numbers decreased by about one order of magnitude in horizon 15 to 20 cm. Prolonged incubation (10 months) of the MPN cultures allowed the detection of slowly growing but more abundant SRBs. Counts increased up to 1.1×10^7 cells per ml. In contrast to the faster growing SRBs, here cells did not grow homogeneously distributed in the medium, but rather formed flocs (Fig. 7 c and d) and aggregates, sometimes similar of the cell packages known from *Desulfosarcina* spp. As with the fast growing SRBs, MPN counts indicated that cells were evenly distributed throughout the horizons, with a decrease in cell numbers only in the last horizon.

Table 4. Identification of SRB by FISH[a] after 1.5 and 10 months of incubation.

	Horizon (cm)								
	0-0.5	**0.5-1**	**1-2**	**2-3**	**3-4**	**4-5**	**5-10**	**10-15**	**15-20**
Hydrogen (CO_2)*									
1.5 months	DSB985	nd	221	221	221	221	nd	221	nd
10 months	DSB985/[b] 221	DSB985	221	221	221	nd	221	221	221
Hydrogen (CO_2+Acetate)*									
1.5 months	DSV698	DSV698	DSV698	DSV698	DSV698	DSV698	DSV698	DSV698	DSV698
10 months	DSV698[c]	DSV698[c]	DSV698[c]	DSV698[c]	DSV698[c]	DSV698[c]	nd	nd	nd
Formate									
1.5 months	nd	nd	nd	nd	DSV698/ DSV214	DSV698/ DSV214	DSV698	nd	DSV698/ DSV214
10 months	nd	nd	DSV698	DSV698	DSV698	DSV698	DSV698	DSV698	nd
Acetate									
1.5 months	DSB985	DSB985	DSB985	DSB985	nd	DSB985	DSB985	DSB985	DSB985
10 months	DSB985/ DSS658	DSB985/ DSS658	DSB985	DSB985	DSB985	DSB985	DSB985	DSB985	DSB985
Lactate									
1.5 months	DSV698	DSV698	DSV698	DSV698	DSV698	DSV698	DSV698	DSV698	DSV698
10 months	DSV698	DSV698	DSV698	DSV698	DSV698	DSV698	DSV698	DSV698	nd
Propionate									
1.5 months	660	660	660	660	nd	nd	660	660	660
10 months	nd	nd	nd	660	nd	nd	660	660	660

nd = not detected; *carbon source

[a] Probes are described in Table 1.

[b] Positive hybridization with more than one probe.

[c] Lower brightness of the signal after 8.5 months incubation.

From the highest dilution steps that still showed growth, samples were recovered for FISH analysis, the results of which are summarized in Table 4.

MPN in agar shakes yielded numbers of colony forming units somewhat higher than those observed with MPNs in liquid media. Colonies from the highest dilution were transferred to fresh liquid media containing the same substrates as used in the corresponding agar shakes.

Subsequent analysis of growth cultures by FISH revealed no other phylogenetic groups as already identified in MPNs with liquid media.

FISH of sediment samples. Total cell counts of the upper 20 cm of the sediment were determined microscopically (Table 5). From top to bottom DAPI counts strongly decreased from 53.9×10^8 in the first cm to 1.0×10^8 cells per cm^{-3} of sediment in the zone below 15 cm.

The microbial community dwelling in Dangast sediments was dominated by Bacteria. In the top 0.5 cm of the sediment, up to 82.3% of the total microorganisms hybridized with the probe EUB338 (Table 5). Archaeal and eucaryal counts remained below the detection limit of 0.1% of the DAPI stained cells.

Recently, the bacterial probe EUB338 was shown not to detect all members of the domain *Bacteria* (Daims et al. 1999). Some bacterial phyla, most notably the *Planctomycetales* and *Verrucomicrobia*, are missed by this probe. Consequently, total bacterial numbers monitored in this study were most probably underestimated. Initial experiments with the newly designed additional probes EUB338-II and -III (Daims et al. 1999) indicated that this underestimation may amount to 4-10% of the total microbial community.

Detection rates with the probe EUB338 decreased exponentially over the vertical profile. Thus, not only the absolute cell numbers decreased with depth, but also the percentage of detectable cells with our FISH protocol.

Group specific probing. A set of 15 different probes specific for SRB of the γ-subclass of *Proteobacteria* were tested (Table 1) with sediment samples from Dangast. Only five of these probes (i.e. 221, DSB985, DSR6512, DSS658, and DSV698) gave counts above the detection limit set at 0.1% of total DAPI counts (Table 5). The target groups of the other probes were either not present in high abundance (>0.1% of the total cell counts) or not detectable by FISH, e.g. due to a low ribosomal content per cell.

The most abundant SRB present in the sediments were members of the *Desulfosarcina-Desulfococcus-Desulfofrigus* group (probe DSS658, Fig. 7 a and b) and *Desulfovibrio* spp. (probe DSV698) with maximum values of 2.9×10^8 and 2.8×10^8 cells per cm^{-3} of sediment, respectively. The counts of these two groups decreased strongly with depth to values of 1.0×10^6 and 0.6×10^6 cells per cm^{-3} of sediment at 20 cm, respectively.

SRB detected with the probe DSR651 specific for *Desulforhopalus* were also counted with decreasing numbers from top to bottom from 1.1×10^8 cells per cm^{-3} of sediment in the first 0.5 cm to 2.7×10^7 cells per cm^{-3} sediment between 10 and 15 cm depth. Cell numbers between 15 and 20 cm depth were below the detection limit.

Desulfobacter spp. (probe DSB985) were found over the whole sediment profile in relatively low abundance. The cell numbers were decreasing from 1.1×10^8 to 0.1×10^6 cells per cm^{-3} throughout the sediment. *Desulfobacterium* detected with the probe 221 were below the detection limit in the sediment except for the layers between 1 and 2 cm depth where between 0.5×10^7 and 1.0×10^7 cells per cm^{-3} were found.

The total SRB community was found to be maximum within the upper 3 cm of the sediment with numbers up to 6.6×10^8 cells per cm^{-3}, accounting for 8.6 to 12.3% of total cells. The number of SRB cells detected by FISH was decreasing over the vertical profile to 1.7×10^6 cells per cm^{-3} (or as low as 1.7% of total cells) at 20 cm depth.

Table 5. Quantification of SRB in Dangast sediments by FISH

	Horizon (cm)								
	0-0.5	**0.5-1**	**1-2**	**2-3**	**3-4**	**4-5**	**5-10**	**10-15**	**15-20**
DAPI absolute cell no. ($x10^8$)[b]	53.9±2.2	53.7±0.9	56.5±1.3	53.5±2.3	33.1±1.5	25.5±2.3	17.0±2.1	9.0±3.3	1.0±4.6
% of hybridized cells[a] (mean±SD)									
Probe EUB338	82.3±14	69.7±13	69.8±5	53.1±3	45.6±4	37.4±6	25.0±3	17.0±2	13.6±6
Probe 221	nd	0.22±0.04	nd[c]	0.15±0.03	nd	nd	nd	nd	nd
Probe DSB985	0.21±0.07	0.18±0.1	nd	nd	0.1±0.08	0.1±0.09	nd	nd	0.1±0.04
Probe DSR651	2.5±1	1.9±0.7	2.1±0.7	1.7±0.6	2.4±0.9	1.7±0.8	0.7±0.3	0.3±0.4	nd
Probe DSS658	2.5±1	4.7±1.3	5.3±0.9	3.8±0.8	2.4±0.7	3.5±0.9	2.2±0.4	1.7±0.5	1.0±0.6
Probe DSV698	4.3±0.9	5.3±1.1	3.9±0.8	4.4±0.4	4.2±0.7	6.2±1.2	3.3±0.3	1.0±0.7	0.6±0.4
Sum of SRB probes	8.6	12.3	11.3	10.0	9.1	11.5	6.3	3.0	1.7

[a]Percent detection compared to DAPI. Numbers have been corrected by subtracting NON338 counts. Means and standard deviations were calculated from the counts of two parallel cores.
[b]Number of cells per cm^{-3}.
[c]Not detected.

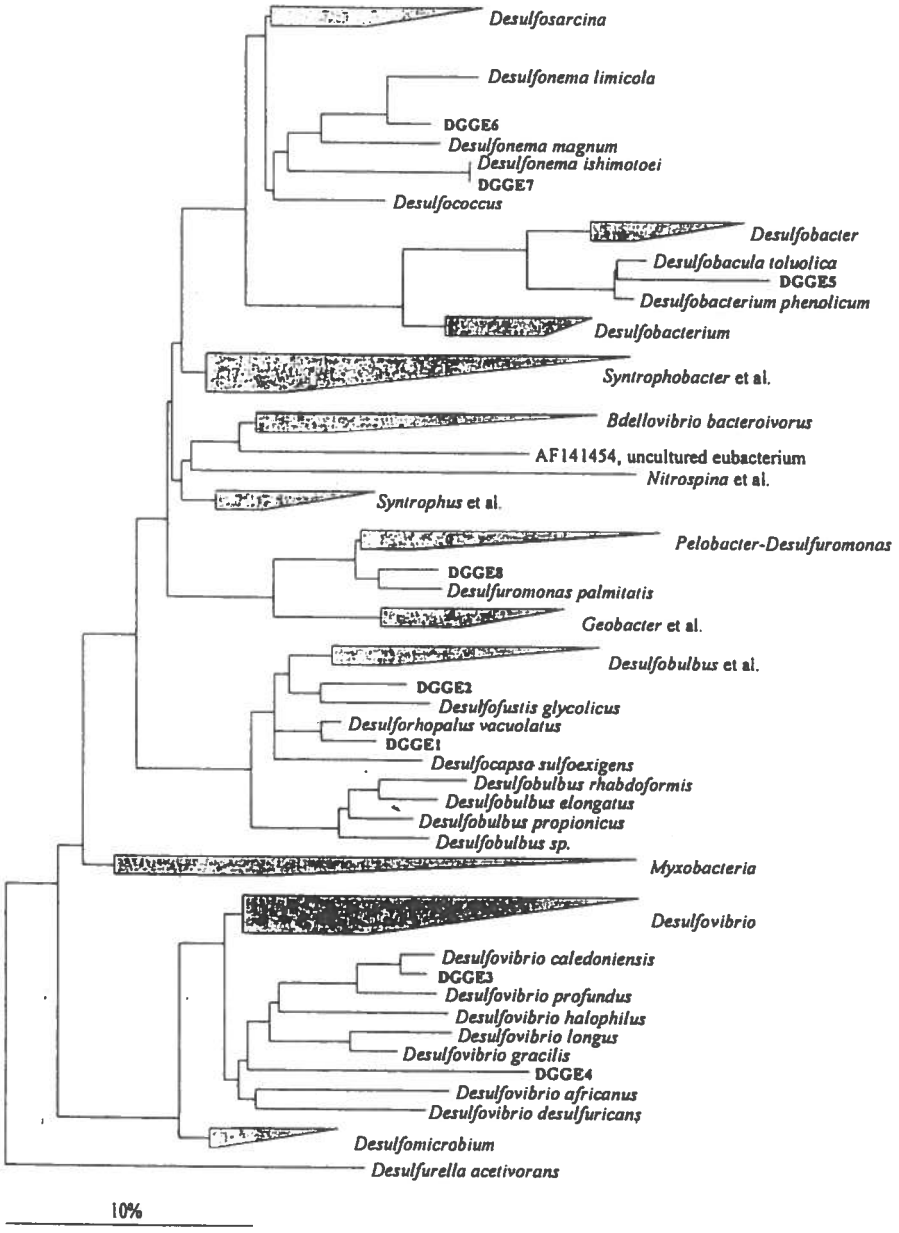

Fig. 6. Phylogenetic affiliation of the sequenced DGGE fragments. The tree is based on the result of the parsimony analysis including only complete or almost complete 16S rRNA sequences of representative bacteria (Maidak et al. 2000). The topology of the tree resulted from the insertion of the partial DGGE sequences into the tree without modifying its topology during sequence positioning (Strunk et al. 1998-2000).

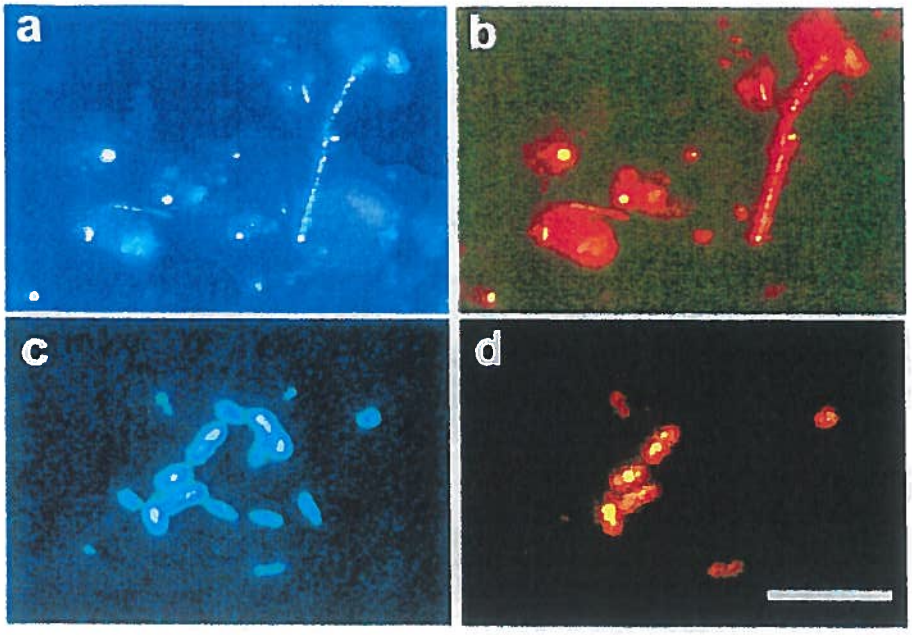

Fig. 7. Microscopic image of sediment samples (a and b) and of cells in a 10^{-7} dilution from an acetate utilizing MPN enrichment culture (c and d). Epifluorescence image using UV filter for DAPI (a and c), and green filter for visualization of cells stained with the Cy3-labelled probe DSS658 (b and d). Scale bar = 10 µm.

Discussion

Biogeochemistry of the sediment. The observed decrease in pore water content with sediment depth is due to changes in the sedimentological and mineralogical properties of the sediment caused by a mixing of minerals of the mud fraction with increasing amounts of the sand grain fraction (essentially quartz). In contrast to quartz, phyllosilicate minerals, and especially smectite, are able to adsorb significant amounts of organic matter (Keil et al. 1994; Mayer 1994). A parallel decrease of pore water and TOC contents with depth has been described earlier for tidal sediments of the German Wadden Sea (Delafontaine et al. 1996; Böttcher et al. 1998; Böttcher et al. 2000; Volkman et al. 2000). Stable carbon isotope measurements on TOC at Site Dangast have additionally shown that the labile fraction, which is of marine origin, decreases with depth due to the preferential biodegradation of marine organic matter (Böttcher et al. 2000). The accumulation of AVS throughout the investigated sediment layers is a clear indication for the dissimilative activity of sulfate-reducing bacteria under anaerobic conditions (e.g., Morse et al. (1987). Solid phase sulfur with higher oxidation states (essentially pyrite with minor elemental sulfur) were found through the sediment core with depth variations essentially decoupled from the AVS trend (Fig. 2C).

The sulfur isotopic discrimination between sulfate and the AVS-fractions is a clear indication that microbial dissimilatory sulfate reduction took place. This is due to a preferential intracellular, enzymatic reduction of $^{32}SO_4^{2-}$ compared to $^{34}SO_4^{2-}$ (e.g., Kaplan and

Rittenberg 1964). The isotope enrichment factors observed in the sediment partly exceeds the maximum found in pure cultures. The enhanced sulfur isotope discrimination may be due to the activity of sulfate reducers in the Dangast sediments that have not been available in previous experiments on isotope discrimination and/or a contribution from the oxidative part of the sulfur cycle. H_2S is to a significant portion reoxidized and sulfur species with intermediate oxidation states may be formed (Jørgensen 1982a). It has been shown experimentally that bacterial disproportionation of the latter leads to the formation of ^{32}S-enriched H_2S (Canfield et al. 1998; Cypionka et al. 1998; Böttcher and Thamdrup 2001) which may contribute to the observed overall isotope effect. Bacteria which are able to disproportionate elemental sulfur, for instance, have been isolated from the Dangast sediments and their ability to discriminate sulfur isotopes has been confirmed experimentally, too (Canfield et al. 1998). Compared to the AVS fraction, the physicochemically most stable phase pyrite from the top 11 cm was generally enriched in ^{34}S (Fig. 2). In the deeper more sandy sediment layers the isotope data of both fractions are close to each others. This may indicate the influence of particle mixing by bioturbation on the pyrite pool in the upper sediment section. Additionally, the pyrite fractions have preserved a change in the depositional environment and the near surface sulfur cycle of the Jade Bay sediments in the last century (Böttcher et al., in prep.).

Pore water composition. The compositions of interstitial waters sensitively mirror the biogeochemical processes in the sediment. According to the typical zonation scheme as proposed by Froelich et al. (1979), organic matter oxidation should be related to the consumption of oxygen followed by nitrate, the build up of Mn(II) and Fe(II) due to the reduction of Mn(IV) and Fe(III) (oxyhydr)oxides, and later to the depletion in sulfate.

Highest oxygen concentration in the photic zone are not directly linked to maximum nitrate production (Fig. 3 and 4). In the top 0.7 mm, where oxygen is produced via photosynthesis and oxygen concentrations are high, nitrate production rates seem to be relatively low. The nitrate concentration profile in the upper part of the sediment is influenced by an advective transport of nitrate resulting from a macrofauna mediated bioturbation. Since an assumption for activity calculations is a diffusion regulated transport and steady state concentrations, the upper part of the profile should be considered as non-informative. Nitrate production is taking place in the depth of 1 mm to approx. 2 mm. Net nitrate consumption was observed below 2 mm depth, where little or no oxygen was present.

Sulfate concentrations at the sediment-water interface correspond well to the measured salinities, indicating that dilution of seawater with sulfate-poor freshwaters took place in the

surface waters of the coastal region. The decrease in sulfate was associated with an enrichment in ^{34}S. This is expected for a system, where the consumption of sulfate by bacteria exceeds the supply of sulfate by transport from the sediment water interface or by reoxidation of sulfide (Hartman and Nielsen 1969).

The measured sulfate reduction rates are within the range observed previously at the same site (Böttcher et al. 2000) and in other fine-grained tidal sediments of the North Sea (Oenema 1990; Kristensen et al. 2000). The downcore variation of SRR found in the present study is essentially identical to results obtained during summer time on the same mudflat 1 year before (Böttcher and Hespenheide unpublished data). The same is also found for the community size and composition of SRB (Böttcher et al. 2000). This indicates a similarity in the seasonal variations of overall boundary conditions through the years. The maximum in bacterial sulfate-reduction within the suboxic zone, together with enhanced dissolved Fe(II) and Mn(II) concentrations and limited accumulation of dissolved sulfide has been observed earlier and is most likely related to reoxidation of sulfide (Moeslund et al. 1994; Thamdrup et al. 1994; Böttcher et al. 2000). Hydrogen sulfide which is produced during bacterial dissimilatory sulfate reduction can react with iron compounds to form iron sulfides and may be re-oxidized by the reaction with Fe(III) or Mn(IV) compounds to intermediate sulfur species or sulfate (Burdige 1993; Thamdrup et al. 1994; Böttcher and Thamdrup 2001). Therefore, the concentrations of dissolved Fe(II) and Mn(II) observed in the pore-waters are not necessarily the result of microbial reduction of the respective oxyhydroxides but may also result from reaction with sulfide produced during dissimilatory sulfate reduction. The observed decrease in SRR near the sediment-water interface is most likely superimposed by reoxidation reactions with reactive metal (oxyhydr)oxides in the surface sediments (Moeslund et al. 1994; Thamdrup et al. 1994). During short-time incubations of coastal sediments, Moeslund et al. (1994) observed 5-fold higher SRR at short compared to long incubation times. They related this phenomenon mainly to the reoxidation of H_2S during incubation with time.

Microbial sulfate reduction depends on the activity of fermentative bacteria which are able to degrade polymeric substances to smaller molecules like VFA, which are the actual substrates of sulfate-reducers (e.g., Sørensen et al. 1981; Christensen 1984). The SRR is, therefore, also linked to the production rate of VFA. The concentrations of volatile fatty acids in interstitial waters, therefore, depend on the balance of production and consumption rates. The reactions responsible for the quantitative balance in different environments, however, are still not understood. In the present study high concentrations of acetate and lactate coincide with low sulfate reduction rates (Fig. 2). This is in agreement with observations by Novelli et

al. (1988) and Holmer and Kristensen (1996). One may speculate that this correlation may be due to changes in sedimentological parameters (e.g., decreased water content), the TOC composition and an overall decrease in the availability of substrates.

The sulfate reduction rates per cell observed in these sediment samples were around 1 fmol $SO_4^{2-} \cdot cell^{-1} \cdot day^{-1}$ (as calculated from FISH and MPN results). This result is in agreement with previous reports (Sahm et al. 1999; Böttcher et al. 2000; Ravenschlag, 2000). They are at the lower end of cellular sulfate reduction rates determined for pure cultures of different psychrophilic, mesophilic, and thermophilic sulfate reducing bacteria (Canfield et al. 2000).

Sulfate reducing bacteria. The distribution of SRB in the vertical profile of the sediment was studied with four independent techniques.

First, the phospholipid fatty acids (PLFA) can be used as biomarkers for specific groups of organisms. The top 0.5 cm of the sediment was dominated by polyunsaturated PLFAs, which are typical for eucaryotic organisms (Vestal and White 1989; Findlay and Dobbs 1993), including protozoa, algae and higher plants. The sediment layers between 0.5 and 15 cm typically contained the PLFA C11 18:1, characteristic of anaerobic bacteria (Findlay et al. 1990; Findlay and Dobbs 1993; Vestal and White 1989). Thus the shift in PLFA pattern around 0.5 cm depth correlates well with the oxygen penetration as observed by the microsensor study (Fig. 3). In the deepest studied horizon (15-20 cm) a pronounced decrease in the diversity of PLFA indicated also a general decrease in microbial diversity. This is in good agreement with the changes of DGGE patterns (Fig. 5) and decrease in viable cell and FISH numbers (Table 3 and 5). Throughout the studied sediment horizons PLFA markers characteristic of SRB genera were identified pointing to an even distribution of SRB in the sediment profile. We found the marker fatty acids 10Me16:0 for *Desulfobacter* (Taylor and Parkes 1983) and i17:1 for *Desulfovibrio/Desulfomicrobium* (Vainshtein et al. 1992) in significant quantities in all horizons. We also found a17:1, a fatty acid which can be detected in *Desulfococcus* and *Desulfosarcina* (Kohring et al. 1994). The highest relative percentage of i17:1 was discovered in 15 to 20 cm depth. Whereas the most relative amount of 10Me16:0 was found between 4.0 and 15 cm depth.

Second, DGGE analysis was carried out to study the diversity of SRB (Fig. 5). A similar electrophoretic pattern was observed in the different horizons within the upper 15 cm of the sediment, showing an even distribution of SRB. Phylogentic identification of the most prominent bands showed diverse phylogenetic affiliation within the δ-subclass of *Proteobacteria*. Interestingly most sequences were closely related to SRBs originating from marine environments, e.g. *Desulfonema ishimotoei* (isolated from Dangast, the present study

area, ref. Fukui et al. 1999) and *Desulfobacula toluolica* (isolated from Eel Pond, Woods Hole, USA, ref. Rabus et al. 1993).

Third, the viable cells in the presence of a variety of substrates typical for SRBs were identified and quantified by combining MPN and FISH studies. Regardless of the MPN substrates used or the length of the incubation period, an even distribution of cells throughout the vertical profile of the sediment was observed (Table 3). Identification of SRB in highest dilution showing growth was performed with FISH (Table 4). With respect to the individual substrates added to the MPN cultures, results were in good agreement with the nutritional properties of known SRBs. For example, cultures with either lactate or H_2/CO_2/acetate known as substrates typical for *Desulfovibrio*, were indeed dominated by members of this species. In addition, H_2/CO_2, the utilization of which requires the capacity for chemolithoautotrophic growth as known from *Desulfobacterium autotrophicum* (Brysch et al. 1987), yielded predominantly members of the genus *Desulfobacterium*. MPN-cultures with acetate, the utilization of which requires the capacity of complete oxidation, were dominated by members of the genera *Desulfobacter* and/or *Desulfobacula*, both known to possess this capacity. During incubation generally two types of populations could be observed under all conditions (i.e. with every substrate used and with every sampling depth tested). After a short incubation time of about six weeks a more rapidly growing population of less abundant cells developed. The more abundant population required a prolonged incubation time of about 10 months. It should be noted that this population type did not grow homogenously in the culture medium but formed flocs (Fig. 7 c and d). The observation that these SRB predominantly grow in flocs may result in an underestimation of the actual cell numbers determined with MPN (Table 3) as compared to FISH (Table 5).

Fourth, the analysis of the active SRB or those with a high ribosome content was performed using FISH and DGGE analysis of reversly transcribed 16S rRNA fragments. DGGE profiles from reversly transcribed 16S rRNA indirectly reflect the ribosome content of cells, rendering this method a possible tool to identify active cells. Except for two sequences (DGGE 4 and 6; Fig. 5) all of those identified by DGGE profiling of 16S rDNA (Fig. 5, Fig. 6) were also found in the DGGE profiles of 16S rRNA. However, in the latter case a discrimination of detectable bands with depth was found. For example, DGGE band 5 is evenly distributed throughout the studied sediment horizons if monitored with the 16S rDNA-based DGGE profiling. In contrast, this band could only be detected in the lower part (below 3 cm) of the sediment, if monitored by 16S rRNA-based DGGE profiling. This could indicate that the *Desulfobacter/Desulfobacula* type SRB represented by DGGE band 5 are most active

in the lower part of the sediment, even though they are also present in the upper part. The opposite was observed for DGGE band 3, representing *Desulfovibrio* type SRB.

The examination of the microbial community in Dangast by FISH showed that SRB account for a significant part of the detectable Bacteria. Up to 6.5×10^8 cells per cm^{-3} were identified as SRB. This number of cells was always higher than the number of viable SRB observed using MPN. Similar discrepancy between MPN counts and for instance DAPI based cell counts have been described (Sievert et al. 1999). The absolute numbers of the different physiological groups might be underestimated since typically less than 1% of the total bacterial population in natural habitats may be accessible by current cultivation dependent methods (Amann et al. 1995). In addition, floc formation and clumping of cells may also lead to an underestimation, since the MPN evaluation assumes that only a single cell is required to initiate growth at the highest dilution.

The *Desulfosarcina-Desulfococcus-Desulfofrigus* group (targeted by probe DSS658) was identified as the most abundant SRB when sediment samples were directly analysed by FISH. This group was also identified by PLFA analysis of sediment samples. However, this group was not identified when MPN and DGGE methods were applied. Possible explanations could be on the one hand that the cultivation conditions used for MPNs do not select for SRBs affiliating with the *Desulfosarcina-Desulfococcus-Desulfofrigus* group, and on the other hand that the primers used for DGGE are not targeting this group (Ravenschlag et al. 2000). The second most abundant group of SRB identified in the sediment samples by FISH belong to the *Desulfovibrio* group. In this case, identification was possible with all three other techniques.

Conclusions

A combined microbiological, molecular, biogeochemical and isotope geochemical approach was applied to gain new information on the relationship between abundance, community structure and activity of the sulfate-reducing bacteria (SRB) of a surface marine sediment. Dissolved oxygen and nitrate were only found within the first few mm of the top sediment with different microscaled zones of formation and consumption, as obtained from profile modeling. Bacterial dissimilatory sulfate reduction was measured through the whole investigated sediment section. However, further reaction and reoxidation of hydrogen sulfide led to the development of sub-oxic conditions in the pore water. Microbial and chemical reactions led to the accumulation of dissolved iron and manganese in the suboxic zone. Maximum sulfate reduction rates (SRR) were found in the top 2 to 10 cm coinciding with a maximum of active SRBs as detected by FISH. Cellular SRR calculated from combined FISH

and volumetric SRR data basically stay constant along the vertical sediment profile. The sulfur isotopic composition of iron mono sulfides (AVS) is in agreement with microbial sulfate reduction at low cellular rates under conditions essentially open with respect to dissolved sulfate with additional contributions from the disproportionation of intermediate sulfur species. Contents and sulfur isotopic compositions of the most stable sulfide species, pyrite, seem to be decoupled from the vertical profile of AVS and significantly influenced by sedimentation conditions and particle mixing (bioturbation).

The combined application of molecular (DGGE of PCR-amplified 16S rDNA) and microbiological (PLFA and MPN analyses) tools demonstrated that diverse SRB are evenly found across the studied vertical sediment profile, regardless of the biogeochemical zonation. Thus presence of cultivatable SRBs in a given horizon cannot always be related to either in situ abundance or cellular activity. However, with respect to the presumably active part of the SRB population, a good correlation was observed between the different techniques. The SRB community in the upper sediment was dominated by SRBs targeted by probes DSS685 and DSV698 and thus most likely by members of the *Desulfosarcina-Desulfococcus-Desulfofrigus* and *Desulfovibrio* groups, as revealed by FISH and in the case of the *Desulfovibrio* group, by DGGE of reversly transcribed 16S rRNA. It is known that these SRB possess the capacity to utilize acetate and lactate. This is in agreement with low concentrations of lactate and acetate in the upper layers of the sediment. Based on the present results we suggest that the *Desulfosarcina-Desulfococcus-Desulfofrigus* group and *Desulfovibrio* spp. - at the sampling time point in June 1999 - are the key populations for the terminal mineralization step in the anaerobic zone of the temperate intertidal mud flat.

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