Molekularbiologische Analyse der Diversität und Struktur mikrobieller Lebensgemeinschaften in marinen, arktischen Sedimenten (Svalbard)

Molecular Analysis of the Microbial Diversity and Community Structure of Marine Arctic Sediments (Svalbard)

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Katrin Ravenschlag

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1. Gutachter: Priv.-Doz. Dr. Rudolf Amann
2. Gutachter: Prof. Dr. Friedrich Widdel

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INHALTSVERZEICHNIS

ZUSAMMENFASSUNG

In marinen Sedimenten spielen mikrobielle Lebensgemeinschaften bei der Mineralisierung organischen Materials eine entscheidende Rolle. Die Kenntnis der Diversität und Struktur dieser mikrobiellen Gemeinschaften ist für das Verständnis der Mineralisierungsprozesse von großer Bedeutung.

Im Rahmen dieser Arbeit wurden mikrobielle Lebensgemeinschaften in permanent kalten marinen Sedimenten von Svalbard (Arktischer Ozean) untersucht. Zur Beschreibung der Diversität wurde eine 16S rDNA Genbank angelegt. Ergebnisse einer Analyse von 353 Klonen zeigten eine hohe bakterielle Diversität im untersuchten Sediment. Eine vergleichende phylogenetische Analyse von 16S rDNA Sequenzen aus verschiedenen marinen Sedimenten zeigte das weit verbreitete Vorkommen bestimmter Gruppen von Bakterien, die eventuell kosmopolitische Gruppen bilden.

Mit Hilfe der Fluoreszenz-*in situ*-Hybridisierung (FISH) und der quantitativen rRNA slot-blot Hybridisierung wurden Abundanz und räumliche Verteilung wichtiger Gruppen untersucht. Vertreter der Bacteria und nicht der Archaea dominierten die mikrobielle Lebensgemeinschaft. Die Gruppe der sulfatreduzierenden Bakterien der δ-Proteobakterien war am häufigsten vertreten mit einem Anteil von 9,3 ± 3,6% der Gesamtzellzahl bzw. 20,1 ± 2,9% der prokaryontischen rRNA. Etwa 75% davon konnte der *Desulfosarcina/Desulfococcus*-Gruppe zugeordnet werden. Die dominante Rolle der *Desulfosarcina/Desulfococcus*-Gruppe zeigte sich in allen vier untersuchten Sedimenten entlang der Küste Svalbards. Innerhalb dieser Gruppe dominierten jedoch nicht die bislang kultivierten Gattungen, sondern eine neue Untergruppe, SVAL1, die mit *Desulfosarcina variabilis* am nächsten verwandt ist (91,2% Sequenzähnlichkeit) und *in situ* mehr als die Hälfte der sulfatreduzierenden Bakterien ausmachte. Die höchste metabolische Aktivität der *Desulfosarcina/Desulfococcus*-Gruppe wurde in der Oberflächenschicht des Sedimentes (0-5 mm Tiefe) gefunden. Andere Gattungen sulfatreduzierender Bakterien wurden nur in einer ca. vier- bis zehnfach niedrigeren Abundanz nachgewiesen.

Neben den sulfatreduzierenden Bakterien waren Cytophagen/Flavobakterien, Planktomyceten und γ-Proteobakterien abundant, wobei die Cytophagen/Flavobakterien eine dominante Rolle in den Svalbard Sedimenten einnehmen.

Psychrophile schwefeloxidierende Bakterien wurden erstmalig isoliert und charakterisiert. Beide isolierten Stämme sind der Gattung *Thiomicrospira* zuzuordnen und stellen eine neue Art der Gattung dar (*Thiomicrospira arctica*).

Methanhydratreiche Sedimente (Cascadia Subduktionszone, Oregon) wurden mittels FISH untersucht, um die an der anaeroben Methanoxidation beteiligten Mikroorganismen zu identifizieren. Biogeochemische Untersuchungen lassen vermuten, daß dieser Prozeß durch Archaea in Syntrophie mit anderen anaeroben Bakterien, wahrscheinlich Sulfatreduzierern, katalysiert wird. In der Zone der anaeroben Methanoxidation wurden in der Tat Konsortien aus Bacteria und Archaea gefunden. Die Archaea konnten der Ordnung Methanomicrobiales und die Bacteria den sulfatreduzierenden Bakterien der *Desulfococcus/Desulfosarcina*-Gruppe zugeordnet werden.

SUMMARY

Microbial communities play a major role in the mineralization of organic matter in marine sediments. Knowledge of the diversity and structure of these microbial communities is of great importance for our understanding of mineralization processes.

In this thesis, microbial communities in permanently cold marine sediments from Svalbard (Arctic Ocean) were investigated. Diversity was studied using a 16S rDNA clone library. Analysis of 353 clones showed high bacterial diversity in these sediments. A comparative phylogenetic analysis of 16S rDNA sequences retrieved from a variety of different marine sediments indicated that certain groups of bacteria, which presumably form cosmopolitan groups, were widely distributed.

The abundance and spatial distribution of several major bacterial groups were investigated by fluorescence-*in situ*-hybridization (FISH) and quantitative rRNA slot-blot hybridization. The microbial community was dominated by members of the Bacteria and not of the Archaea. The most abundant group identified were sulfate-reducing bacteria belonging to the δ-proteobacteria, which accounted for 9.3 ± 3.6% of total cell counts and 20.1 ± 2.9% of prokaryotic rRNA. Seventy-five percent could be affiliated with the *Desulfosarcina/Desulfococcus* group, which predominated at all four stations investigated along the coast of Svalbard. A new subgroup (SVAL1), rather than any cultivated genera, dominated this group and made up more than 50% of total sulfate-reducing bacteria *in situ*. Maximal metabolic activity of *Desulfosarcina/Desulfococcus* cells occurred close to the surface (0-0.5 cm depth). Other genera of sulfate-reducing bacteria were found at four- to ten-fold lower abundance.

Planctomycetales, γ-proteobacteria, and particularly *Cytophaga*/Flavobacteria were also abundant in the Svalbard sediments.

For the first time, psychrophilic sulfur-oxidizing bacteria were isolated and characterized. The new strain SVAL-E is affiliated with the genus *Thiomicrospira* and represents a new species of this genus (*Thiomicrospira arctica*).

Methane-rich sediments from the Cascadia convergent margin off Oregon were also investigated by FISH, to identify the microorganisms involved in anaerobic oxidation of methane. Geochemical studies have suggested that this process is catalyzed by Archaea in synthrophy with other anaerobic bacteria, presumably sulfate-reducing bacteria. In the Cascadia sediments, consortia of Bacteria and Archaea were identified in the zone of anaerobic oxidation of methane. The Archaea were affiliated with the order Methanomicrobiales and the Bacteria with the *Desulfosarcina/Desulfococcus* group.

Teil I:

Darstellung der Ergebnisse im Gesamtzusammenhang

A Einleitung

1 Mikrobieller Abbau von organischem Material in marinen Sedimenten

Marine Sedimente sind Orte, in denen ein wesentlicher Teil der marinen Nahrungskette abläuft. Sie spielen eine wichtige Rolle bei der Mineralisierung organischen Materials. Der größte Teil des organischen Kohlenstoffs, der in marinen Sedimenten mineralisiert wird, stammt aus der marinen Primärproduktion in der photischen Zone (Jørgensen, 1983). Ein Teil der organischen Partikel wird bereits beim Absinken in der Wassersäule abgebaut. In Schelfregionen erreichen bis zu 50% der Primärproduktion den Meeresboden, in der Tiefsee hingegen wird der größte Teil des organischen Materials schon in der Wassersäule abgebaut (Jørgensen, 1983; Wollast, 1991).

Bei der Mineralisierung des organischen Materials und der Regeneration anorganischer Nährstoffe im Sediment spielen mikrobielle Lebensgemeinschaften eine entscheidende Rolle. Ein Teil der eingetragenen organischen Substanz kann nicht mineralisiert werden und wird für immer im Sediment "begraben", der größte Teil jedoch wird reoxidiert, und zwar hauptsächlich durch Mikroorganismen (Wollast, 1991). Am mikrobiellen Abbau sind verschiedene Bakteriengruppen beteiligt. Unter oxischen Bedingungen können organische Verbindungen häufig von einem einzigen Organismus komplett mineralisiert werden (Capone und Kiene, 1988; Gibson, 1984). In Schelfsedimenten jedoch ist Sauerstoff nur in den obersten wenigen Millimetern verfügbar (Revsbech *et al.*, 1980).

Im anoxischen Bereich erfolgt der Abbau von organischem Material schrittweise unter Beteiligung verschiedener physiologischer Gruppen von Bakterien ("anaerobe Nahrungskette", Abb.1). Das Endprodukt der einen Gruppe von Organismen dient einer anderen Gruppe als Wachstumssubstrat, bis das organische Material vollständig zu CO_2 oxidiert worden ist. In anaeroben Lebensgemeinschaften ist eine Vielfalt von fermentierenden Bakterien für die Hydrolyse organischer Polymere verantwortlich, bei der Wasserstoff, Acetat, andere Fettsäuren, Alkohole und andere niedermolekulare Verbindungen als Endprodukte gebildet werden. Denitrifikanten, Eisenreduzierer, Sulfatreduzierer, Homoacetogene und Methanogene nutzen diese Fermentationsendprodukte als Elektronendonatoren und Kohlenstoffquellen zur anaeroben Respiration unter Verwendung

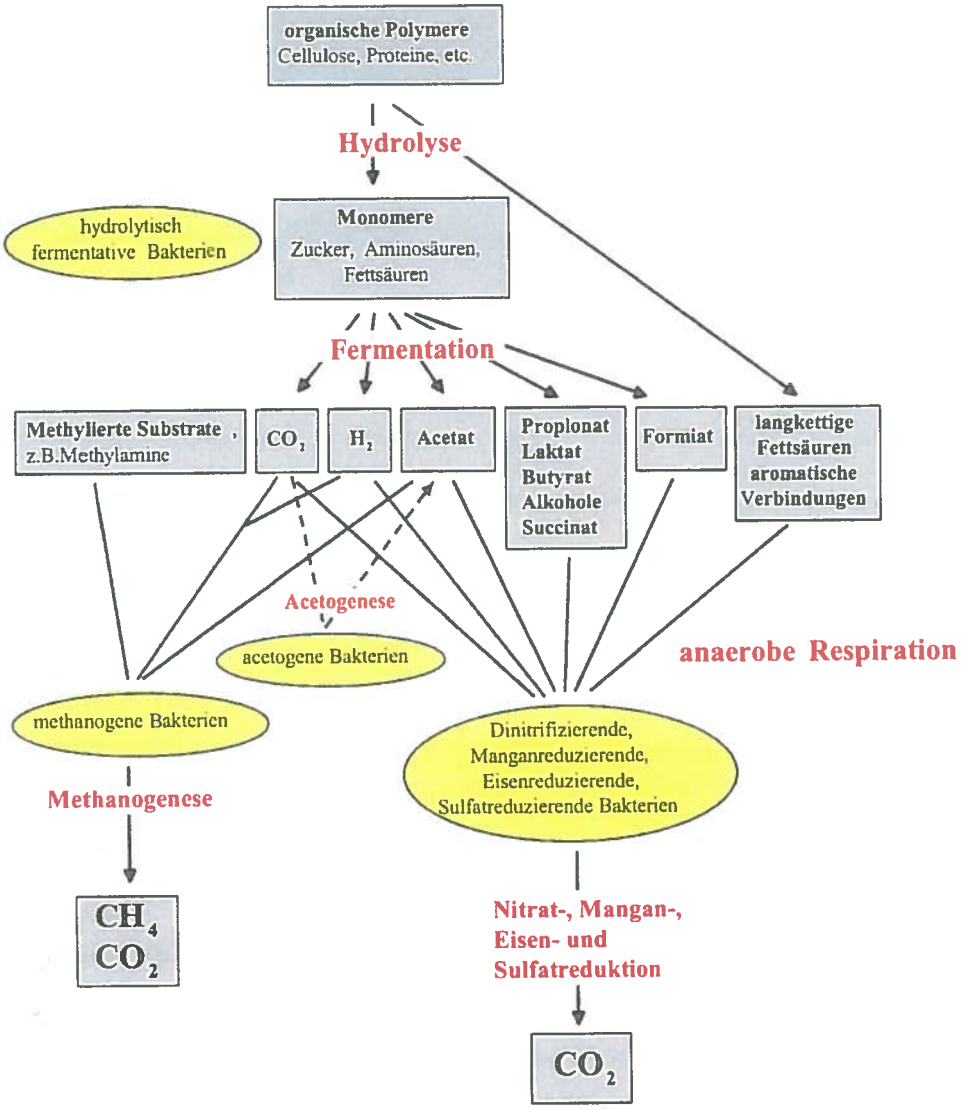


Abbildung 1: Vereinfachte Darstellung der verschiedenen Prozesse des anaeroben Abbaus organischen Materials in Sedimenten und der wichtigsten Kohlenstoffquellen für diese Prozesse (nach Fenchel (1995) und Capone (1988), verändert)

unterschiedlicher Elektronenakzeptoren.

In marinen Sedimenten tritt eine vertikale Abfolge der von Bakterien genutzten Elektronenakzeptoren auf (Abb.2), die in etwa den abnehmenden Redoxpotentialen und den möglichen Energiegewinnen folgt (Thauer *et al.*, 1977; Fenchel und Finlay, 1995). In der suboxischen Zone (anoxisch, aber mit positivem Redoxpotential) sind Nitrat, Mangan (IV) und Eisen (III) die Hauptelektronenakzeptoren. Im darunterliegenden sulfidischen Bereich überwiegt die Sulfatreduktion. Eine strikte vertikale Abgrenzung der unterschiedlichen Respirationsprozesse ist jedoch nicht möglich (Canfield *et al.*, 1993).

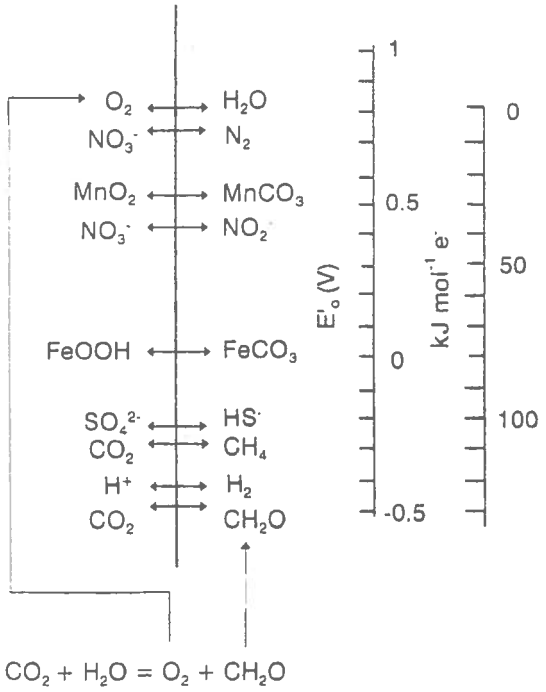


Abbildung 2: Standard Redoxpotentiale einiger wichtiger Redoxpaare (pH 7) und Änderungen der freien Energie (aus: Fenchel *et al.*, 2000)

Sulfatreduktion. In marinen Sedimenten kann aufgrund der hohen Sulfatkonzentration von 28 mM im Meerwasser die Sulfatreduktion für bis zu 50% der gesamten Mineralisierung des organischen Materials verantwortlich sein (Jørgensen, 1982a; Canfield *et al.*, 1993). In Süßwasserhabitaten, in denen Sulfat nur in geringen Konzentrationen zur Verfügung steht, hat die Methanogenese im allgemeinen den größten Anteil an der anaeroben Mineralisierung (Capone und Kiene, 1988).

Sulfatreduzierende Bakterien (SRB). Die Fähigkeit zur assimilatorischen Sulfatreduktion ist eine weitverbreitete Eigenschaft von Bakterien, jedoch sind nur wenige Gruppen zur dissimilatorischen Sulfatreduktion befähigt (Widdel und Hansen, 1992a). Dissimilatorisch sulfatreduzierende Bakterien können Sulfat als terminalen Elektronenakzeptor für eine anaerobe Respiration verwenden (Widdel und Bak, 1992b). Als Kohlenstoffquellen und Elektronendonatoren können sie eine Reihe von löslichen und langkettigen Fettsäuren (C_1-C_{18}), Aminosäuren, Alkoholen und aromatischen Verbindungen nutzen, nicht jedoch Polysaccharide oder andere polymere Substanzen. Manche sulfatreduzierende Bakterien, wie z.B. Vertreter der Gattung *Desulfosarcina*, sind auch zu autotrophem Wachstum mit CO_2 als einziger Kohlenstoffquelle fähig (Widdel und Bak, 1992b).

Einige Sulfatreduzierer sind nicht auf Sulfat angewiesen und können Nitrat (Widdel und Hansen, 1992a) oder Eisen (III) (Coleman *et al.*, 1993; Lovley und Phillips, 1994) als alternative Elektronenakzeptoren nutzen oder andere anorganische Schwefelverbindungen reduzieren oder disproportionieren (Krämer und Cypionka, 1989; Janssen *et al.*, 1996) . Auch das Wachstum unter fermentativen Bedingungen, z.B. mit Pyruvat oder Malat, wurde beschrieben (Widdel und Bak, 1992b).

Physiologisch lassen sich sulfatreduzierende Bakterien in zwei Gruppen einteilen. Organismen, die organisches Substrat vollständig zu CO_2 oxidieren, werden als vollständige

Oxidierer (z.B. Vertreter der Desulfobacteraceae) bezeichnet. Organismen, die organisches Substrat nur unvollständig zu Acetat oxidieren, werden als unvollständige Oxidierer bezeichnet (z.B. Vertreter der Desulfovibrionaceae).

Aus heutiger Sicht sind die sulfatreduzierenden Bakterien eine der vielseitigsten physiologischen Gruppen anaerober Bakterien, sowohl in bezug auf die Verwertung von Elektronendonatoren und Kohlenstoffquellen als auch auf die möglichen Elektronenakzeptoren. Diese vielfältigen physiologischen Eigenschaften ermöglichen es ihnen, verschiedene Lebensräume zu besiedeln. Die größte Diversität von sulfatreduzierenden Bakterien wurde bislang in marinen Sedimenten gefunden, in denen Sulfat kein limitierender Faktor ist (Widdel und Bak, 1992b). Andere typische Lebensräume sind limnische Ökosysteme (z.B. Bak und Pfennig, 1991; Sass *et al.*, 1997; Sass *et al.*, 1998), Biofilme (z.B. Ramsing *et al.*, 1993; Raskin *et al.*, 1996; Santegoeds *et al.*, 1998) und Kläranlagen (z.B. Manz *et al.*, 1998; Schramm *et al.*, 1999).

Die Vorstellung eines strikt anaeroben Lebensraumes für sulfatreduzierende Bakterien wurde gelockert, nachdem diese auch in oxischen Bereichen mariner Sedimente und mikrobieller Matten gefunden wurden (Jørgensen und Bak, 1991; Visscher *et al.*, 1992; Sahm *et al.*, 1999a; Minz *et al.*, 1999). Auch wurde metabolische Aktivität in Gegenwart von Sauerstoff beobachtet (Jørgensen, 1977; Dilling und Cypionka, 1990; Canfield und DesMarais, 1991a; Dannenberg *et al.*, 1992; Fründ und Cohen, 1992; Marschall *et al.*, 1993; Krekeler *et al.*, 1998), Wachstum jedoch bisher nicht gefunden.

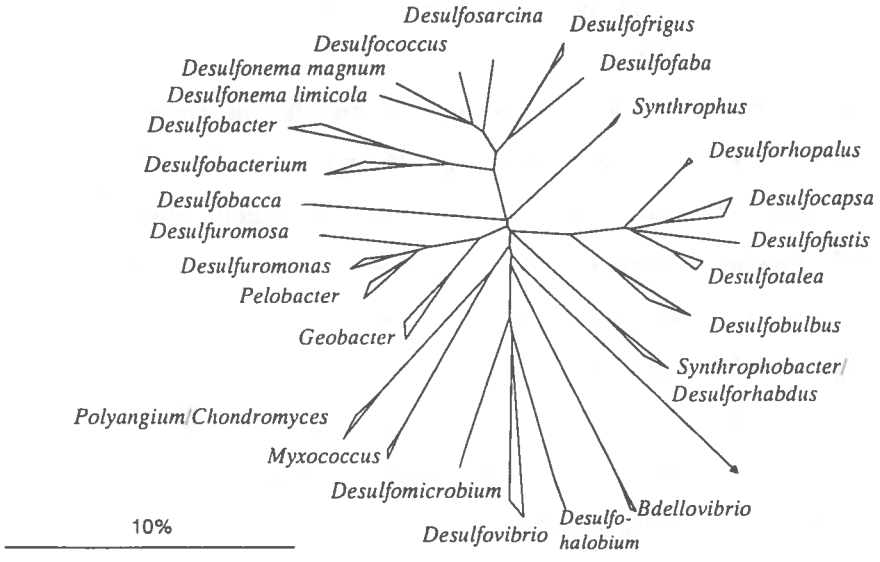


Abbildung 3: Phylogenetischer Baum der δ-Proteobakterien, der aus 16S rDNA Sequenzen ausgesuchter Vertreter der gezeigten Gattungen rekonstruiert wurde. Der Balken gibt 10% Sequenzunterschied an.

Sulfatreduzierende Bakterien können vier verschiedenen phylogenetischen Gruppen zugeordnet werden: Die meisten Sulfatreduzierer sind Gram-negativ und befinden sich innerhalb der δ-Proteobakterien (Abb.3). Derzeit (Jahr 2000) werden dieser Gruppe gemäß "Bergey's Manual of Systematic Bacteriology" 27 Gattungen sulfatreduzierender Bakterien zugeordnet.

Weitere Gattungen finden sich unter den Gram-positiven Bakterien (*Desulfotomaculum* und *Desulfosporosinus*) und in der Domäne Archaea (*Archaeoglobus*). Die Gram-negativen thermophilen Sulfatreduzierer der beiden verwandten Gattungen *Thermodesulfobacterium* und *Thermodesulfovibrio* sind weder eng mit den sulfatreduzierenden Bakterien der δ-Proteobakterien noch mit denen der Gram-positiven Bakterien verwandt.

2 Identifizierung und Quantifizierung von Mikroorganismen

Die Identifizierung von Mikroorganismen und damit auch die Beschreibung mikrobieller Diversität als Grundlage für die Analyse von mikrobiellen Lebensgemeinschaften war über lange Zeit von deren Kultivierung abhängig. Die Reinkulturen wurden aufgrund phänotypischer, physiologischer und biochemischer Eigenschaften klassifiziert. Diese Form der Identifizierung ist oft sehr zeitaufwendig und die Bedeutung nicht kultivierter Organismen bleibt unklar. Oft läßt sich nur ein kleiner Teil der mikroskopisch sichtbaren Bakterien eines natürlichen Lebensraum in Kultur bringen (Jannasch, 1958; Amann *et al.*, 1995). Die Häufigkeit der kultivierten Bakterien spiegelt nicht unbedingt ihre Abundanz im natürlichen Lebensraum wider (Wagner *et al.*, 1993).

In den frühen achtziger Jahren haben Woese und Mitarbeiter die Untersuchung von Bakterien basierend auf vergleichender Sequenzanalyse ihrer ribosomalen RNA-Moleküle (5S, 16S, 23S rRNA) eingeführt und damit für "tiefe Abzweigungen" eine stabile, die Evolution reflektierende Systematik ermöglicht (Woese, 1987). rRNA Moleküle haben sich als geeignet herausgestellt, um phylogenetische Verwandtschaftsverhältnisse zu rekonstruieren. rRNA ist in allen Organismen universell vorhanden, funktionell gleich und besitzt Regionen, die unterschiedlich stark konserviert sind. Heute (Juli 2000) sind in den großen Datenbanken GenBank (http://www.ncbi.nlm.nih.gov), EMBL (http://www.embl-heidelberg.de) und DDBJ (http://www.ddbj.nig.ac.jp) mehr als 47000 16S und 18S rDNA Sequenzen hinterlegt. Das Potential der vergleichenden 16S rRNA-Analyse für kultivierungsunabhängige Untersuchungen zur Diversität und Ökologie von

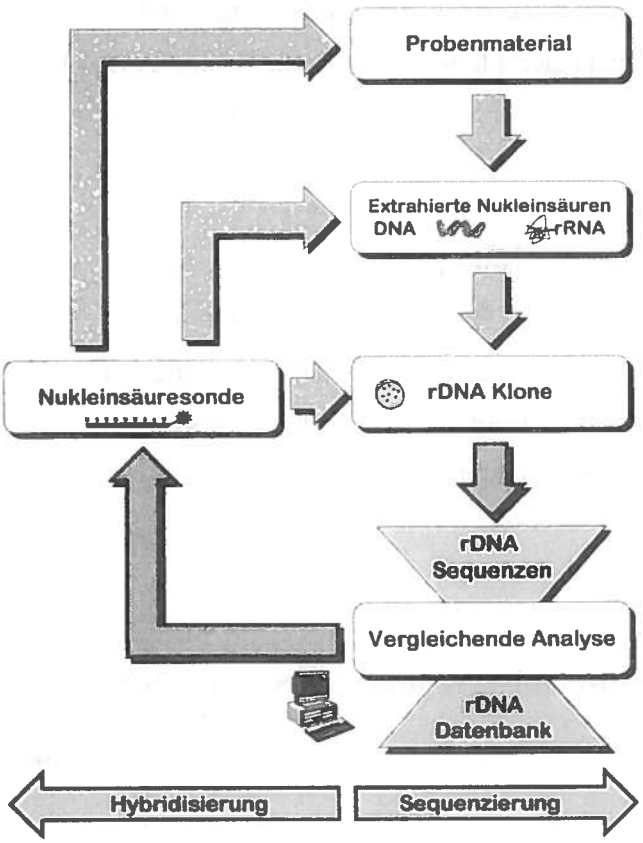


Abbildung 4: Der rRNA-Ansatz zur kultivierungsunabhängigen Untersuchung mikrobieller Lebensgemeinschaften in Umweltproben
(aus: Glöckner, 1999, modifiziert von Amann, 1995)

Mikroorganismen in Umweltproben wurde schnell erkannt, und das Verfahren wird heute als rRNA-Ansatz (Abb. 4) bezeichnet (Olsen *et al.*, 1986; Pace, 1996).

Die Klonierung von 16S rRNA Genen aus DNA, die direkt aus Umweltproben isoliert wurde, deckte in den vergangenen Jahren eine Vielzahl neuer Sequenzen auf, die in der Regel nicht in den Datenbanken vorhanden waren und damit auch nicht den Sequenzen aus kultivierten Bakterien entsprachen, sofern deren 16S rDNA Sequenz bestimmt war (z.B. Ward *et al.*, 1990; Giovannoni *et al.*, 1990; DeLong, 1992; Fuhrman *et al.*, 1992; Devereux und Mundfrom, 1994). Reassoziationsanalysen von DNA aus Sedimenten zeigten, daß etwa 10000 unterschiedliche Genome in der Probe enthalten waren (Torsvik *et al.*, 1996). Die Zahl der bisher beschriebenen Arten liegt mit ca. 4300 (Ende 1999, Bergey's Manual) deutlich unter diesem Wert.

Die 16S rDNA Genbanken können einen guten Überblick über die prokaryontische Artenvielfalt ("species richness") in einem Lebensraum geben, von der Repräsentativität einer Genbank darf jedoch nicht ausgegangen werden. Das Verhältnis der PCR-Produkte kann aufgrund möglicher selektiver DNA-Extraktion oder PCR-Amplifikation und der Amplifikation bis zu einer Sättigungskonzentration nicht dem der Ausgangsprobe entsprechen (Reysenbach *et al.*, 1992; Farrelly *et al.*, 1995; Suzuki und Giovannoni, 1996). Ebenso kann nicht zwischen aktiven und inaktiven oder autochthonen und allochthonen Fraktionen der Gemeinschaft unterschieden werden. Hybridisierungstechniken sind für die Quantifizierung von Bakterien besser geeignet: Mit Hilfe der quantitativen rRNA slot-blot Hybridisierung (Stahl *et al.*, 1988) kann spezifische rRNA im Verhältnis zur gesamten, extrahierten rRNA

quantifiziert werden, und mittels der Fluoreszenz-*in situ*-Hybridisierung (FISH) können intakte fixierte Zellen identifiziert und direkt gezählt werden (Amann *et al.*, 1995). FISH ermöglicht nicht nur eine Identifizierung und Quantifizierung von Bakterien, sondern auch Aussagen über Morphologie sowie die räumliche Verteilung und Interaktion von Bakteriengruppen (Amann *et al.*, 1995).

Der Einsatz der *in situ*-Hybridisierung in marinen Sedimenten ist aufgrund eines in tieferen Sedimentschichten oft sehr geringen zellulären rRNA-Gehaltes der vorkommenden Bakterien und möglicher Autofluoreszenz von organischer Substanz schwierig. Erst 1998 gelang es nach Einführung neuer Fluoreszenzfarbstoffe, verbesserter Filtersätze und einer Optimierung des Protokolls erstmalig, FISH in marinen Sedimenten erfolgreich anzuwenden (Llobet-Brossa *et al.*, 1998).

3 Prokaryontische Lebensgemeinschaften in marinen Sedimenten

3.1 Diversität

Die Beschreibung der mikrobiellen Diversität ist Grundlage für Strukturanalysen von Lebensgemeinschaften und für die Quantifizierung von Bakterien in ihren natürlichen Lebensräumen. Das Wissen über die bakterielle Diversität in marinen benthischen Lebensgemeinschaften wurde in den letzten Jahren durch intensive Untersuchungen, die auf der Analyse von 16S rDNA Sequenzen beruhen, stark erweitert (Tab.1).

Tabelle 1: Bakterielle 16S rDNA Genbanken aus marinen Sedimenten (k.A. keine Angabe)

Sediment	Probennahmestelle	Tiefe	Klone	Accession-no.	Referenz
Küstensediment	Puget Sound, USA 47°37'N, 122°30'W	13 m	EH-*	U43630-U43651	Gray & Herwig, 1996
Küstensediment	Tokyo Bay 35°21,0'N, 139°47,3'O	15 m	TIHP302-* 0-2 cm Tiefe TIHP368-* 6-8 cm Tiefe	AB031590-AB031662	Urakawa *et al.*, unveröffentlicht
Küstensediment	Taynaya Bay, Antarktis 68°46'S, 78°29'O	32 m	TAYNAYA*	AF142950-AF142975	Bowman *et al*, 2000
Küstensediment	Santa Rosa Sound, USA (k.A.)	k.A.	A* (nur SRB)	U08385-U08397	Devereux *et al.*, 1994
Küstensediment	Wattenmeer, Sylt	-	L*	-	Kolb, 2000

Sediment	Probennahmestelle	Tiefe	Klone	Accession-no.	Referenz
Küstensediment (Seegras-besiedelt)	Bassin d'Archachon (k.A.)	k.A.	B2M*	AF223253-AF223307	Cifuentes *et al.*, 2000
Küstensediment (Brackwasser)	k.A.	k.A.	CE* MT*	AF211258-AF211331	Tanner *et al.*, unveröffentlicht
Ästuarsediment	Papua, Neu Guinea (k.A.)	k.A.	n* a*	AF194185-AF194214; AF193560-AF193570	Todorov *et al.*, unveröffentlicht
Küsten- und Tiefseesediment	Sagami Bay SB: 35°4,0'N, 139°14,5'O SA: 35°0,2'N, 139°20,5'E Tokyo Bay 35°20,8'N, 139°47,1'E	 1159 m 1516 m 43 m	 SA* SB* TK*	AB022607-AB022642	Urakawa *et al.*, 1999
Schelfsediment	Black Sea	k.A.	A3b3, A3b2, B4b1, D1Mn, D1a1	AJ271653-AJ271657	Thamdrup *et al.*, 2000
Schelfsediment	Black Sea 43°53'N, 29°58'O	77 m	BS*	AJ011657-AJ011668	Rosselló-Mora *et al.*, 1999
Tiefseesedimente	Suruga Bay 35°02'N, 138°37'O 34°55'N, 138°39'O Ryukyu Trench 28°10'N, 130°23'O 28°29'N, 130°11'O 27°53'N, 130°32'O Izu-Bonin Trench 30°55'N, 141°49'O Japan Trench 40°06'N, 144°11'O	 1159 m 1521 m 2339 m 3671 m 6482 m 4031 m 6379 m	 BD1-* BD2-* BD3-* BD4-* BD6-* BD5-* BD7-*	AB015514-AB015588	Li *et al.*, 1999a
Tiefseesediment	Nankai Trough 33°39'N, 137°56'O	3843 m	NKB*	AB013253-AB013271	Li *et al.*, 1999b
Tiefseesediment (unterschiedliche Probenbehandlung)	Japan Sea 37°02'N, 134°48'O	900 m	JAP*	U07035; U09570-75; U09773-80; U09826-30 U09845	Rochelle *et al.*, 1994
Tiefseesediment (Anreicherung)	Guaymas Basin	k.A.	SB*	AF029039-AF029050	Phelps *et al.*, 1998
"Cold-Seep" Sediment	Japan Trench 40°06'N 144°11'O	6400 m	JTB*	AB01540-AB015272	Li *et al.*, 1999c
"Cold-Seep" Sediment	k.A.	k.A.	BPC*	AF154080-AF154099	O'Neill *et al.*, unveröffentlicht
Methanhydrat-reiche Sedimente und Fluids	Bioherm "Champagne Hill" und "Dead Clam" 44°40.51'N, 125°07.41'W 44°40.45'N 125°07.37'W Cascadia Margin (Fluid) 44°40.54'N, 125°07.09'W	~675m	ODP8-* ODPB-*	AF121082-AF121091	Bidle *et al.*, 1999

Es wurden neue phylogenetische Gruppen entdeckt, von denen viele bisher nur Sequenzen unkultivierter Organismen enthalten. Bislang konnte keine Sequenz mit 100% Sequenzähnlichkeit zu einem kultivierten Bakterium in den Genbanken gefunden werden. Eine Übersicht und phylogenetische Einordnung/Gruppierung aller 16S rDNA Sequenzen, die aus Genbanken mariner Sedimente erhalten wurden, ist im Anhang zu finden.

Der größte Anteil der aus marinen Sedimenten erhaltenen 16S rDNA Sequenzen befindet sich in den Gruppen der γ- und δ-Proteobakterien. Innerhalb der γ-Proteobakterien zeigen sich sechs Gruppen von Sequenzen. Drei Gruppen sind mit endosymbiontisch-lebenden schwefeloxidierenden Bakterien verwandt. Sie weisen eine Sequenzähnlichkeit von 91-95%, 89-94% bzw. 87-94% zu den Symbionten des Röhrenwurms *Riftia pachyptila* bzw. der Muscheln *Lamellibrachia columna* und *Codakia costata* auf. Sie wurden in nahezu allen bislang untersuchten Küsten- und Tiefseesedimenten gefunden, aber auch in Ästuaren (z.B. Gray und Herwig, 1996; Urakawa *et al.*, 1999; Li *et al.*, 1999a,c; Cifuentes *et al.*, 2000). Andere häufig gefundene Sequenzen der γ-Proteobakterien sind eng mit *Pseudomonas* spp. (97-99%) oder entfernter mit *Methylophaga marina* (85-96%) und *Alcanovorax borkumii* (82-92%) verwandt. Vereinzelt gefunden wurden aber auch 16S rDNA Sequenzen, die ihre höchste Ähnlichkeit zu den Sequenzen typischer aerober Bakterien der Gattungen *Marinobacter*, *Aeromonas*, *Oceanospirillum* und *Methylobacter* haben (Rochelle *et al.*, 1994; Gray und Herwig, 1996; Urakawa *et al.*, 1999; Cifuentes *et al.*, 2000).

In der Gruppe der sulfat- und schwefelreduzierenden Bakterien der δ-Proteobakterien findet sich die größte Anzahl der aus marinen Sedimenten erhaltenen Sequenzen, die verwandt sind mit denen bisher kultivierten Gattungen, wie z.B. *Desulfococcus*, *Desulfosarcina*, *Desulforhopalus*, *Desulfotalea*, *Desulfobacterium*, *Desulfobulbus* oder *Desulfuromonas*, und in nahezu allen 16S rDNA Genbanken zu finden waren (z.B. Devereux und Mundfrom, 1994; Phelps *et al.*, 1998; Bidle *et al.*, 1999; Li *et al.*, 1999a,b,c; Urakawa *et al.*, 1999; Bowman *et al.*, 2000; Cifuentes *et al.*, 2000). In den letzten zwei Jahren hat sich besonders die Vielfalt der Sequenzen in der *Desulfosarcina/Desulfococcus*-Gruppe sehr vergrößert. Die Sequenzen in dieser Gruppe weisen jedoch alle nicht mehr als 95% Sequenzähnlichkeit zu *Desulfosarcina variabilis* bzw. zu *Desulfococcus multivorans* auf (siehe Anhang).

Innerhalb der ε-Proteobakterien zeigen sich drei große phylogenetische Gruppen, von denen eine mit *Arcobacter* spp. verwandt ist (93-95%) und die beiden anderen mit den Epibionten von *Alvinella pompejana* (89-91%; Phelps *et al.*, 1998; Li *et al.*, 1999a,b,c; Urakawa *et al.*, 1999; Cifuentes *et al.*, 2000).

Auch Sequenzen der *Cytophaga/Flavobacterium*-Gruppe und der Ordnungen Planctomycetales und Verrucomicrobiales wurden häufiger gefunden (Rochelle *et al.*, 1994; Gray und Herwig, 1996; Urakawa *et al.*, 1999; Li *et al.*, 1999a,c; Bowman *et al.*, 2000; Kolb, 2000). Innerhalb der *Cytophaga/Flavobacterium*-Gruppe sind die Sequenzen sehr verschieden, meist mit weniger als 90% Ähnlichkeit zu ihren nächsten Verwandten. Auch innerhalb der Planctomycetales und Verrucomicrobiales liegen die Sequenzähnlichkeiten zu den nächsten kultivierten Verwandten bei weniger als 90%, jedoch sind die Sequenzen phylogenetisch nicht über die ganze Ordnung verteilt, sondern haben innerhalb der Planctomycetales mit *Pirellula marina* (74-89%) bzw. innerhalb der Verrucomicrobiales mit *Verrucomicrobium spinosum* (74-87%) denselben nächsten Verwandten. Die erhaltenen Sequenzen haben aber häufig auch <90% Sequenzähnlichkeit innerhalb der Ordnung.

Innerhalb der Klasse Actinobacteria (ehemals Gram-positive Bakterien mit hohem DNA GC-Gehalt) wurde häufig eine Gruppe von Sequenzen gefunden, die mit *Microthrix parvicella* entfernt verwandt (75-84%) ist (Gray und Herwig, 1996; Li *et al.*, 1999a,c; Bowman *et al.*, 2000). Innerhalb der Gram-positiven Bakterien mit niedrigem DNA GC-Gehalt befindet sich eine relativ hohe Anzahl an Sequenzen, von denen eine Gruppe entfernt verwandt ist mit *Acetohalobium arabaticum* (84-87%), einem anaeroben, halophilen, homoacetogenen Bakterium (Kevbrin *et al.*, 1995). Desweiteren sind vereinzelt Sequenzen erhalten worden, die mit den Gattungen *Bacillus*, *Clostridium*, *Microbacterium* und *Clavibacter* verwandt sind.

Sequenzen der α- und β-Proteobakterien, zwei physiologisch sehr diversen Gruppen, die häufig in planktonischen Lebensgemeinschaften limnischer und mariner Ökosysteme gefunden werden (Glöckner *et al.*, 1999b; Mullins *et al.*, 1995; González und Moran, 1997), wurden nur vereinzelt aus Sedimenten erhalten (Gray und Herwig, 1996; Li *et al.*, 1999a,c; Bowman *et al.*, 2000; Cifuentes *et al.*, 2000).

3.2 Struktur

Über die quantitative Zusammensetzung benthischer prokaryontischer Lebensgemeinschaften sowie über die räumliche Verteilung verschiedener Gruppen ist noch immer wenig bekannt. Autochthone, aber auch allochthone Organismen sind im Sediment zu finden. Daher ist neben der Quantifizierung unterschiedlicher Gruppen die Identifikation des aktiven Teils der prokaryontischen Gemeinschaft für das Verständnis metabolischer Prozesse im Sediment von großer Bedeutung.

Im allgemeinen werden schlickige Sedimente von einer höheren Anzahl an

Prokaryonten besiedelt als sandige. Ein Vergleich der Gesamtzellzahlen aus arktischen, antarktischen, gemäßigten und tropischen Regionen zeigt, daß die Zellzahlen mehr durch den Sedimenttyp als durch den geographischen Bereich geprägt werden (Meyer-Reil, 1993). Für schlickige Sedimente werden prokaryontische Gesamtzellzahlen gefunden, die in den oberen Sedimentschichten in der Größenordnung von 1×10^9 bis 1×10^{10} pro ml Sediment liegen und mit zunehmender Sedimenttiefe abnehmen (siehe z.B. Jørgensen und Revsbech, 1989; van Duyl und Kop, 1990; Parkes *et al.*, 1994; Wellsbury *et al.*, 1996; Llobet-Brossa *et al.*, 1998; Sahm und Berninger, 1998). In sandigen Sedimenten liegen die Zellzahlen bei etwa 10^8 bis 10^9 pro ml Sediment (Epstein und Rossel, 1995; Epstein *et al.*, 1997; Llobet-Brossa *et al.*, 1998; Kolb, 2000). Der Grund für die Abnahme der Gesamtzellzahlen mit zunehmender Sedimenttiefe ist vor allem in einer Verringerung des Nährstoffangebotes und der verfügbaren Energie zu suchen (Meyer-Reil, 1993).

Bacteria dominierten gegenüber Archaea in den bisher untersuchten marinen Sedimenten. Llobet-Brossa und Mitarbeiter konnten in Wattenmeer-Sedimenten mittels FISH mit einer bakteriellen Sonde bis zu 73% bzw. 54% aller DAPI-gefärbten Zellen der Domäne Bacteria zuordnen, Archaea konnten nicht detektiert werden (Llobet-Brossa *et al.*, 1998). In zwei arktischen Sedimenten sowie einem dänischen Küstensediment konnte nur ein relativer Archaea rRNA-Anteil von höchstens 4% der prokaryontischen rRNA nachgewiesen werden (Sahm und Berninger, 1998). Die höchste relative Abundanz von Archaea rRNA fanden bisher Vetriani und Mitarbeiter mit bis zu 8% an prokaryontischer rRNA in einem Tiefseesediment (Vetriani *et al.*, 1999).

Nur zwei kultivierungsunabhängige Studien zur Abundanz und Tiefenverteilung verschiedener bakterieller Großgruppen wurden bislang im Meeressediment durchgeführt. Beide Untersuchungen basieren auf FISH und charakterisieren die bakteriellen Lebensgemeinschaften in Wattenmeersedimenten von Dangast (Llobet-Brossa *et al.*, 1998) bzw. Sylt (Kolb, 2000). Die quantitativ bedeutendste Gruppe war in beiden Untersuchungen *Cytophaga/Flavobacterium*. Llobet-Brossa und Mitarbeiter (1998) konnten in der oberen oxischen Sedimentschicht bis zu 18% aller DAPI-angefärbten Zellen und in anoxischen Bereichen noch 6 bis 16% dieser Gruppe zuordnen. Kolb (2000) detektierte 2 bis 11% mit einem Maximum in 2 bis 3 cm Tiefe. Kultivierte Vertreter der *Cytophaga/Flavobacterium* Gruppe sind meist aerobe oder mikroaerophile Organismen, die durch ihre Fähigkeit zur Synthese polymerabbauender Enzyme wie Cellulasen, Chitinasen oder Proteasen auf den Abbau komplexer Makromoleküle spezialisiert sind (Holmes, 1991; Reichenbach, 1991) und häufig in hohen Zahlen in der marinen Wassersäule gefunden wurden (Glöckner et al., 1999b;

Eilers *et al.*, 2000). Durch die hohe Abundanz von *Cytophaga/Flavobacterium* in anaeroben Bereichen der Sedimente und der Aktivierung dieser Gruppe unter anaeroben Bedingungen nach Zugabe von komplexen organischen Substraten (Rosselló-Mora *et al.*, 1999) wird eine ökologische Relevanz dieser Bakterien als hydrolytisch fermentative Organismen vermutet (Rosselló-Mora *et al.*, 1999).

Auch die Planktomyceten könnten für den Polymerabbau in marinen Sedimenten wichtig sein. Charakteristisch für die Planktomyceten ist die Vermehrung durch Knospung und das Fehlen von Peptidoglykan als Bestandteil der Zellwand (Staley *et al.*, 1991). Die Fähigkeit aquatischer Planktomyceten, N-Acetylglukosamin als C- und N-Quelle zu verwenden, könnte ihnen eine Schlüsselrolle im Chitinabbau zuweisen (Schlesner, 1994; Zarda *et al.*, 1997). Weiterhin ist N-Acetylglukosamin ein wesentlicher Bestandteil der bakteriellen Zellwand. Diese Gruppe der Planctomycetales macht ebenfalls einen bedeutenden Teil der prokaryontischen Lebensgemeinschaft der untersuchten Wattenmeersedimente von bis zu 7% (Sylt) und 6% (Dangast) aus.

Insgesamt 7-13% der DAPI-gefärbten Zellen in den untersuchten Wattenmeersedimenten von Dangast sind den α-, β- und γ-Proteobakterien zuzuordnen (Llobet-Brossa *et al.*, 1998). Eine spezielle Rolle bei der Mineralisierung von organischem Material kann diesen Gruppen aufgrund der physiologischen Vielfalt der beinhalteten Organismen noch nicht zugewiesen werden. In der gamma-Gruppe der Proteobakterien finden sich z.B. Bakterien, die am oxidativen Teil des Schwefelkreislaufs beteiligt sind.

Untersuchungen der sulfatreduzierenden Bakterien waren in den vergangenen Jahren zum überwiegenden Teil auf Kultivierungstechniken angewiesen. „Most-Probable-Number" (MPN) Techniken ermittelten Zellzahlen von 2×10^5 bis 2×10^6 Zellen pro ml Sediment (Bak und Pfennig, 1991; Jørgensen und Bak, 1991; Teske *et al.*, 1996a; Vester und Ingvorsen, 1998; Knoblauch *et al.*, 1999a). Eine ähnliche Abundanz fand Lillebaek (1995) mit einem polyklonalen Antikörperserum gegen sulfatreduzierende Bakterien. Berechnet man basierend auf diesen Zellzahlen zelluläre Sulfatreduktionsraten, liegen die Geschwindigkeiten beim 10 bis 100-fachen exponentiell wachsender Reinkulturen (Jørgensen, 1978a; Knoblauch *et al.*, 1999a) unrealistisch hoch. Abschätzungen mittels quantitativer slot-blot Hybridisierung extrahierter rRNA deuteten auf eine wesentlich höhere Abundanz sulfatreduzierender Bakterien hin (Sahm *et al.*, 1999a). In einem dänischen Küstensediment lagen die so berechneten Zellzahlen sulfatreduzierender Bakterien bei 2 bis 6×10^8 Zellen pro ml Sediment (Sahm *et al.*, 1999a). Die ersten Zählungen mittels FISH stammen aus der Arbeit

von Llobet-Brossa und Mitarbeitern (1998). Mit Zahlen von 0,4 bis 2,9 x 10^8 Zellen pro ml bestätigen sie die Abschätzungen von Sahm. Inzwischen liegen aus zwei Diplomarbeiten weitere Werte vor, die abhängig von der Sedimenttiefe im Bereich von 0,1 bis 8 x 10^8 Zellen pro ml liegen (Mußmann, 1999; Kolb, 2000).

Devereux und seine Mitarbeiter entwickelten 1992 einen Satz von insgesamt 6 Oligonukleotidsonden, mit denen ein Großteil der zum damaligem Zeitpunkt bekannten Gram-negativen sulfatreduzierenden Bakterien detektiert werden konnte (Devereux *et al.*, 1992). Mit Hilfe dieser Sonden wurden Sedimente aus Salzmarschen, Ästuaren und Küstenregionen mittels rRNA slot-blot Hybridisierung (Devereux *et al.*, 1996a; Rooney-Varga *et al.*, 1997; Hines *et al.*, 1999; Sahm *et al.*, 1999a; Sahm *et al.*, 1999b) oder rDNA-Hybridisierung (Edgcomb *et al.*, 1999) untersucht. In allen Untersuchungen zeigte sich eine Stratifizierung der unterschiedlichen Gruppen sulfatreduzierender Bakterien. Die detektierte rRNA nimmt dem Trend der gesamten prokaryontischen rRNA folgend generell mit zunehmender Tiefe ab (Devereux *et al.*, 1996a; Sahm *et al.*, 1999a). Der Anteil der SRB-rRNA an der prokaryontischen rRNA lag in den untersuchten Sedimenten bei 20,4 ± 3,1% (Sahm *et al.*, 1999a), 9,4 ± 4,5% und 22,3 ± 15,5% (Sahm *et al.*, 1999b) bzw. 5% (Devereux *et al.*, 1996a). Es dominieren Vertreter der Gattung *Desulfovibrio* (Devereux *et al.*, 1996a; Sahm *et al.*, 1999a) oder der Familie der Desulfobacteraceae (Rooney-Varga *et al.*, 1997; Edgcomb *et al.*, 1999; Hineş *et al.*, 1999; Sahm *et al.*, 1999a). Für Sedimente aus Salzmarschen berichteten Hines und Mitarbeiter (1999) einen relativen Anteil von bis zu 34% Desulfobacteraceae rRNA an der bakteriellen rRNA und Rooney-Varga und Mitarbeiter (1997) zeigten Abundanzen von 3,4 ± 2,1% und 7,5 ± 3,5% für bislang unkultivierte Bakterien, die mit *Desulfosarcina variabilis* bzw. *Desulfococcus multivorans* (96,3 bzw. 89,1% Sequenzähnlichkeit) verwandt sind. Sahm et al. (1999a) konnten einen Anteil von 16,5 ± 2,8% *Desulfosarcina/Desulfococcus/Desulfobotulus* rRNA an prokaryontischer rRNA in einem dänischen Küstensediment nachweisen. In zwei arktischen Sedimenten von Svalbard lag rRNA dieser Gruppe jedoch unter der Nachweisgrenze (Sahm *et al.*, 1999b). Vertreter der Desulfobacteraceae, insbesondere Mitglieder der *Desulfosarcina/Desulfococcus*-Gruppe, haben ein breites Substratspektrum und können organisches Substrat komplett zu CO_2 oxidieren. Diese Vielseitigkeit kann ein großer Vorteil in natürlichen Lebensräumen sein, in denen eine diverse Lebensgemeinschaft ein weites Spektrum an Kohlenstoffquellen entlang der Nahrungskette produziert.

Desulfobulbus spp. konnten nur in Sedimenten aus Salzmarschen in Abundanzen deutlich über der Nachweisgrenze gezeigt werden (Edgcomb *et al.*, 1999; Hines *et al.*, 1999)

und scheinen gut an diese Habitate adaptiert zu sein. *Desulfobulbus propionicus* kann Energie außer durch diverse Gärungsreaktionen auch über die Disproportionierung von elementarem Schwefel konservieren (Lovley und Phillips, 1994), der in hohen Konzentrationen in Salzmarschen vorkommt (Luther *et al.*, 1991).

In arktischen Sedimenten konnte eine hohe relative Abundanz von *Desulfotalea* rRNA (7,2 ± 6,4% der prokaryontischen rRNA) gezeigt werden (Sahm *et al.*, 1999b). *Desulfotalea* gehört neben *Desulfofrigus* und *Desulfofaba* zu den ersten, erst kürzlich beschriebenen Gattungen psychrophiler sulfatreduzierender Bakterien (Knoblauch *et al.*, 1999b).

Ein verbesserter Satz von insgesamt 12 Oligonukelotidsonden wurde 1998 von Manz und Mitarbeitern entwickelt (Manz *et al.*, 1998). Mittels FISH wurde in zwei Diplomarbeiten die Gemeinschaft der sulfatreduzierenden Bakterien in Wattenmeersedimenten von Dangast (Mußmann, 1999) und Sylt (Kolb, 2000) unter Verwendung dieses Sondensatzes detailliert untersucht. Die Gesamtzahl der detektierten sulfatreduzierenden Bakterien lag je nach Sedimenttiefe zwischen 2 und 15% aller DAPI-gefärbten Bakterien. Vertreter der Gattungen *Desulfovibrio* und der *Desulfococcus/Desulfosarcina*-Gruppe waren auch hier neben *Desulforhopalus* die abundanten Gruppen. Vertreter der komplett oxidierenden Gattungen *Desulfobacterium* und *Desulfobacter* lagen nahe der Nachweisgrenze.

4 Permanent kalte marine Sedimente

Marine Sedimente als Lebensraum vieler Mikroorganismen sind durch niedrige Temperaturen charakterisiert; 90% des Meeresbodens weisen permanent Temperaturen von weniger als 4°C auf (Levitus und Boyer, 1994). Die Polarregionen machen etwa 15% der Erdoberfläche aus (Morita, 1975; Russel, 1990).

Mikrobielle Lebensgemeinschaften und Physiologie bei permanent kalten Temperaturen ("Svalbard-Projekt")

Seit 1995 werden am Max-Planck-Institut für Marine Mikrobiologie in Bremen in einem interdisziplinären Projekt verschiedene Aspekte des mikrobiellen Lebens bei permanent kalten Temperaturen untersucht. Die vorliegende Arbeit beschäftigt sich mit der Diversität und Zusammensetzung der bakteriellen Lebensgemeinschaft in arktischen Sedimenten und ist als Teil dieses Projektes zu verstehen. Im Rahmen dieses Projektes wurden Sedimente vor der Küste Spitzbergens beprobt (Abb.5). Spitzbergen ist die Hauptinsel der Inselgruppe Svalbard

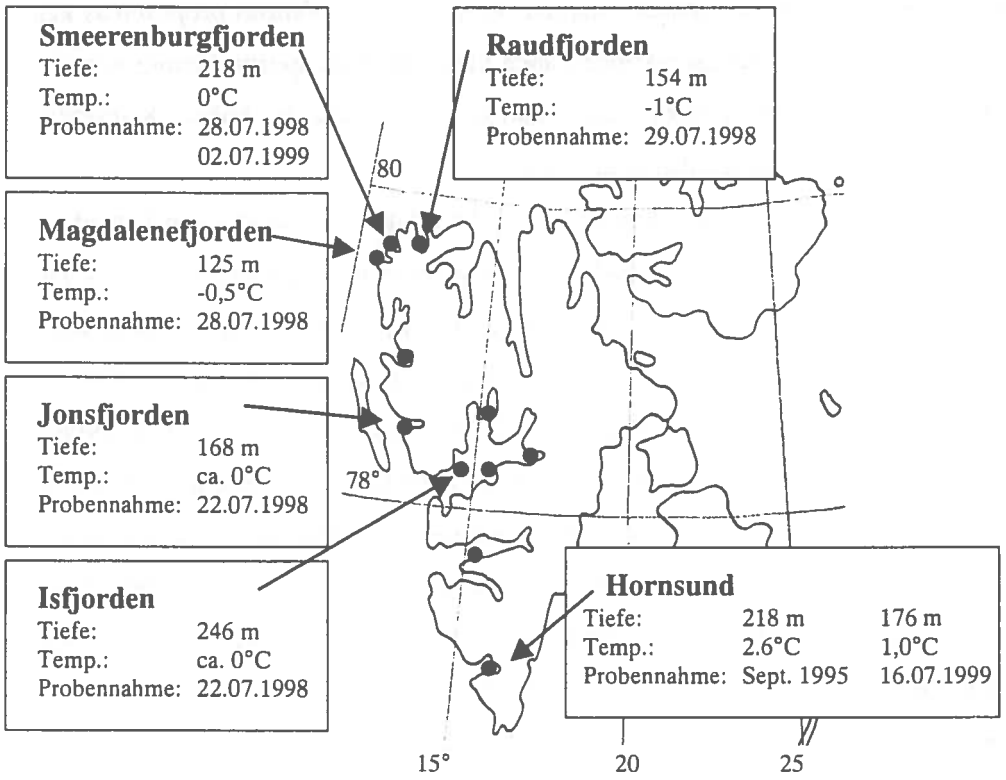


Abbildung 5: Probennahmegebiete vor Spitzbergen. Die schwarzen Punkte zeigen die im Rahmen dieses Projektes bereits beprobten Regionen. Die näher beschriebenen Sedimente wurden in dieser Arbeit untersucht.

im Arktischen Ozean (etwa 77 bis 81°N, 11 bis 17°O).

Im Rahmen dieses Projektes konnte gezeigt werden, daß der mikrobielle Stoffumsatz in permanent kalten Regionen mit gleicher Geschwindigkeit abläuft wie in Lebensräumen gemäßigter Breiten (Arnosti *et al.*, 1998; Glud *et al.*, 1998; Sagemann *et al.*, 1998; Thamdrup und Fleischer, 1998; Knoblauch und Jørgensen, 1999c). Arnosti und Mitarbeiter (Arnosti *et al.*, 1998) bestimmten die Temperaturabhängigkeit des mikrobiellen Abbaus organischer Substanzen und zeigten, daß der Kohlenstoffumsatz (pro Volumen und Zeit) in kalten Sedimenten bei *in situ*-Temperaturen nicht langsamer abläuft als in gemäßigten Lebensräumen. Auch die Sulfatreduktionsraten (Sagemann *et al.*, 1998) und Raten (pro Fläche und Zeit) für die benthische Kohlenstoffmineralisation (Glud *et al.*, 1998; Kostka *et al.*, 1999) waren bei Standorttemperaturen mit den Raten in gemäßigten Habitaten vergleichbar.

Hinweise auf das Vorkommen psychrophiler sulfatreduzierender Bakterien gaben MPN-Zahlen, die bei 10°C höher als bei 20°C waren (Knoblauch *et al.*, 1999a). Mehrere Stämme psychrophiler sulfatreduzierender Bakterien konnten isoliert und drei neue Gattungen, *Desulfotalea*, *Desulfofrigus* und *Desulfofaba*, beschrieben werden (Knoblauch *et al.*, 1999b).

Alle neu isolierten Stämme konnten noch bei *in situ*-Temperatur von -1,7°C wachsen und sind an die permanent kalten Temperaturen durch hohe relative Wachstumsraten und hohen Wachstumsertrag bei *in situ* Bedingungen angepaßt (Knoblauch und Jørgensen, 1999c).
In Sedimenten von Storfjord, aus denen *Desulfotalea psychrophila* und *Desulfotalea arctica* isoliert wurden, konnte ein quantitativ bedeutender Anteil von rRNA aus *Desulfotalea* mit 7,2 ± 6,4% an der prokaryontischen rRNA gezeigt werden (Sahm *et al.*, 1999b), in Sedimenten von Hornsund jedoch war der Anteil mit 1,4 ± 1,2% deutlich geringer (Sahm *et al.*, 1999b).

5 Zielsetzung dieser Arbeit

Die Zusammensetzung und Diversität benthischer prokaryontischer Lebensgemeinschaften wurde bislang nur wenig untersucht. Im Falle mariner Sedimente basiert das vorhandene Wissen zu einem großen Teil auf Untersuchungen mit Reinkulturen oder auf kultivierungsabhängigen Studien, die, wie bereits beschrieben, nur in einem sehr begrenzten Maße die natürliche Diversität und Abundanz im Lebensraum widerspiegeln. Zum besseren Verständnis der mikrobiellen Prozesse und der Ökologie in marinen Sedimenten ist die Kenntnis der Abundanz und räumlichen Verteilung funktionell wichtiger mikrobieller Gruppen jedoch unerläßlich.

Ein Ziel dieser Arbeit war daher eine möglichst umfassende Beschreibung der bakteriellen Diversität in permanent kalten Sedimenten (Svalbard, Arktischer Ozean) mit kultivierungsunabhängigen Methoden. Einen weiteren Schwerpunkt dieser Arbeit bildete die Charakterisierung der Zusammensetzung der prokaryontischen Lebensgemeinschaft und die Quantifizierung und räumliche Verteilung der Organismen, die hinter den gefundenen 16S rDNA Sequenzen stehen. Die für die anaerobe Mineralisierung in marinen Sedimenten so bedeutende Gruppe der sulfatreduzierenden Bakterien wurde besonders detailliert untersucht. Eine solche Strukturanalyse verspricht nicht nur für die untersuchten permanent kalten Sedimente Aufschlüsse über mikrobielle Prozesse und die Ökologie der beteiligten Organismen, sondern trägt auch zum allgemeinen Verständnis benthischer Lebensgemeinschaften bei.

Gegenüber der Sulfatreduktion ist der oxidative Teil des Schwefelkreislaufs weniger gut verstanden. Dies liegt zum großen Teil an der höheren Komplexität, weil es neben der biogenen Schwefeloxidation auch eine rein chemische Oxidation reduzierter

Schwefelverbindungen gibt, sowie an der, verglichen mit der Sulfatreduktion, Beteiligung mehrerer Bakteriengruppen. Unbekannt ist auch, ob die bisher kultivierten Bakterien auch *in situ* abundante schwefeloxidierende Bakterien sind. Psychrophile schwefeloxidierende Bakterien sind bislang noch nicht isoliert worden. Auch dies war ein Ziel dieser Arbeit.

Viele Studien, in denen mikrobielle Gruppen in Umweltproben quantifiziert wurden, basieren entweder auf der *in situ*-Hybridisierung (FISH) (z.B. Ramsing *et al.*, 1996; Snaidr *et al.*, 1997; Llobet-Brossa *et al.*, 1998; Glöckner *et al.*, 1999b) oder der rRNA slot-blot Hybridisierung (z.B. Raskin *et al.*, 1994b; Devereux *et al.*, 1996a; MacGregor *et al.*, 1997; Rooney-Varga *et al.*, 1997; Sahm *et al.*, 1999a,b). Bislang lagen noch keine Studien vor, die beide Methoden kombinierten. Es war daher unklar, wie stark unterschiedliche Charakteristika der Methoden die Vergleichbarkeit der Ergebnisse beeinflussen (Tab.2). In dieser Arbeit wurde deshalb parallel mit beiden Methoden die Zusammensetzung der bakteriellen Lebensgemeinschaft in permanent kalten Sedimenten charakterisiert und die Vergleichbarkeit der Ergebnisse analysiert.

Tabelle 2: Vergleich von Fluoreszenz-*in situ*-Hybridisierung (FISH) und rRNA slot-blot Hybridisierung

	***In situ*-Hybridisierung (FISH)**	**slot-blot Hybridisierung**
Detektion abhängig von	**Einzelzell-Ebene** rRNA-Gehalt pro Zelle	**rRNA-Pool** rRNA-Gehalt pro Zelle + Anzahl der Zielzellen
Daten	% aller DAPI-gefärbten Zellen oder Zellen/ml	% prokaryontischer rRNA oder ng/ml
Detektionsgrenze	**0,5% - 1%** aller DAPI-gefärbten Zellen	0,5% **der prokaryontischen rRNA** **aber**: spezifische rRNA **oft noch detektierbar**, wenn zellulärer rRNA-Gehalt unter Detektionsgrenze für FISH
Zellzahlen	+	**nicht bestimmbar**
Zellmorphologie	+	**nicht bestimmbar**
Räumliche Verteilung	+	**nur begrenzt bestimmbar**
Sondenposition/ Sondenzugänglichkeit	**in situ Zugänglichkeit** der Zielregionen bestimmt die Fluoreszenzintensität der Sonde	**keine Einschränkungen**
Einschränkungen	**Autofluoreszenz** (z.B. von Sedimenten) **Impermeabilität** der Zellwände (z.B. von Gram-positiven)	Spezies-abhängige Unterschiede in **Zelllysis-Effizienz**

B

Ergebnisse und Diskussion

B Ergebnisse und Diskussion

Im folgenden werden die Ergebnisse der einzelnen Publikationen zusammengefaßt und im Zusammenhang diskutiert. Dieser Abschnitt kann allerdings nicht die detaillierten Diskussionen der Einzelpublikationen ersetzen, auf die deshalb hier verwiesen wird. Ausführlicher werden hier Aspekte erläutert, die in den Publikationen nicht oder nur kurz behandelt werden.

1 Prokaryontische Lebensgemeinschaften in marinen Sedimenten

Marine Sedimente bieten eine Vielzahl an Habitaten und Mikronischen, die potentiell von einer großen Zahl metabolisch unterschiedlicher Mikroorganismen besiedelt werden können. Eine umfassende Kenntnis der Zusammensetzung der mikrobiellen Gemeinschaften ist für das Verständnis metabolischer Prozesse erforderlich.

1.1 Bakterielle Diversität in permanent kalten Sedimenten

Zur Beschreibung der bakteriellen Diversität in permanent kalten Sedimenten wurde zunächst eine 16S rDNA Genbank aus Sedimenten von Hornsund (Svalbard) erstellt (Publikation 1).
Die Diversität bezüglich der Artenvielfalt war in den untersuchten Sedimenten sehr hoch. Über eine "Rarefaction Analyse" konnte gezeigt werden, daß die Charakterisierung von 353 Klonen nicht ausreichend war, um die tatsächliche Diversität in der Genbank zu erfassen. Die gezeigte Diversität ist daher als "Mindestdiversität" zu verstehen.

Auch in anderen kalten Sedimenten, wie in denen vom Antarktischen Ozean (Bowman *et al.*, 2000) und aus der Tiefsee vor Japan (Li *et al.*, 1999a,b,c), ist die Diversität hoch. Der Befund der hohen Biodiversität in kalten Sedimenten wird durch Daten unterstützt, die zeigen, daß die Temperatur nur einen begrenzten Einfluß auf die prokaryontische benthische Produktivität hat (Nedwell *et al.*, 1993; Rivkin *et al.*, 1996; Arnosti *et al.*, 1998; Glud *et al.*, 1998; Kostka *et al.*, 1999). Allerdings darf man nicht außer acht lassen, daß die über Genbanken bestimmte Artenvielfalt solcher Sedimente auch allochthonen Eintrag aus der Wassersäule beinhalten kann. Inwieweit diese planktonischen Bakterien auch aktive Mitglieder einer benthischen Lebensgemeinschaft sein können, ist ein Punkt, der in Zukunft nähere Betrachtung verdient. Sicher ist, daß der Artenreichtum das Potential einer mikrobiellen Gemeinschaft, auf Umweltänderungen zu reagieren, reflektiert. Zu bestimmten

Zeitpunkten kann ein selten detektierter Organismus eine wichtige Rolle in dem Habitat spielen.

In der vorliegenden Svalbard Genbank waren etwa 70% der Klonsequenzen mit Bakterien des Schwefelkreislaufs verwandt: 50% der Sequenzen wurden verschiedenen Sulfatreduzierern zugeordnet, wobei die größte Klon-Gruppe 93-100% Sequenzähnlichkeit zu *Desulfotalea* spp. und anderen nahe verwandten Psychrophilen hatte. Etwa 20% der Sequenzen hatte als nächsten Verwandten symbiontisch oder freilebende Schwefeloxidierer der γ-Proteobakterien. Weiterhin wurden je 5% der Sequenzen der *Cytophaga/Flavobacterium*-Gruppe, der Planctomycetales und der Gram-positiven Bakterien erhalten. Sequenzen der α- und β-Proteobakterien wurden nur sehr selten (2/353 Klonen) bzw. gar nicht in der Genbank gefunden.

1.2 Vergleichende phylogenetische Analyse von 16S rDNA Sequenzen

Mit dieser Arbeit liegen derzeit (Juli 2000) Sequenzdaten aus 31 verschiedenen Küsten-, Tiefsee-, "cold-seep"- und Ästuarsedimenten in den Datenbanken vor (z.B. Devereux und Mundfrom, 1994; Gray und Herwig, 1996; Rooney-Varga *et al.*, 1997; Phelps *et al.*, 1998; Bidle *et al.*, 1999; Urakawa *et al.*, 1999; Li *et al.*, 1999a,b,c; Rosselló-Mora *et al.*, 1999; Bowman *et al.*, 2000; Cifuentes *et al.*, 2000; Thamdrup *et al.*, 2000).

Im marinen und limnischen Bakterioplankton gibt es mehrere Beispiele für Habitat-spezifische phylogenetische Gruppen (Giovannoni *et al.*, 1990; Mullins *et al.*, 1995) (Glöckner *et al.*, eingereicht); dazu gehören z.B. die "SAR cluster", deren Sequenzen man bislang nur in marinen Planktonproben findet. Zur Identifizierung Sediment-spezifischer phylogenetischer Gruppen in marinen Systemen wurden die derzeit in den Datenbanken verfügbaren 16S rDNA-Klonsequenzen analysiert und vorläufige phylogenetische Bäume durch Addieren der Sequenzen zu einem Parsimony-Baum mit anschließender lokalen und globalen Optimierung unter Parsimony-Kriterien erstellt. Auf Grund dieser Einordnung konnten zahlreiche phylogenetische Gruppen identifiziert werden, die bislang ausschließlich oder vorwiegend Sequenzen aus marinen Sedimenten oder anderen marinen Systemen enthalten (Abb. 6-15). Kriterium für die Definition einer phylogenetischen Gruppe war die Anwesenheit von Sequenzen aus mindestens drei Genbanken unterschiedlicher Sedimente. Die gebildeten Gruppen basieren auf phylogenetischer Verwandtschaft und nicht auf Ähnlichkeitswerten. Eine auf Ähnlichkeitswerten beruhende Abgrenzung durch Definition eines Schwellenwertes war nicht möglich. Häufig standen nur Partialsequenzen zur

Verfügung, mit denen eine zuverlässige Bestimmung von Ähnlichkeitswerten nicht möglich war. Bei der Interpretation der Gruppierungen ist zu beachten, daß die Gruppen unterschiedliche phylogenetische Tiefen aufweisen. Innerhalb der einzelnen Gruppen ist im allgemeinen eine hohe Diversität zu finden, die durch z.T. weniger als 90% Sequenzähnlichkeit zu den nächstverwandten Klonen angezeigt wird. Vereinzelt sind in den gefundenen Gruppen auch kultivierte Organismen zu finden.

Auf der Grundlage des derzeit zur Verfügung stehenden Datensatzes läßt sich die Verbreitung der identifizierten Gruppen nicht auf bestimmte Lebensräume begrenzen. In Küstensedimenten wurden im allgemeinen dieselben phylogenetischen Gruppen gefunden wie in Tiefseesedimenten, in Sedimenten von "cold-seeps" oder in Süßwasser-beeinflußten Ästuarsedimenten. In nahezu allen identifizierten Gruppen befinden sich auch Sequenzen aus den Svalbard Sedimenten. Eine Erklärung für die weite globale Verbreitung einzelner Gruppen könnte das Vorhandensein unzähliger Mikronischen in marinen Sedimenten liefern. Einige Nischen könnten dabei den mikrobiellen Gemeinschaften ähnliche Lebensbedingungen wie in anderen Sedimenten ermöglichen. Die weite Verbreitung der phylogenetischen Gruppen könnte auch die Stabilität des Ökosystems "marines Sediment" auf einer für die bakterielle Zusammensetzung entscheidenden Ebene reflektieren. Die oft sehr hohe phylogenetische Tiefe in den Gruppen läßt zudem noch ausreichend Raum für physiologische Anpassungen, wie z.B. die Druckanpassung barophiler Bakterien in Tiefseesedimenten.

Die sich abzeichnende globale Verbreitung bestimmter Gruppen gibt wichtige Hinweise auf ubiquitäre Gruppen in marinen Sedimenten. Über die Funktion und physiologischen Eigenschaften der beschriebenen Gruppen läßt sich oft nur spekulieren, weil die Ähnlichkeiten auf Ebene der 16S rDNA häufig unter 90% zum nächsten kultivierten Verwandten liegt. In zukünftigen Untersuchungen sollte es das Ziel sein, die relative Häufigkeit der als wichtig erkannten Populationen zu bestimmen, z.B. über FISH oder quantitative slot-blot Hybridisierung. Weiterhin sind funktionelle Untersuchungen erforderlich, um die Physiologie und ökologische Bedeutung der Gruppen besser verstehen zu können. Auf dem Weg dorthin sollte die Isolierung von Vertretern der einzelnen Gruppen mit anschließender "klassischer" Charakterisierung ein Hauptziel bleiben. Andere Ansätze umfassen die Erstellung von großen Genbanken aus Umwelt-DNA ("Environmental Genomics") mit der Identifizierung funktioneller Gene (Stein *et al.*, 1996; Rondon *et al.*, 2000) oder die Kombination aus Mikroautoradiographie und FISH, bei denen die Aufnahme von Tracern durch spezifische phylogenetische Gruppen beobachtet werden kann (Lee *et al.*, 1999; Ouverney und Fuhrman, 1999).

γ-Proteobakterien. Innerhalb der γ-Proteobakterien konnten drei stabile Gruppen identifiziert werden (Abb.6). In diesen Gruppen (GamSI - GamSIII) befinden sich fast ausschließlich Sequenzen aus marinen Sedimenten unterschiedlicher Habitate. Die Sequenzen wurden aus verschiedenen Küsten- und Tiefseesedimenten, "cold-seep"-Sedimenten und Ästuaren erhalten. Eine Sequenz aus der Wassersäule eines Ästuars und eine aus dem Sediment eines antarktischen Sees mit mariner Salinität wurden ebenso in GamSII bzw. GamSIII gefunden. Die Svalbard-Genbank trägt mit einer beträchtlichen Anzahl an Sequenzen zur Stabilität dieser Gruppe bei. Die nächsten Verwandten von GamSI-III sind chemoautotrophe schwefeloxidierende Endosymbionten verschiedener Muscheln und Würmer, wie z.B. von *Solemya velum*, *Riftia pachyptila* oder *Codakia costata* (maximale Sequenzähnlichkeit 94,6%). Diese Symbiosen zwischen chemoautotrophen Bakterien und Evertebraten findet man weitverbreitet in vielen marinen Habitaten, wie z.B. Tiefseehydrothermalquellen und Küstensedimenten (Fisher, 1990; Cavanaugh, 1994). Mittels FISH konnten in Svalbard Sedimenten freilebende Bakterien detektiert werden, die hinter diesen Sequenzen stehen (siehe S.40-41). Unklar aber bleibt, ob diese Bakterien eine Rolle bei der Oxidation reduzierter Schwefelverbindungen spielen.

δ-Proteobakterien. Mit 47 Sequenzen aus marinen Sedimenten ist DelSI die größte aller identifizierten Gruppen. Diese Gruppe enthält, von wenigen Ausnahmen abgesehen, nur Sequenzen aus marinen Sedimenten und beinhaltet die kultivierten Gattungen *Desulfosarcina*, *Desulfococcus* und *Desulfonema* (Abb.7). Die Ähnlichkeit der Sequenzen liegt bei maximal 94,7% zu einem der kultivierten Organismen. Mit der Verfügbarkeit weiterer Sequenzinformationen werden die sich bereits abzeichnenden Untergruppen vermutlich weiter stabilisiert werden.

Beim Vergleich der Sedimente, aus denen die Sequenzen erhalten wurden, fällt auf, daß trotz der hohen Anzahl von Sequenzen in der Gruppe DelSI kein Klon aus Tiefseesedimenten zu finden war. Generell sind aus den untersuchten Tiefseesedimenten nur wenig Sequenzen von sulfatreduzierenden Bakterien erhalten worden (6 Sequenzen, Abb. 7-10). Sulfatreduktion tritt in Tiefseesedimenten aufgrund der hohen Sauerstoffeindringtiefe im allgemeinen erst in tiefen Sedimentschichten auf (Lochte, 1993). Deshalb werden die Sulfatreduktionszonen mit den oberflächlichen Beprobungsmethoden nicht erfaßt, was das weitgehende Fehlen von SRB-Sequenzen in Tiefseesedimenten erklären würde. Einige der untersuchten Tiefseesedimente (z.B. Japan Trench, Nankai Trench) sind keine "typischen" Tiefseesedimente, in denen von einer tiefen oxischen Schicht ausgegangen werden kann (Boetius, pers. Mitteilung), so daß weitere Daten benötigt werden, um das Fehlen der SRB-Sequenzen zu klären.

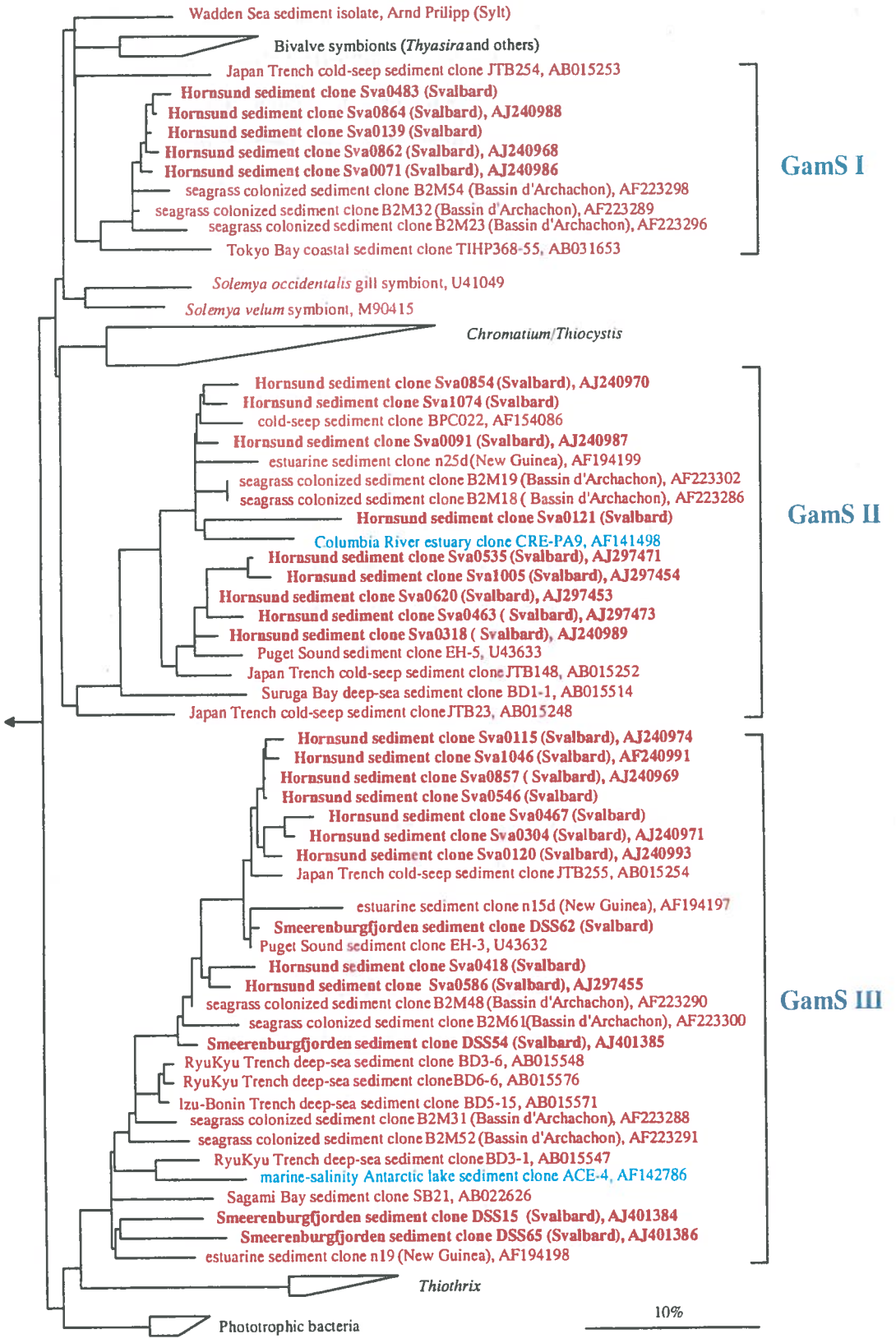


Abbildung 6. Vorläufiger 16S rDNA phylogenetischer Baum, der für marine Sedimente spezifische Gruppen innerhalb der γ-Proteobakterien zeigt. Rot gedruckt sind Sequenzen, die aus marinen Sedimenten erhalten wurden, rot & fett die Sequenzen der Svalbard-Genbank und blau die Sequenzen aus anderen, nicht sedimentären marinen Systemen. Der Balken gibt 10% geschätzte Sequenzunterschiede an.

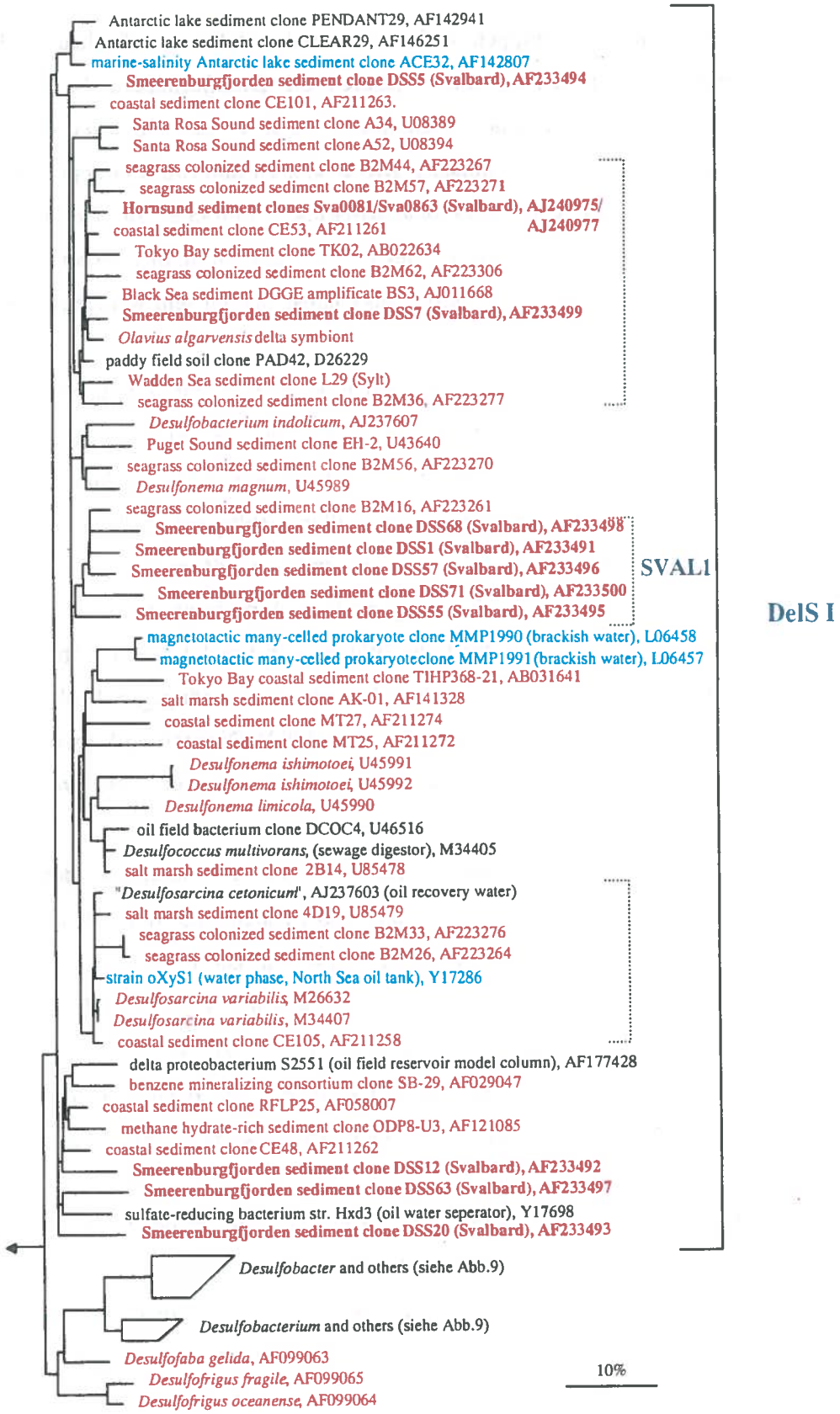


Abbildung 7. Vorläufiger 16S rDNA phylogenetischer Baum, der für marine Sedimente spezifische Gruppen innerhalb der δ-Proteobakterien zeigt. Rot gedruckt sind Sequenzen, die aus marinen Sedimenten erhalten wurden, rot & fett die Sequenzen der Svalbard-Genbank und blau die Sequenzen aus anderen, nicht sedimentären marinen Systemen. Der Balken gibt 10% geschätzte Sequenzunterschiede an.

Zu den weiteren identifizierten Gruppen sulfatreduzierender Bakterien, die hauptsächlich Sequenzen aus marinen Systemen enthalten, gehören die *Desulforhopalus/Desulfotalea*-Gruppe (90-94% zu *Desulforhopalus vacuolatus,* 90-95% zu *Desulfotalea* spp.), *Desulfofustis*-Gruppe (90-96% zu *Desulfofustis glycolicus*), *Desulfocapsa*-Gruppe (90-94% zu *Desulfocapsa sulfexigens*) und *Desulfobulbus*-Gruppe (90-93% zu *Desulfobulbus propionicus*) (Abb.8). Obwohl viele *Desulfobacter* spp. und *Desulfobacterium* spp. aus marinen Sedimenten isoliert worden sind, wurden aus Genbanken nur sehr wenige Sequenzen dieser Gruppen erhalten (Abb. 9).

Weitere Gruppen innerhalb der δ-Proteobakterien waren rein marine Gruppen, die mit *Desulfobacterium anilini* (DelSII, 86-94%) bzw. *Nitrospina gracilis* (83-92%), einem nitritoxidierenden Bakterium (Watson und Waterbury, 1971), verwandt sind (Abb.10). In der Gruppe der Myxobacteria sind Sequenzen nahezu aller Genbanken enthalten, die mit Vertretern der Gattungen *Myxococcus*, *Polyangium* und *Chondromyces* verwandt sind (80-87%, Abb.11). Myxobacteria sind als streng aerobe, chemoheterotrophe terrestrische Bakterien beschrieben, die in der Natur durch die Bildung von Fruchtkörpern auffallen. Die Isolierung aus marinen Küstensedimenten wurde auf Grund ihrer niedrigen Salztoleranz auf ruhende Zellen zurückgeführt (Reichenbach und Dworkin, 1992). Die meisten Myxobakterien können andere Bakterien mit Hilfe von Exoenzymen lysieren; daher könnte diese Gruppe eine Rolle bei der Kontrolle der bakteriellen Abundanz spielen.

ε-Proteobakterien. Innerhalb der ε-Proteobakterien wurden drei Gruppen identifiziert (*Arcobacter*-Gruppe, EpSI, EpSII; Abb.11). EpSI und EpSII haben eine Ähnlichkeit von 89-91% zu einem Epibionten des Polychaeten *Alvinella pompejana*. Die Epibionten werden auf dem dorsalen Integument des Wurmes gefunden, dessen klassischer Lebensraum Hydrothermalquellen der Tiefsee sind (Haddad *et al.*, 1995). Die physiologischen Eigenschaften der ε-Episymbionten sind bislang ungeklärt. Für *Arcobacter* spp. konnte die Fähigkeit zur Nitratreduktion und zur Schwefeloxidation gezeigt werden (Teske *et al.*, 1996b; Telang *et al.*, 1999).

Verrucomicrobiales. Innerhalb der Verrucomicrobiales konnte keine phylogenetische Gruppe beschrieben werden, die überwiegend Klonsequenzen aus marinen Sedimenten umfaßt (Abb.13). Neben Sequenzen aus marinen Sedimenten finden sich auch solche aus dem Pansen sowie aus Süßwassersedimenten. Die Sequenzen haben eine sehr niedrige Ähnlichkeit untereinander (72-91%) und zeigen die höchste Ähnlichkeit zu *Verrucomicrobium spinosum* mit 74 bis 87%. Eine Untergruppe jedoch zeichnet sich ab, die derzeit aus sechs Svalbard-

Sequenzen sowie zwei weiteren Sequenzen aus einem Küstensediment besteht und sich in weiteren Untersuchungen weiter stabilisieren könnte.

***Cytophaga/Flavobacterium*/Planctomycetales.** Innerhalb der *Cytophaga/Flavobacterium*-Gruppe konnte ebenfalls keine phylogenetische Gruppe identifiziert werden, die hauptsächlich in marinen Sedimenten zu finden ist (Abb.14). Die Diversität innerhalb dieser Gruppe ist sowohl unter den kultivierten Organismen als auch unter den erhaltenen Klonen ausgesprochen groß und zeigt deutlich die noch große Unkenntnis über diese Gruppe. Gleiches gilt auch für die Planctomycetales (Abb.13). Die Sequenzähnlichkeit liegt bei beiden Gruppen häufig bei weniger als 90% zu einem kultivierten Bakterium.

Gram-positive Bakterien. Nur eine Gruppe konnte innerhalb der Gram-positiven Bakterien identifiziert werden, die abgesehen von dem nicht weiter beschriebenen Isolat Koll13 aus einer Kläranlage nur Sequenzen aus marinen Sedimenten enthält (Abb.15). Die Ähnlichkeit der Sequenzen zu den nächsten Verwandten Stamm Koll13 und *Microthrix parvicella* bei 87 bis 96% bzw. 76 bis 84%.

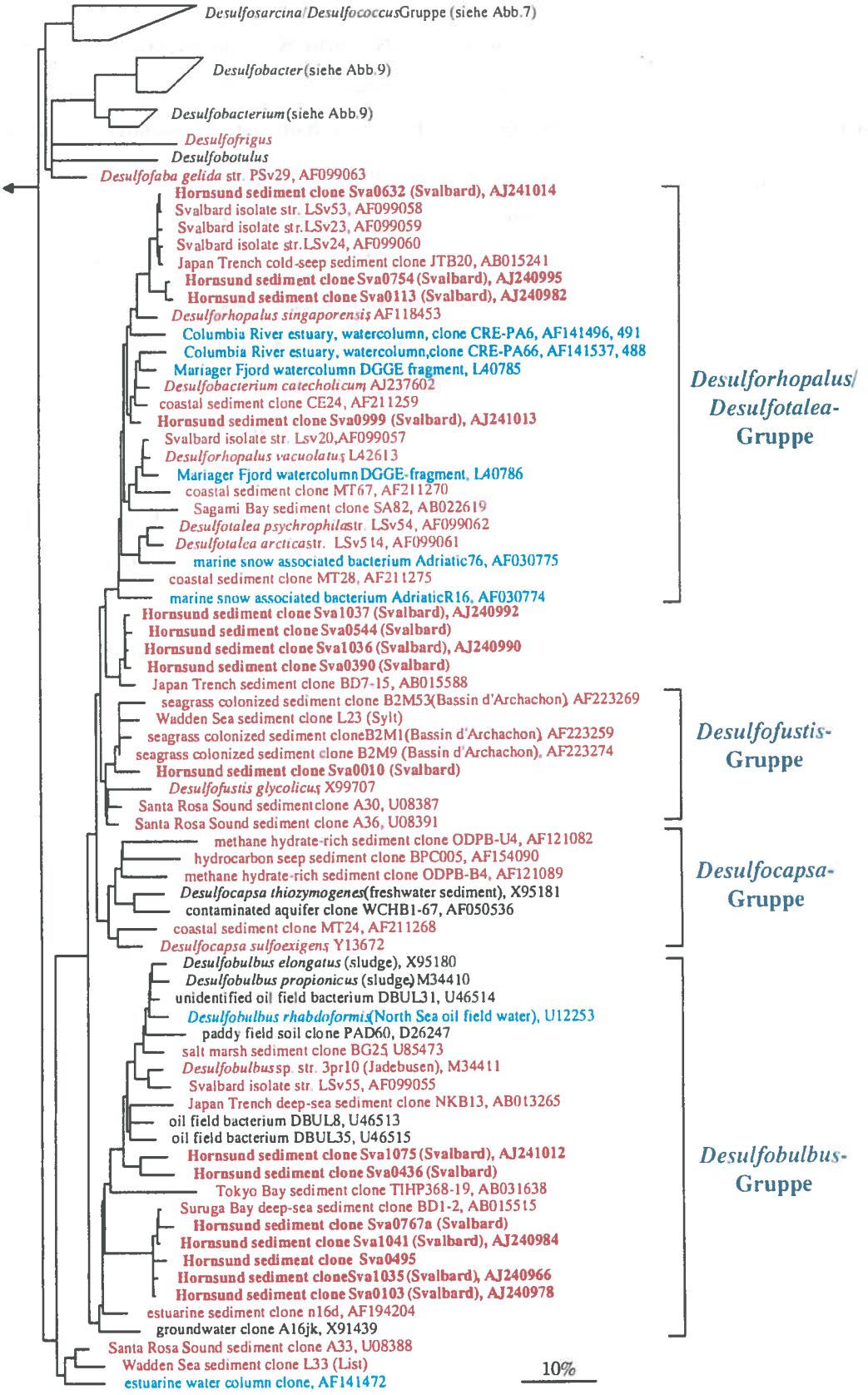


Abbildung 8. Vorläufiger 16S rDNA phylogenetischer Baum, der für marine Sedimente spezifische Gruppen innerhalb der δ-Proteobakterien zeigt. Rot gedruckt sind Sequenzen, die aus marinen Sedimenten erhalten wurden, rot & fett die Sequenzen der Svalbard-Genbank und blau die Sequenzen aus anderen, nicht sedimentären marinen Systemen. Der Balken gibt 10% geschätzte Sequenzunterschiede an.

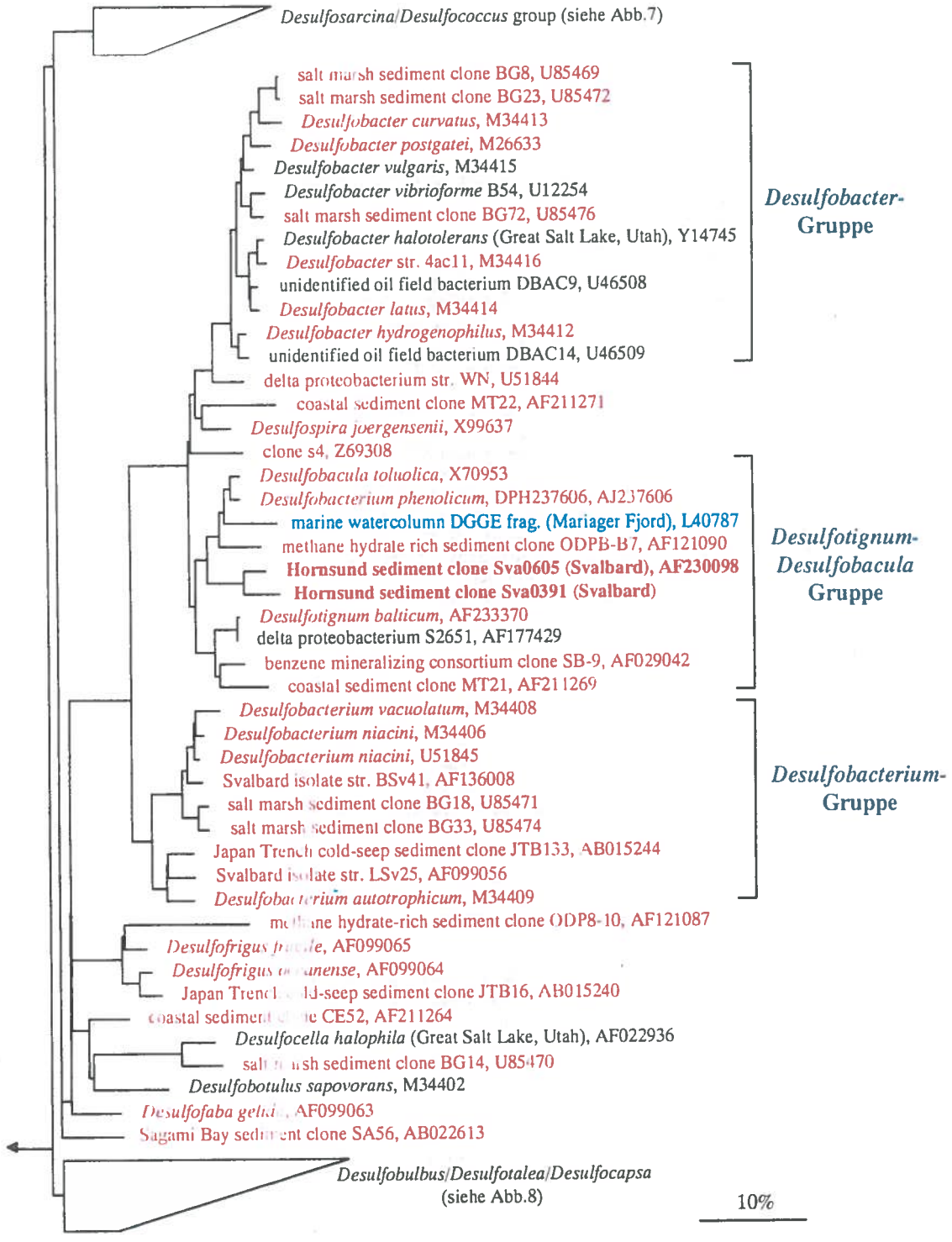


Abbildung 9. Vorläufiger 16S rDNA phylogenetischer Baum, der für marine Sedimente spezifische Gruppen innerhalb der δ-Proteobakterien zeigt. Rot gedruckt sind Sequenzen, die aus marinen Sedimenten erhalten wurden, rot & fett die Sequenzen der Svalbard-Genbank und blau die Sequenzen aus anderen, nicht sedimentären marinen Systemen. Der Balken gibt 10% geschätzte Sequenzunterschiede an.

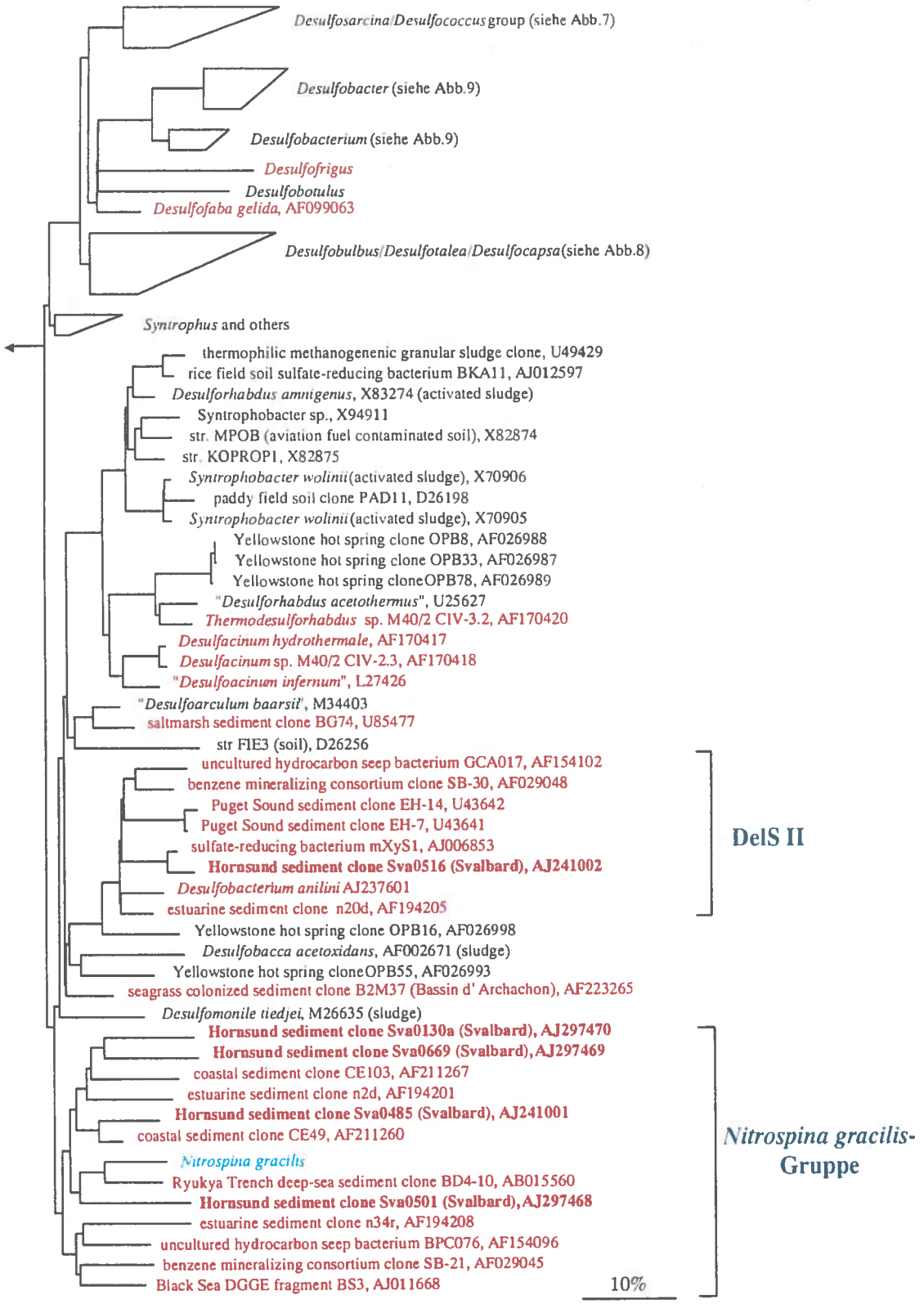


Abbildung 10. Vorläufiger 16S rDNA phylogenetischer Baum, der für marine Sedimente spezifische Gruppen innerhalb der δ-Proteobakterien zeigt. Rot gedruckt sind Sequenzen, die aus marinen Sedimenten erhalten wurden, rot & fett die Sequenzen der Svalbard-Genbank und blau die Sequenzen aus anderen, nicht sedimentären marinen Systemen. Der Balken gibt 10% geschätzte Sequenzunterschiede an.

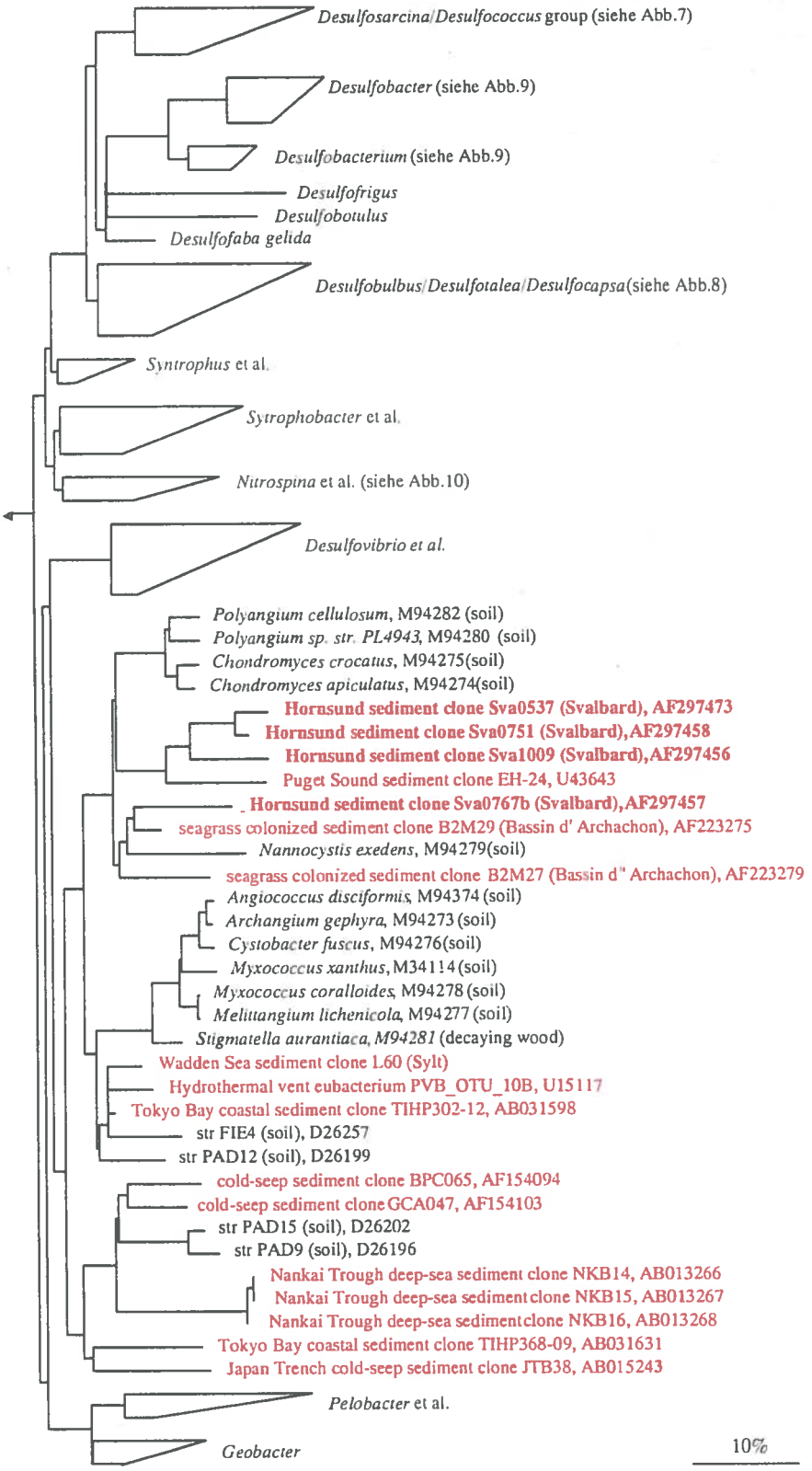


Abbildung 11. Vorläufiger 16S rDNA phylogenetischer Baum, der für marine Sedimente spezifische Gruppen innerhalb der δ-Proteobakterien zeigt. Rot gedruckt sind Sequenzen, die aus marinen Sedimenten erhalten wurden, rot & fett die Sequenzen der Svalbard-Genbank und blau die Sequenzen aus anderen, nicht sedimentären marinen Systemen. Der Balken gibt 10% geschätzte Sequenzunterschiede an.

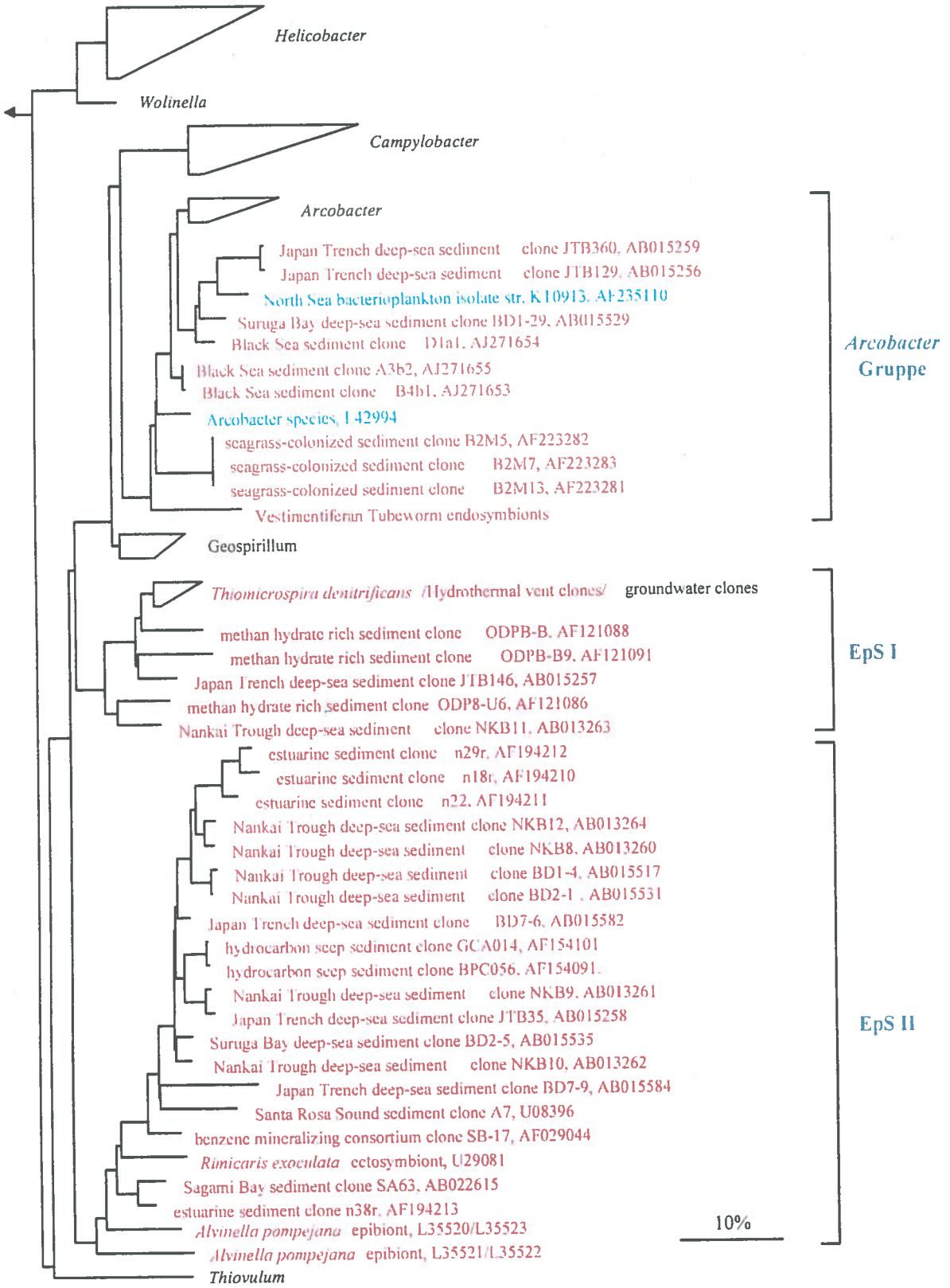


Abbildung 12. Vorläufiger 16S rDNA phylogenetischer Baum, der für marine Sedimente spezifische Gruppen innerhalb der ε-Proteobakterien zeigt. Rot gedruckt sind Sequenzen, die aus marinen Sedimenten erhalten wurden, rot & fett die Sequenzen der Svalbard-Genbank und blau die Sequenzen aus anderen, nicht sedimentären marinen Systemen. Der Balken gibt 10% geschätzte Sequenzunterschiede an.

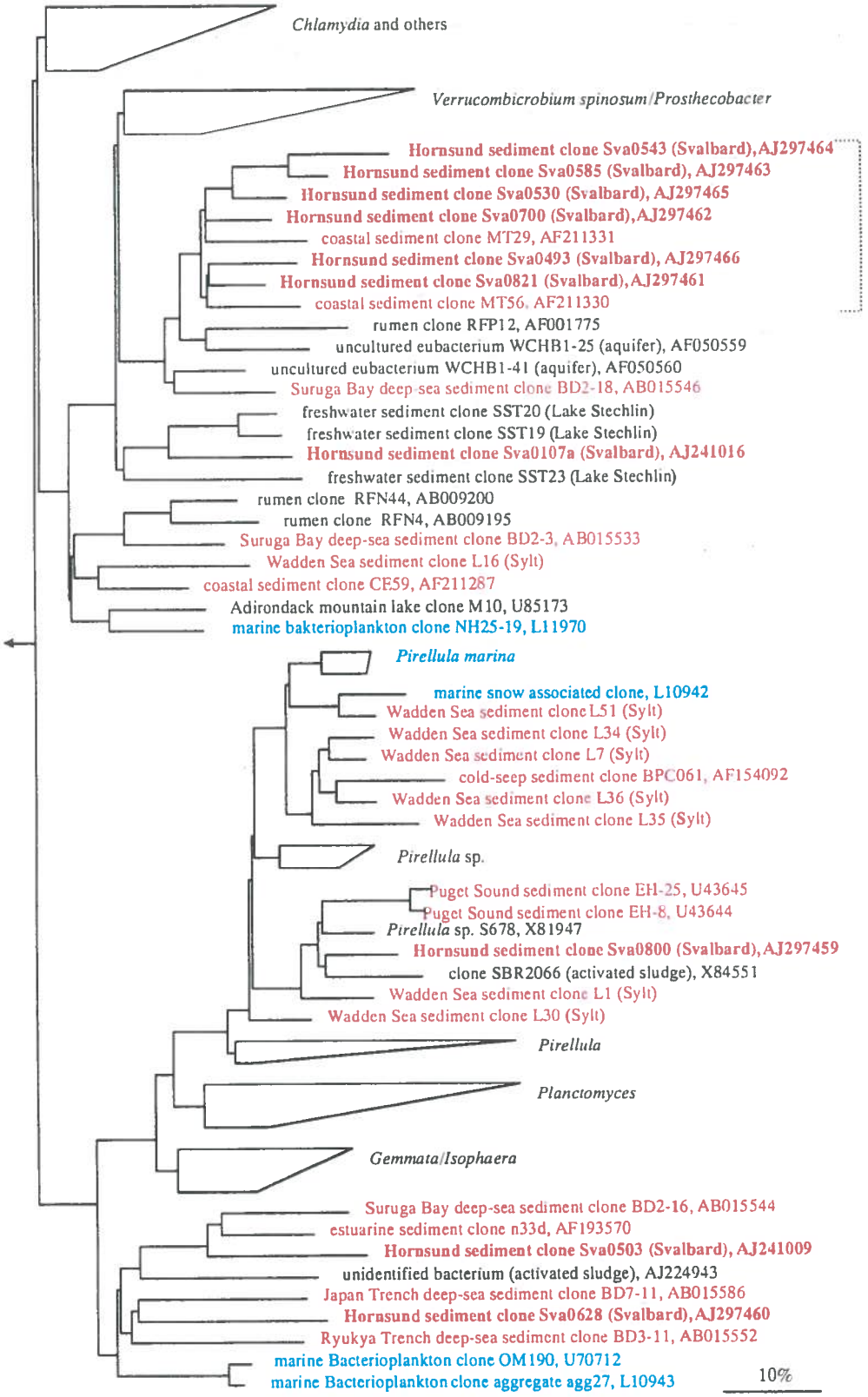
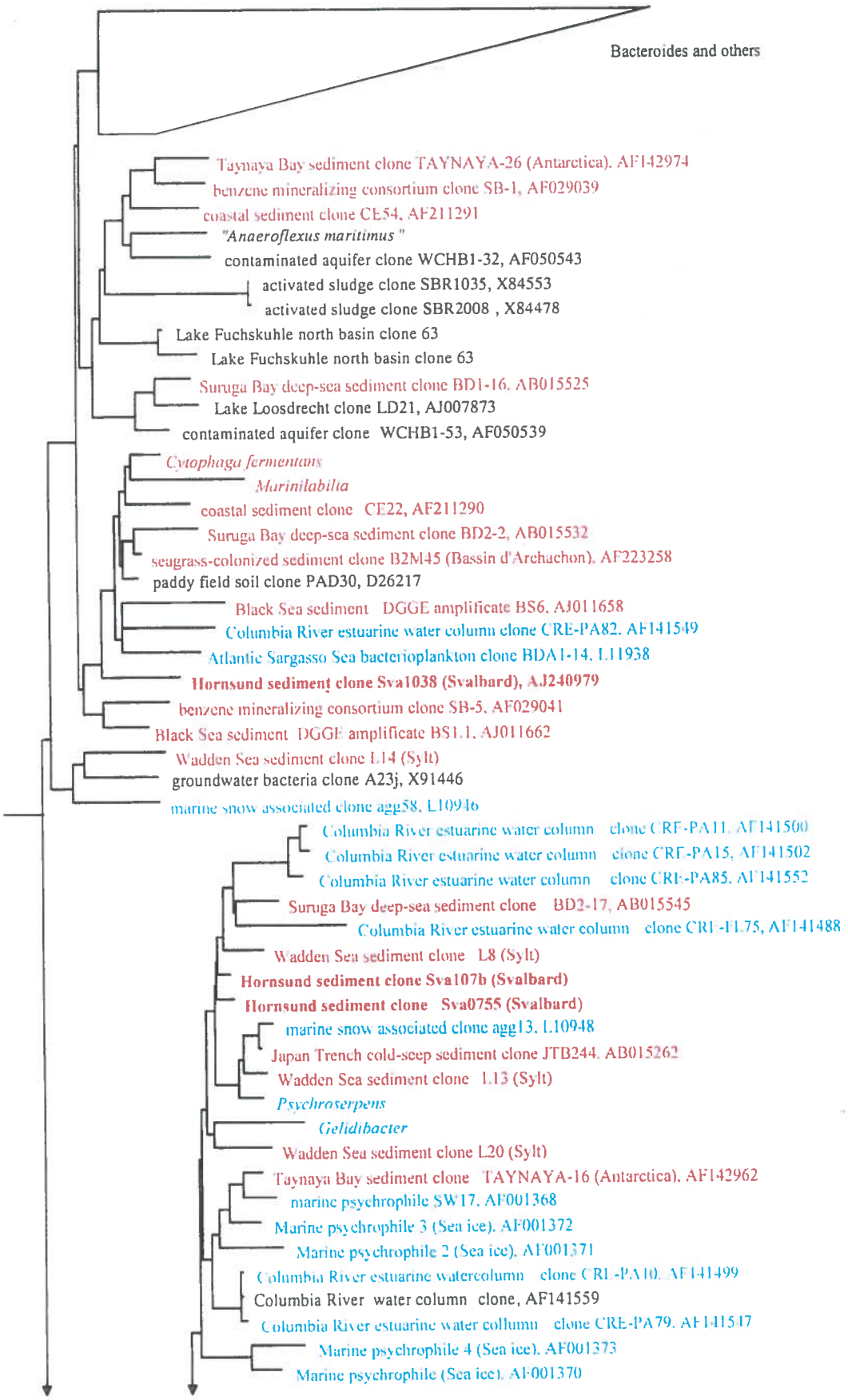


Abbildung 13. Vorläufiger 16S rDNA phylogenetischer Baum, der für marine Sedimente spezifische Gruppen innerhalb der Verrucomicrobiales und Planctomycetales zeigt. Rot gedruckt sind Sequenzen, die aus marinen Sedimenten erhalten wurden, rot & fett die Sequenzen der Svalbard-Genbank und blau die Sequenzen aus anderen, nicht sedimentären marinen Systemen. Der Balken gibt 10% geschätzte Sequenzunterschiede an.


Bacteroides and others
Taynaya Bay sediment clone TAYNAYA-26 (Antarctica), AF142974
benzene mineralizing consortium clone SB-1, AF029039
coastal sediment clone CE54, AF211291
"Anaeroflexus maritimus"
contaminated aquifer clone WCHB1-32, AF050543
activated sludge clone SBR1035, X84553
activated sludge clone SBR2008 , X84478
Lake Fuchskuhle north basin clone 63
Lake Fuchskuhle north basin clone 63
Suruga Bay deep-sea sediment clone BD1-16, AB015525
Lake Loosdrecht clone LD21, AJ007873
contaminated aquifer clone WCHB1-53, AF050539
Cytophaga fermentans
Marinilabilia
coastal sediment clone CE22, AF211290
Suruga Bay deep-sea sediment clone BD2-2, AB015532
seagrass-colonized sediment clone B2M45 (Bassin d'Archachon), AF223258
paddy field soil clone PAD30, D26217
Black Sea sediment DGGE amplificate BS6, AJ011658
Columbia River estuarine water column clone CRE-PA82, AF141549
Atlantic Sargasso Sea bacterioplankton clone BDA1-14, L11938
Hornsund sediment clone Sva1038 (Svalbard), AJ240979
benzene mineralizing consortium clone SB-5, AF029041
Black Sea sediment DGGE amplificate BS1.1, AJ011662
Wadden Sea sediment clone L14 (Sylt)
groundwater bacteria clone A23j, X91446
marine snow associated clone agg58, L10946
Columbia River estuarine water column clone CRE-PA11, AF141500
Columbia River estuarine water column clone CRE-PA15, AF141502
Columbia River estuarine water column clone CRE-PA85, AF141552
Suruga Bay deep-sea sediment clone BD2-17, AB015545
Columbia River estuarine water column clone CRE-FL75, AF141488
Wadden Sea sediment clone L8 (Sylt)
Hornsund sediment clone Sva107b (Svalbard)
Hornsund sediment clone Sva0755 (Svalbard)
marine snow associated clone agg13, L10948
Japan Trench cold-seep sediment clone JTB244, AB015262
Wadden Sea sediment clone L13 (Sylt)
Psychroserpens
Gelidibacter
Wadden Sea sediment clone L20 (Sylt)
Taynaya Bay sediment clone TAYNAYA-16 (Antarctica), AF142962
marine psychrophile SW17, AF001368
Marine psychrophile 3 (Sea ice), AF001372
Marine psychrophile 2 (Sea ice), AF001371
Columbia River estuarine watercolumn clone CRE-PA10, AF141499
Columbia River water column clone, AF141559
Columbia River estuarine water collumn clone CRE-PA79, AF141547
Marine psychrophile 4 (Sea ice), AF001373
Marine psychrophile (Sea ice), AF001370

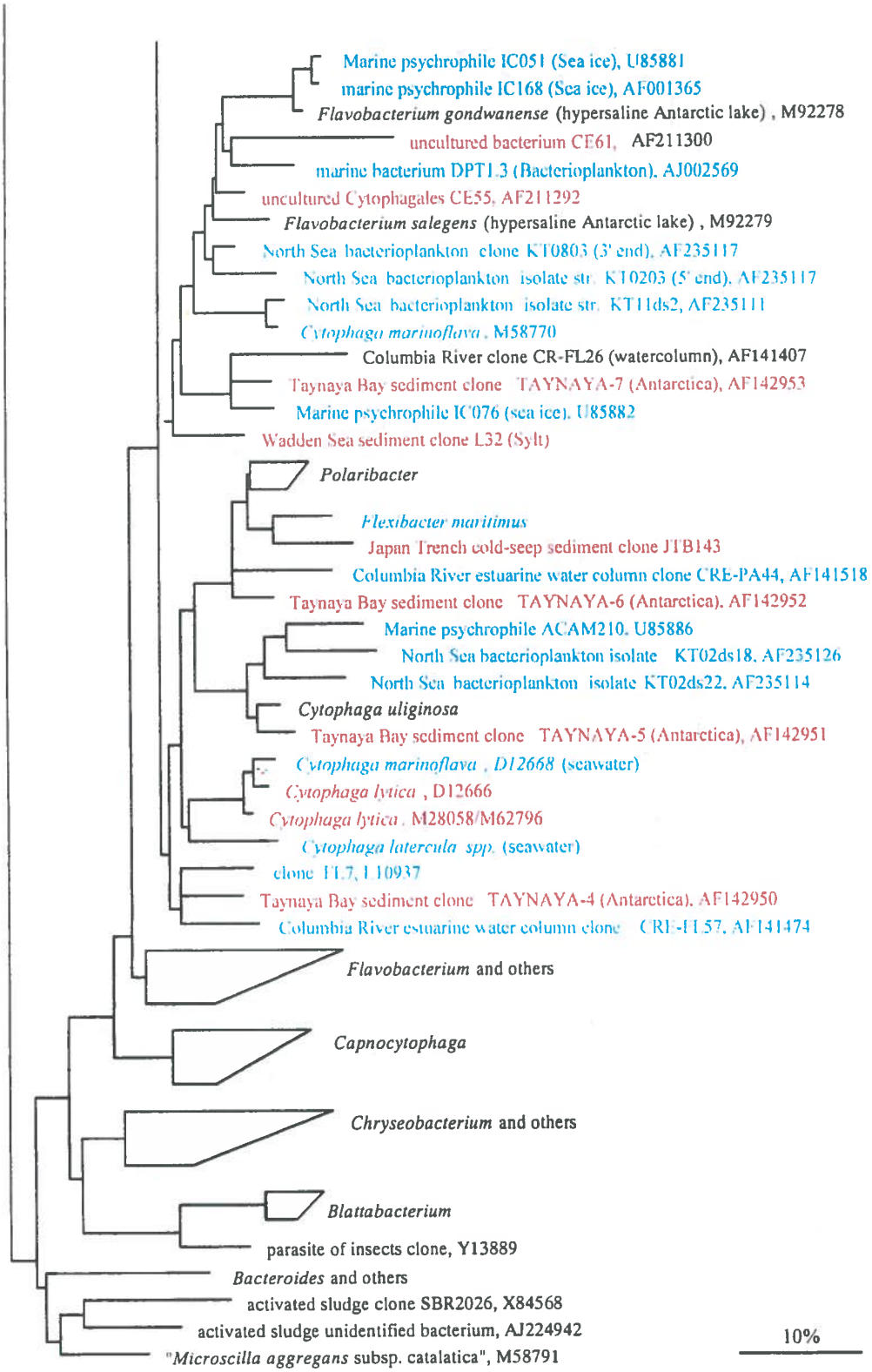


Abbildung 14. Vorläufiger 16S rDNA phylogenetischer Baum, der für marine Sedimente spezifische Gruppen innerhalb der *Cytophaga/Flavobacterium*-Gruppe zeigt. Rot gedruckt sind Sequenzen, die aus marinen Sedimenten erhalten wurden, rot & fett die Sequenzen der Svalbard-Genbank und blau die Sequenzen aus anderen, nicht sedimentären marinen Systemen. Der Balken gibt 10% geschätzte Sequenzunterschiede an.

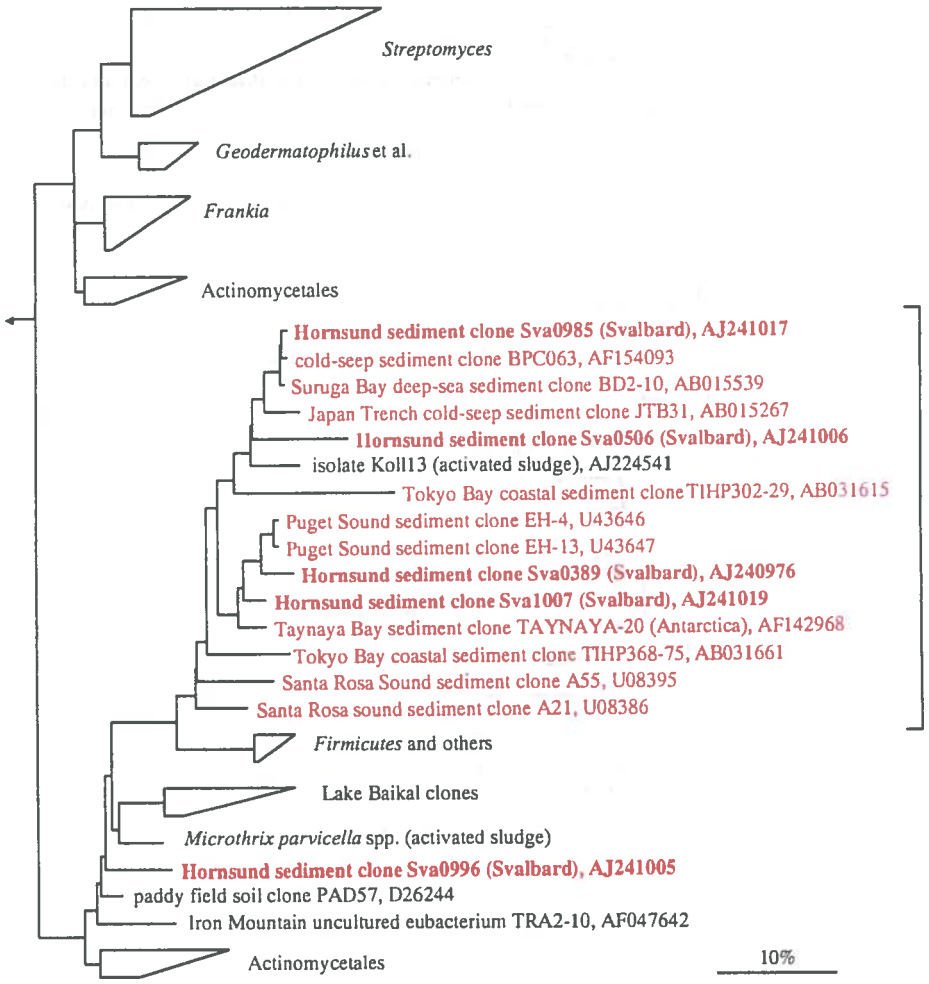


Abbildung 15. Vorläufiger 16S rDNA phylogenetischer Baum, der die für marine Sedimente spezifische Gruppe innerhalb der Actinobacteria (Gram-positive Bakterien mit hohem DNA GC Gehalt) zeigt. Rot gedruckt sind Sequenzen, die aus marinen Sedimenten erhalten wurden, und rot & fett die Sequenzen der Svalbard-Genbank. Der Balken gibt 10% geschätzte Sequenzunterschiede an.

1.3 Strukturanalysen prokaryontischer Lebensgemeinschaften

Die Zusammensetzung der prokaryontischen Lebensgemeinschaft in Smeerenburgfjorden Sedimenten (Station J) wurde mittels FISH und quantitativer slot-blot Hybridisierung unter Berücksichtigung der in der Genbank gefundenen phylogenetischen Gruppen charakterisiert (Publikationen 2 und 3). Der Schwerpunkt dieser Untersuchung lag in der Charakterisierung der sulfatreduzierenden Bakterien (Publikation 2).

In den untersuchten Sedimenten wurden sulfatreduzierende Bakterien (SRB) am häufigsten mit $9,3 \pm 3,6\%$ der Gesamtzellzahl und $20,1 \pm 2,9\%$ der detektierten prokaryontischen rRNA gefunden. Die Gemeinschaft der Sulfatreduzierer wurde dominiert durch Vertreter der *Desulfosarcina/Desulfococcus*-Gruppe. Diese Gruppe erfaßt die als DelSI beschriebene Gruppe und schließt zusätzlich die beiden Gattungen psychrophiler sulfatreduzierender Bakterien, *Desulfofaba* und *Desulfofrigus*, ein. Bis zu 11,7% aller DAPI-färbbaren Zellen und bis zu 16,8% der prokaryontischen rRNA konnten dieser Gruppe zugeordnet werden. Damit liegt der Anteil dieser Gruppe bezüglich der anderen sulfatreduzierenden Bakterien bei bis zu 73% der SRB-Zellzahlen und bis zu 70% der SRB rRNA. Eine weitere Probennahme im darauffolgenden Jahr bestätigte die hohe Abundanz sowie die räumliche Verteilung in den Smeerenburgfjorden Sedimenten. Eine hohe Abundanz dieser Gruppe konnte auch für drei weitere Stationen vor der Küste Svalbards gezeigt werden. Mit neu entwickelten gattungsspezifischen Sonden konnten die detektierten Zellen jedoch keiner der kultivierten Gattungen *Desulfosarcina*, *Desulfococcus*, *Desulfofrigus* oder *Desulfofaba* zugeordnet werden. Mittels spezifischer PCR und anschließender Klonierung wurden 16S rDNA-Sequenzen der *Desulfosarcina/Desulfococcus*-Gruppe erhalten, die maximal 91,2% Sequenzähnlichkeit zu *Desulfosarcina variabilis* aufwiesen. Eine neue Untergruppe (SVAL1) konnte beschrieben werden (Abb.7), die auch *in situ* mit 54,8% aller detektierten SRB-Zellen als dominierend nachgewiesen wurde. Eine hohe Abundanz von SVAL1 konnte als gemeinsames "Merkmal" der untersuchten Sedimente entlang der Küste Svalbards festgestellt werden. Die identifizierten Zellen zeigten eine sarcinenähnliche Zellmorphologie mit einem Durchmesser von etwa 1 µm und traten in den untersuchten Sedimenten als typische Sarcina-Tetraden, in Gruppen von acht und mehr Zellen oder auch sehr häufig als Diplokokken auf. In der *Desulfosarcina/Desulfococcus*-Gruppe sind bislang nur wenige kultivierte Bakterien zu finden. Die kultivierten Vertreter dieser Gruppe zeichnen sich durch ein breites Substratspektrum in bezug auf mögliche Elektronendonatoren aus und können organische Verbindungen vollständig zu CO_2 oxidieren (Widdel und Bak, 1992b).

Mögliche Gründe für die bislang seltene Isolierung von Vertretern dieser Gruppe könnte die Verwendung von zu "einfachen" Substraten zur Isolierung sein, wie Laktat oder Propionat, langsames Wachstum und die Eigenschaft zur Aggregatbildung oder zum Anheften an Partikel sein, welche die Isolierung aus Anreicherungskulturen erschwert. Die Vielseitigkeit der *Desulfosarcina/Desulfococcus*-Gruppe könnte im Wettbewerb um limitierte Kohlenstoffquellen einen Vorteil bieten. Die Detektion mittels FISH von Vertretern der Gattung *Desulfovibrio* lag in Smeerenburgfjorden Sedimenten nur knapp über der Nachweisgrenze. Aus 16S rDNA Genbanken von mehr als 30 verschiedenen marinen Sedimenten konnte bislang keine Sequenz erhalten werden, die mit *Desulfovibrio* spp. näher verwandt war. Vertreter der Gattung *Desulfovibrio* spp. sind jedoch häufig aus Sedimenten isoliert worden und sind als effiziente Wasserstoff-Verwerter bekannt (Widdel und Bak, 1992b). Neben Wasserstoff können sie nur einfache organische Säuren und in einigen Fällen Ethanol als Elektronendonator verwenden.

Vertreter der Gattung *Desulforhopalus* bildeten mit wesentlich geringerer Abundanz von 1,8 ± 0,6% aller DAPI-färbbaren Zellen und 2,6±0,7% der prokaryontischen rRNA die zweithäufigste Gruppe innerhalb der Sulfatreduzierer. Die in der Genbank am häufigsten gefundene Gruppe, die mit psychrophilen, aus dem gleichen Habitat isolierten Sulfatreduzierern der Gattung *Desulfotalea* (Knoblauch *et al.*, 1999b) verwandt war, konnte hier jedoch nur in relativ niedrigeren Zahlen von 0,9±0,6% bzw. 1,7±0,9% der prokaryontischen rRNA detektiert werden und spielt damit in den untersuchten Sedimenten vermutlich nur eine untergeordnete Rolle für die Mineralisierung organischen Materials.

Eine große Gruppe von Sequenzen der Svalbard-Genbank war phylogenetisch am nächsten mit *Desulfuromonas palmitatis* (bis zu 93,7%) verwandt. Für *Desulfuromonas palmitatis* wurde die Fähigkeit zur Eisenreduktion (Coates *et al.*, 1995) und für andere Arten innerhalb dieser Gattung die zur Schwefelreduktion beschrieben (Widdel und Pfennig, 1992c). Mit bis zu 2,2% aller DAPI-färbbaren Zellen und bis zu 6,4% der gesamten prokaryontischen rRNA kann der Beitrag dieser Gruppe zum Schwefel- oder Eisenkreislauf in den Smeerenburgfjorden Sedimenten von Bedeutung sein.

γ-Proteobakterien waren neben den sulfatreduzierenden Bakterien die zweithäufigste Gruppe. Die höchste Abundanz wurde in der Sedimentoberflächenschicht mit 10,6% aller Zellen und 18,5% der prokaryontischen rRNA detektiert. Die in zahlreichen Genbanken gefundenen Gruppen GamSI und GamSIII (Abb.5) konnten mit einer neu entwickelten Sonde mit 2,1±0,7% aller DAPI-färbbaren Zellen (1,1 x 10^8 Zellen pro ml) *in situ* detektiert werden. In der rRNA slot-blot Hybridisierung lag der relative rRNA Anteil sogar deutlich darüber

(12,8±2,1% der prokaryontischen rRNA). Durch den *in situ* Nachweis der Gruppe konnte gezeigt werden, daß die klonierten Sequenzen von freilebenden Bakterien stammen könnten. Unklar bleibt jedoch weiterhin, ob diese Gruppe am oxidativen Teil des Schwefelkreislaufes beteiligt ist.

Eine wesentliche Rolle bei der Mineralisierung des organischen Materials spielt die *Cytophaga/Flavobacterium*-Gruppe. Der relative Anteil dieser Gruppe lag in den Smeerenburgfjorden Sedimenten bei bis zu 12,8% der DAPI-färbbaren Zellen und 6,1% der prokaryontischen RNA an der Oberfläche, aber auch in anoxischen Sedimentschichten wurden noch mehr als 1,5 x 10^8 Zellen pro ml gefunden. Diese hohe Abundanz stützt die Hypothese, daß Vertreter dieser hoch diversen Gruppe in marinen Sedimenten, einem hauptsächlich anaeroben Habitat, eine ökologische Bedeutung als hydrolytisch fermentative Bakterien haben (Rosselló-Mora *et al.*, 1999) und zeigt, daß die Bakterien nicht nur allochthoner Eintrag aus der Wassersäule in das Sediment sind, sondern auch in den anoxischen Bereichen der Sedimente aktiv sind. Der Anteil der Planctomycetales lag zwischen 1,3 und 3,9% der DAPI-färbbaren Zellen, eine vertikale Zonierung wurde dabei nicht beobachtet. Die bislang in Reinkultur vorliegenden Vertreter dieser Ordnung sind aerobe Organismen (Staley *et al.*, 1991). Kürzlich jedoch konnte auch eine Beteiligung an einem anaeroben Stoffwechselweg gezeigt werden: Der für die anaerobe Ammoniumoxidation ("Anammox") in Kläranlagen verantwortliche Organismus ist ein Vertreter der Planctomycetales, der bislang jedoch noch nicht kultiviert werden konnte (Strous *et al.*, 1999).

Gram-positive Bakterien scheinen in den untersuchten Svalbard Sedimenten nur eine untergeordnete Rolle zu spielen. Der mittels quantitativer slot-blot Hybridisierung bestimmte relative rRNA-Anteil variierte zwischen 0 und 2,6% der prokaryontischen rRNA. Obwohl Vertreter der Gattungen *Clostridium* und *Bacillus* bereits mehrfach aus marinen Sedimenten isoliert werden konnten (Takami *et al.*, 1997), dürften Vertreter dieser Gattungen keine große Bedeutung bei der Mineralisierung in diesen Sedimenten haben. Fermentative Gruppen der Gattung *Clostridium* sind als ausgezeichnete Polysaccharid- und Polymerabbauer beschrieben (Coughlan und Mayer, 1991; Hippe *et al.*, 1991). Das Gleiche gilt für die im Boden so wichtigen aeroben *Bacillus* spp. (Coughlan und Mayer, 1991; Slepecky und Hemphill, 1991).

Die Quantifizierungen der phylogenetischen Gruppen mittels FISH und slot-blot Hybridisierung gaben vergleichbare Ergebnisse. Diese Vergleichbarkeit ist ermutigend. Ein Vergleich von Untersuchungen, gleich ob auf FISH oder slot-blot Hybridisierung basierend, ist weitgehend ohne Einschränkungen möglich, obwohl beide Methoden unterschiedliche

Limitationen haben (Amann *et al.*, 1995).

Die Kombination von FISH und slot-blot Hybridisierung ermöglicht es, den zellulären rRNA Gehalt einzelner phylogenetischer Gruppen zu kalkulieren (Publikation 2). Eine gute Korrelation wurde zwischen dem zellulären rRNA Gehalt von *Desulfococcus/Desulfosarcina* Zellen und den zellulären Sulfatreduktionsraten gefunden. Die höchste metabolische Aktivität dieser Gruppe fand sich in den obersten 5 mm des Sedimentes. Für die Zukunft wäre die Untersuchung weiterer natürlicher Systeme bezüglich dieser Korrelation von zellulärer Aktivität und rRNA Gehalt interessant. Die Kenntnis über die maximale Sauerstoffeindringtiefe in dem untersuchten Sediment wäre wichtig gewesen, da so leider ungeklärt bleibt, ob die gezeigte höchste metabolische Aktivität der *Desulfosarcina/Desulfococcus*-Gruppe in den obersten 5 mm des Sedimentes unter oxischen oder bereits anoxischen Bedingungen auftrat. Zuvor wurden bereits mehrfach sulfatreduzierende Bakterien in oxischen Bereichen mariner Sedimente und mikrobieller Matten gefunden (Jørgensen und Bak, 1991; Visscher *et al.*, 1992; Minz *et al.*, 1999; Sahm *et al.*, 1999a) und sogar metabolische Aktivität in Gegenwart von Sauerstoff beobachtet (Jørgensen, 1977; Dilling und Cypionka, 1990; Canfield und DesMarais, 1991a; Dannenberg *et al.*, 1992; Fründ und Cohen, 1992; Marschall *et al.*, 1993; Krekeler *et al.*, 1998). Wachstum in Gegenwart von Sauerstoff konnte bislang jedoch noch nicht gezeigt werden

2 Isolierung und Charakterisierung psychrophiler schwefeloxidierender Bakterien

In marinen Sedimenten liegen die *in situ*-Temperaturen zwischen weniger als 0°C in polaren Regionen und mehr als 100°C in geothermisch erwärmten Sedimenten. Schwefeloxidierende Bakterien wurden vielfach aus Hydrothermalquellen und Küstensedimenten isoliert (z.B. Kuenen und Veldkamp, 1972; Jannasch *et al.*, 1985; Durand *et al.*, 1993; Brinkhoff *et al.*, 1999b; Sievert und Kuever, 2000). Psychrophile schwefeloxidierende Bakterien wurden bislang jedoch noch nicht beschrieben.

In der vorliegenden Arbeit konnte ein neuer Stamm der Gattung *Thiomicrospira* aus Svalbard Sedimenten isoliert werden (Publikation 4). Der Temperaturbereich, in dem Wachstum beobachtet wurde, liegt für den neuen Stamm SVAL-E zwischen –2°C und +20,8°C mit einer optimalen Wachstumstemperatur von 11,5 bis 13,2°C. Eine weitgehend akzeptierte Definition von Morita (Morita, 1975) sagt, daß psychrophile Bakterien eine

optimale Wachstumstemperatur von ungefähr 15°C, eine maximale Wachstumstemperatur um 20°C und eine minimale Wachstumstemperatur von 0°C und weniger haben. Danach ist der isolierte Stamm SVAL-E als psychrophil zu bezeichnen. Bezüglich seiner Wachstumstemperatur unterscheidet sich SVAL-E erheblich von den bisher beschriebenen *Thiomicrospira*-Stämmen (Wachstumsoptima 28-40°C), er ist aber wie alle anderen Vertreter der Gattung auch durch aerobes, obligat chemolithoautotrophes Wachstum charakterisiert. Die Ähnlichkeit des Stammes SVAL-E liegt auf 16S rDNA Ebene bei < 97% zu allen bislang beschriebenen Arten und auf DNA-DNA-Ebene bei höchstens 55,6% zum nächsten Verwandten *Thiomicrospira chilensis*. Aufgrund der phäntotypischen und genotypischen Unterschiede wird SVAL-E als neue Art vorgeschlagen (*Thiomicrospira arctica*).

Vertreter der Gattung *Thiomicrospira* sind bereits aus vielen verschiedenen Habitaten global isoliert bzw. nachgewiesen worden, darunter sind unterschiedliche marine Systeme, aber auch ein Süßwasserhabitat (z.B. Kuenen und Veldkamp, 1972; Ruby und Jannasch, 1982; Wood und Kelly, 1993; Jannasch *et al.*, 1985; Brinkhoff und Muyzer, 1997). Mit der Isolierung des neuen psychrophilen Stammes kann der Lebensraum der Gattung *Thiomicrospira* auch auf kalte Sedimente ausgedehnt werden. Das Vorkommen der Gattung scheint hauptsächlich auf die Anwesenheit reduzierter Schwefelverbindungen zurückzuführen zu sein, wobei sich ihre Vertreter gut an verschiedene Umweltbedingungen anpassen können. Die Häufigkeit der Organismen dieser Gattung bleibt noch zu untersuchen.

3 Anaerobe Methanoxidation in marinen Sedimenten

Die anaerobe Oxidation von Methan in marinen Sedimenten ist ein mikrobieller Prozeß von globaler Bedeutung bei der Kontrolle des Methanflusses in die Atmosphäre (Valentine und Reeburgh, 2000). Geochemische Beweise für einen Methanverbrauch in anoxischen Sedimenten basieren auf Methanprofilen (Martens und Berner, 1977), Tracer-Experimenten (Iversen und Jørgensen, 1985) und Daten von stabilen Kohlenstoffisotopen (Reeburgh, 1982; Alperin *et al.*, 1988). Bislang sind jedoch weder der Abbauweg noch die daran beteiligten Mikroorganismen bekannt. Neuere Untersuchungen zeigen, daß die anaerobe Methanoxidation durch Archaea in Syntrophie mit anderen anaeroben Bakterien, wahrscheinlich Sulfatreduzierern, katalysiert wird (Hoehler *et al.*, 1994; Hansen *et al.*, 1998; Hinrichs *et al.*, 1999; Thiel *et al.*, 1999; Pancost *et al.*, 2000).

In der vorliegenden Arbeit wurden methanhydratreiche Sedimente (Cascadia

Subduktionszone, Oregon) mittels FISH untersucht, um die an der anaeroben Methanoxidation beteiligten Mikroorganismen zu identifizieren (Publikation 5). In der Zone der anaeroben Methanoxidation konnten in der Tat Konsortien aus Bacteria und Archaea gefunden werden. Die untersuchte Zone war charakterisiert durch extrem hohe Raten auf Methan basierender Sulfatreduktion sowie sehr negative $\delta^{13}C$–Isotopenwerte der mikrobiellen Biomarker. Mit spezifischen Sonden wurden die Archaea der Ordnung Methanomicrobiales, und die Bacteria den sulfatreduzierenden Bakterien (SRB) der *Desulfosarcina/Desulfococcus*-Gruppe (DelSI) zugeordnet. Interessanterweise gehörten diese Vertreter der *Desulfosarcina/Desulfococcus*-Gruppe auch der in den Svalbard Sedimenten dominierenden Untergruppe SVAL1 an.

Ein durchschnittliches Archaea/SRB-Konsortium besteht aus einem inneren Kern, der etwa 100 Archaea enthält, und einer äußeren Hülle von etwa 200 sulfatreduzierenden Bakterien um diesen Kern. Die Größe der Aggregate variierte zwischen 1 und 11 µm bei einem durchschnittlichen Durchmesser von 3,2 ± 1,5 µm. Die höchsten Zahlen von bis zu 7×10^7 Aggregaten pro ml wurden in oberflächlichen Schichten des Sedimentes gefunden. Methanoxidierende Archaeabakterien, die wie in den gefundenen Konsortien in engem räumlichen Kontakt mit sulfatreduzierenden Bakterien stehen, haben bezüglich eines effektiven Transfers von Intermediaten über molekulare Diffusion einen klaren Vorteil gegenüber freilebenden Zellen (Boone *et al.*, 1989). Daher ist es sehr wahrscheinlich, daß die beschriebenen abundanten und stark ^{13}C abgereicherten Konsortien Methan anaerob oxidieren können. Postulierte Intermediate sind Wasserstoff und/oder Acetat (siehe Publikation 5). Der letzte Beweis auf funktioneller Ebene für die anaerobe Oxidation von Methan durch diese Konsortien sowie die Identifizierung der von den Sulfatreduzierern verwendeten Intermediate muß jedoch noch folgen, z.B. über eine Kombination aus FISH und Mikroautoradiographie.

Die für marine Sedimente gezeigte weitverbreitete hohe Abundanz und Diversität der *Desulfosarcina/Desulfococcus*-Gruppe unterstreicht deren große ökologische Bedeutung. Ein Schwerpunkt in künftigen Untersuchungen sollte auch im bereits beginnenden "Environmental Genomics"-Zeitalter auf der Isolierung von Vertretern dieser Gruppe liegen, um über Studien von Reinkulturen deren Physiologie und ökologische Bedeutung in unterschiedlichen Lebensräumen besser verstehen zu können. Das Ziel der Isolierung der Bakterien sollte auch für alle anderen beschriebenen phylogenetischen Gruppen gelten, die bislang nur aus klonierten 16S rDNA-Sequenzen bestehen oder nur entfernte kultivierte Verwandte haben.

C

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Teil II:

Publikationen

A Publikationsliste mit Erläuterungen

Diese Dissertation basiert zu einem großen Teil auf fünf Publikationen. Die Beiträge der verschiedenen Autoren zu den Publikationen werden im Folgenden erläutert.

1 **Katrin Ravenschlag, Kerstin Sahm, Jakob Pernthaler, and Rudolf Amann.** 1999. High Bacterial Diversity in Permanently Cold Marine Sediments. Appl. Environ. Microbiol. **65**: 3982-3989

Entwicklung des Konzeptes von K.R. und K.S., Durchführung der Experimente von K.R., "Rarefaction"-Analyse durch J.P., Erstellen des Manuskriptes von K.S. unter der redaktionellen Mitarbeit von K.R. und R.A.

2 **Katrin Ravenschlag, Kerstin Sahm, Christian Knoblauch, Bo Barker Jørgensen, and Rudolf Amann.** 2000. Community Structure, Cellular rRNA Content and Activity of Sulfate-Reducing Bacteria in Marine Arctic Sediments. Appl. Environ. Microbiol. **66**: 3592-3602

Entwicklung des Konzeptes von K.R. und K.S., Durchführung der Experimente von K.R. mit Ausnahme der Messung der Sulfatreduktionsraten (C.K. und B.B.J.), Erstellen des Manuskriptes von K.R. unter der redaktionellen Mitarbeit von K.S. und R.A.

3 **Katrin Ravenschlag, Kerstin Sahm, Rudolf Amann.** Quantitative Molecular Analysis of the Microbial Community in Marine Arctic Sediments (Svalbard). Submitted to Appl. Environ. Microbiol.

Entwicklung des Konzeptes von K.R. und K.S., Durchführung der Experimente von K.R., Erstellen des Manuskriptes von K.R. unter der redaktionellen Mitarbeit der Co-Autoren.

4 **Katrin Ravenschlag, Jan Kuever, Ruth Meinke, Rudolf Amann, and Thorsten Brinkhoff.** Isolation of a Psychrophilic Strain of *Thiomicrospira* spp. from Marine Arctic Sediments and Description of *Thiomicrospira arctica* sp. nov. In preparation.

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5 **Antje Boetius, Katrin Ravenschlag, Carsten Schubert, Dirk Rickert, Friedrich Widdel, Armin Gieseke, Rudolf Amann, Bo Barker Jørgensen, Ursula Witte, Olaf Pfannkuche.** A Marine Microbial Consortium Apparently Mediating Anaerobic Oxidation of Methane. Nature 407: 05 October 2000; in press.

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High bacterial diversity in permanently cold marine sediments

Katrin Ravenschlag, Kerstin Sahm, Jakob Pernthaler, and Rudolf Amann

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High Bacterial Diversity in Permanently Cold Marine Sediments

KATRIN RAVENSCHLAG, KERSTIN SAHM,* JAKOB PERNTHALER, AND RUDOLF AMANN
Molecular Ecology Group, Max-Planck-Institute for Marine Microbiology, D-28359 Bremen, Germany

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A 16S ribosomal DNA (rDNA) clone library from permanently cold marine sediments was established. Screening 353 clones by dot blot hybridization with group-specific oligonucleotide probes suggested a predominance of sequences related to bacteria of the sulfur cycle (43.4% potential sulfate reducers). Within this fraction, the major cluster (19.0%) was affiliated with *Desulfotalea* sp. and other closely related psychrophilic sulfate reducers isolated from the same habitat. The cloned sequences showed between 93 and 100% similarity to these bacteria. Two additional groups were frequently encountered: 13% of the clones were related to *Desulfuromonas palmitatis*, and a second group was affiliated with *Myxobacteria* spp. and *Bdellovibrio* spp. Many clones (18.1%) belonged to the γ subclass of the class *Proteobacteria* and were closest to symbiotic or free-living sulfur oxidizers. Probe target groups were further characterized by amplified rDNA restriction analysis to determine diversity within the groups and within the clone library. Rarefaction analysis suggested that the total diversity assessed by 16S rDNA analysis was very high in these permanently cold sediments and was only partially revealed by screening of 353 clones.

Coastal and shelf sediments play a significant role in the remineralization of organic matter. In shelf areas, an estimated 32 to 46% of the primary production settles to the sea floor (54). While part of it is permanently buried, the majority of this detrital material is reoxidized, mainly through the action of prokaryotes (54). Steep redox gradients provide niches for a wide variety of metabolically diverse microorganisms, and O_2, NO_3^-, manganese and iron oxides, and SO_4^{2-} have been identified as the most important electron acceptors in marine sediments (3, 19). The various processes of microbial carbon mineralization can be quantified by tracer techniques, and their importance for biogeochemical cycles in the marine environment is recognized; however, little is known about the microbial community responsible for them.

Few cultivation-independent studies of microbial diversity in marine sediments have been conducted (6, 15, 22, 43). The sequences recovered in these studies revealed the presence of mainly unknown organisms only distantly related to known isolates. To further uncover microbial diversity in marine shelf sediments and to identify potentially dominant groups in this habitat, we constructed a 16S ribosomal DNA (rDNA) clone library using general bacterial primers to amplify the almost complete gene.

The screening process was tested by statistical analysis to evaluate whether we had covered total diversity in our clone library by screening 353 clones. Species diversity can be considered to be composed of two components: species richness (the number of species in a community) and species evenness (the distribution of levels of abundance among the species). Two types of analyses have been used to assess diversity. Rarefaction is a statistical technique for different applications in an ecological context and gives an estimation of the decrease in apparent species richness of a community with decreasing subsample size (50). A second approach to evaluate whether diversity within a subsample approaches diversity within a sample of infinite size is to calculate coverage (14). Coverage (C) values are calculated by the equation $C = 1 - (n/N) \times 100$, where n is the number of unique clones and N is the total number of clones examined.

We chose to study permanently cold sediments because 90% of the sea floor has temperatures below 4°C (25). During a cruise to the Arctic Ocean in September and October of 1995, several studies of different aspects of microbial life in this habitat, such as the determination of prokaryotic abundance and the profiling of prokaryotic rRNA (47, 48), were conducted. Temperature dependence was determined, rates of polysaccharide hydrolysis (2) and sulfate reduction were measured (46), and psychrophilic sulfate reducers were enriched (23). Furthermore, benthic exchange and mineralization rates were determined (12, 24). All the above-described studies indicated an active microbial community with metabolic rates comparable to those of temperate habitats. Forty-two percent of total benthic mineralization was due to sulfate reduction at the station sampled for the clone library (46). Here and in the accompanying paper (48), we describe the phylogenetic affiliation and diversity of the prokaryotic community and quantify the contribution of sulfate-reducing bacteria (SRB) to the total microbial community.

MATERIALS AND METHODS

Study site. Sediment samples were collected at Hornsund off the coast of Spitsbergen, Arctic Ocean, in September and October of 1995. The bottom water temperature was 2.6°C (47), and sediments were anoxic below a depth of approximately 8 mm (12). For a detailed description of the sampling procedure, see the report of Sahm and Berninger (47).

DNA extraction and purification. Total community DNA was extracted directly from the sediment as described by Zhou et al. (55). The protocol encompassed three cycles of freezing and thawing, chemical lysis in a high-salt extraction buffer (1.5 M NaCl) by heating of the suspension in the presence of sodium dodecyl sulfate (SDS) and hexadecyltrimethylammonium bromide, and a proteinase K step. It was slightly modified by performing only two SDS extraction steps.

Aliquots of 2 g of wet sediment of different sections (0 to 2, 3 to 6, and 8 to 11 cm) from duplicate cores were used for DNA extraction. Extracted DNAs were finally combined. The crude DNA was purified with the WIZARD DNA Clean Up System (Promega, Madison, Wis.). DNA yield was quantified photometrically. Per cubic centimeter of wet sediment, 11.5 μg of DNA was recovered. High-molecular-weight DNA was cut out of an agarose gel and extracted with a GeneClean II Kit (Bio 101 Inc., La Jolla, Calif.) by following the manufacturer's instructions. Approximately 40% of the crude DNA was recovered after this step.

Cell lysis efficiency. Cell lysis efficiency of the DNA extraction procedure was checked by enumerating the total number of 4′,6-diamidino-2-phenylindole

* Corresponding author. Present address: Biotechnology I, Technical Microbiology, Technical University Hamburg-Harburg, Denickestr. 15, D-21073 Hamburg, Germany. Phone: 49 (0)40 42878 3336. Fax: 49 (0)40 42878 2909. E-mail: ksahm@mpi-bremen.de.

TABLE 1. Oligonucleotide probes used in this study

Probe	Specificity	Sequence (5'→3')	Position[a]	T_d (°C)	Reference
EUB338	Bacteria	GCTGCCTCCCGTAGGAGT	338–355	57	1
ALF968	α subclass of the *Proteobacteria*, several members of the δ subclass of *Proteobacteria*, and most *Pelobacter-Geobacter* spp.	GGTAAGGTTCTGCGCGTT	968–985	58	35
687	*Desulfovibrio* and some species of *Geobacteriaceae*	TACGGATTTCACTCCT	687–702	48	5
660	*Desulfobulbus*	GAATTCCACTTTCCCCTCTG	660–679	56	5
804	*Desulfobacterium*, *Desulfobacter*, *Desulfobotulus*, *Desulfosarcina*, *Desulfococcus*	CAACGTTTACTGCGTGGA	804–821	52	5
Sval428	*Desulfotalea*, *Desulfofustis*	CCATCTGACAGGATTTTAC	428–446	56	48
GP	Most gram-positive bacteria	Unpublished		41	28
CF319a	*Cytophaga-Flavobacterium* cluster	TGGTCCGTGTCTCAGTAC	319–336	58	30
Gamma598	16S rDNA clone sequences affiliated with endosymbionts and some other species in the γ subclass of *Proteobacteria*	CGGATGTGAAAGCCCTGG	598–615	58	This study

[a] Position in the 16S rRNA of *E. coli*.

(DAPI)-stained cells in aliquots of sediments taken before and after cell lysis. Ninety-three percent ± 3.4% of the microorganisms were lysed.

PCR amplification of 16S rDNAs. Two universal bacterial primers, EUB008 (17) and EUB1492 (20), were used to amplify 16S rDNAs from the extracted and purified chromosomal DNAs. PCR was performed with a model PHC-3 Temperature Cycler (Techne, Cambridge, United Kingdom) as follows: 50 pmol of each primer, 2.5 μmol of each deoxyribonucleoside triphosphate, 300 μg of bovine serum albumin, 1× PCR buffer, and 1 U of Super *Taq* DNA polymerase (HT Biotechnology, Cambridge, United Kingdom) were adjusted to a final volume of 100 μl with sterile water. Template DNA (80 to 500 ng) was added to the reaction mixture (preheated to 70°C) to avoid nonspecific annealing of the primers to nontarget DNA. The cycles used were as follows: 1 cycle at 70°C for 1 min; 33 cycles at 95°C for 1 min, 40°C for 1 min, and 72°C for 3 min; and 1 final cycle at 95°C for 1 min, 40°C for 1 min, and 72°C for 10 min. The number of amplification cycles during PCR was reduced as much as possible to reduce PCR biases (52), chimera formations (53), and *Taq* polymerase error rates; however, 34 cycles were needed to yield sufficient product.

Clone library construction. Products of three parallel PCRs were combined and precipitated to concentrate the DNAs for cloning. DNA was ligated in the pGEM-T-Easy vector by using the protocol of the manufacturer (Promega). Ligation reaction mixtures were purified and used for electroporation of *Escherichia coli* XL1 Blue (Stratagene GmbH, Heidelberg, Germany) or *E. coli* JM-109 cells (Promega) as described by Flohr (8). Recombinant transformants were selected by blue and white screening.

Dot blot hybridization. Plasmid DNA was prepared from overnight cultures with a WIZARD Mini Prep Purification Kit (Promega) by following the manufacturer's recommendations. Plasmids were checked for insert presence on agarose gels. All plasmids known to contain the correctly sized insert of 1.5 kb were used for dot blot hybridization.

For blotting, plasmid DNA was denatured for 5 min at 95°C and cooled immediately on ice. Aliquots of 100 to 400 ng of DNA were spotted onto a prewetted nylon membrane (Hybond-N+; Amersham, Little Chalfont, Buckinghamshire, United Kingdom) with a Bio-Rad (Munich, Germany) dot blot apparatus. For additional denaturation, blots were placed on filter paper soaked with 0.4 M NaOH–0.6 M NaCl for 15 min. Finally, membranes were equilibrated with 2× SSC (0.3 M NaCl, 0.03 M sodium citrate [pH 7.0]) for 10 min. For immobilization of the DNA, the membrane was baked at 80°C for 2 h. Oligonucleotide probes were 5' end labeled with [γ-^{32}P]ATP by using T4 polynucleotide kinase according to the recommendation of the manufacturer (New England Biolabs, Schwalbach, Germany). The unincorporated [γ-^{32}P]ATP was removed from the labeled probes by using Sephadex columns (NAP columns; Pharmacia Biotech, Freiburg, Germany) according to the manufacturer's protocol.

Membranes were prehybridized for 1 h at 40°C in hybridization solution (10× Denhardt solution, 4× SSC, 0.1% SDS, 2 mM EDTA [pH 8.0], 50 μg of salmon sperm DNA per ml [32]) before ^{32}P-labeled probes were added. Hybridization was carried out at 40°C (except with membranes for hybridization with a gram-positive probe, for which the temperature was 30°C) for 14 to 16 h. Thereafter, the membranes were washed twice for 30 min with washing buffer (2× SSC, 0.1% SDS) at hybridization temperature. To eliminate nonspecific binding, the membranes were washed two more times for 15 min at the dissociation temperature (T_d), which had been determined according to the method of Raskin et al. (39). Probes and T_ds used in this study are given in Table 1. Control 16S rDNAs different in sequence from each particular probe by one nucleotide were also spotted on membranes and hybridized as well to check the stringency of washing conditions. Hybridization signals were analyzed with a PhosphorImager (Molecular Dynamics, Sunnyvale, Calif.).

ARDRA and rarefaction analysis. Amplified rDNA restriction analysis (ARDRA) was performed to analyze the diversity of clones within each group defined by dot blot hybridization. Isolated plasmid DNAs of 16S rDNA clones were used as templates for insert amplification. The PCR was performed as described above, except that the primer annealing temperature was higher (44°C). PCR products were purified, and aliquots of 200 to 400 ng of the amplified insert were digested with 7.5 U of the restriction endonuclease *Hae*III (Promega) for 3 h at 37°C. The resulting fragments were analyzed on an 8% polyacrylamide gel, and restriction patterns within each group were compared. Diversity of the clone library was further investigated by rarefaction analysis (16, 18, 50). Rarefaction curves were produced by using the analytical approximation algorithm of Hurlbert (18) and 95% confidence intervals estimated as described by Heck et al. (16). Calculations were performed on a personal computer with the freeware program aRarefactWin (17a).

Sequencing and phylogenetic analysis. Representatives of all major ARDRA pattern groups were chosen for sequencing. Plasmid DNAs from selected 16S rDNA clones were sequenced (partially or in full) by *Taq* Cycle Sequencing with universal rRNA-specific primers with a model ABI377 (Applied Biosystems, Inc.) or a Li-Cor (MWG Biotech, Ebersberg, Germany) sequencer. A total of 116 clones were sequenced partially or fully. All sequences were checked for chimera formation with the CHECK_CHIMERA software of the Ribosomal Database Project (29), and the phylogenetic affiliations of their 5' and 3' ends were compared. By this procedure seven potential chimeras (6.0%) were detected. This figure probably underestimates the real chimera fraction because it is more difficult to detect chimera formation of two closely related sequences (53). Potential chimeras were eliminated before phylogenetic trees were constructed.

Sequence data were analyzed with the ARB software package (51). Phylogenetic trees were calculated by parsimony, neighbor-joining, and maximum-likelihood analysis with different sets of filters. For tree reconstruction, only full-length sequences were considered.

Nucleotide sequence accession numbers. The nucleotide sequence data reported in this paper will appear in the EMBL, GenBank, and DDBJ nucleotide sequence databases under the accession no. AJ240966 to AJ241022. Only sequences of more than 1,000 bases in length were submitted.

RESULTS

Initial clone library analysis. A sample of 30 clones was initially selected for sequencing and phylogenetic analysis to get a first overview of the quality of and the diversity in the 16S

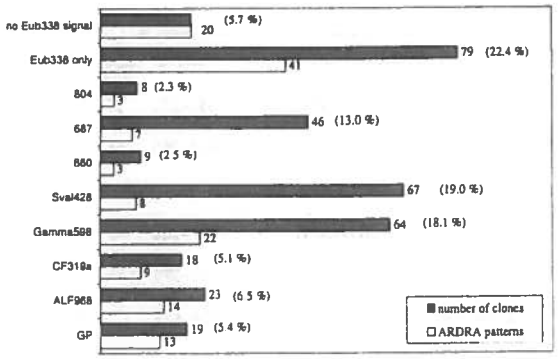

FIG. 1. Dot blot hybridization and ARDRA of 16S rDNA clones. Three hundred fifty-three clones were screened by dot blot hybridization with different probes. The diversity within each group was further investigated by ARDRA with one restriction endonuclease (*Hae*III). The filled bars represent the numbers of clones detected with specific probes, and the open bars show the numbers of different ARDRA patterns after digestion with *Hae*III. Probe GP is specific for gram-positive bacteria (28), ALF968 is specific for members of the α subclass of *Proteobacteria* (35), CF319 is specific for the *Cytophaga-Flavobacterium* group (30), Gamma598 targets three gene clusters affiliated with sulfur-oxidizing bacteria in the γ subclass of *Proteobacteria*, Sval428 is specific for psychrophilic sulfate reducers isolated from the same site (48), probe 660 targets *Desulfobulbus* species (5), 687 is specific for *Desulfovibrio* and some species of *Geobacteraceae* (5), and 804 targets *Desulfobacter*, *Desulfobacterium*, and *Desulfobotulus* species (5). "EUB338 only" indicates the clones which hybridized only with the universal eubacterial probe (1). No EUB338 signal describes clones with a correctly sized insert of 1.5 kb but no hybridization signal at all.

rDNA clone library. Within the 30 clones, we detected 21 different sequences. Two major groups became evident: approximately 50% of the 16S rDNA clone sequences were related to gram-negative SRB and other members of the δ subclass of the class *Proteobacteria*, and approximately 40% were affiliated with the γ subclass of *Proteobacteria*, most closely with sulfur-oxidizing bacteria. Additional sequences were related to *Cytophaga* spp. (one sequence) and gram-positive bacteria (two sequences).

Grouping of clones by dot blot hybridization and ARDRA. On the basis of the initial sequence analysis, we developed a new probe (Gamma598) and used this probe, in addition to others, for dot blot hybridization (for an overview of probes used, see Table 1).

Of the screened clones, 94.3% hybridized with the bacterial probe EUB338 and contained a 16S rDNA insert. The remaining 5.7% had no hybridization signal with EUB338 (Fig. 1), although all of them had a correctly sized insert of 1.5 kb. ARDRA of this group resulted in 20 different patterns, with each pattern being represented by a single clone (see Fig. 2J). Fourteen of these clones were sequenced, and 12 fell into the division *Planctomycetes-Verrucomicrobiales*. This result is in agreement with the work of Neef et al., who showed that *Planctomycetales* spp. have at least one nucleotide that is different from the sequence of probe EUB338 (36). The other clones were distantly related to low-G+C-content gram-positive and green nonsulfur bacteria. Of the EUB-positive clones, 71.9% bound one of the group-specific probes used.

δ-Proteobacteria. The most abundant group of clones was affiliated with the δ subclass of *Proteobacteria*. A total of 36.8% of the clones hybridized with different probes specific for SRB. The majority of these clones were targeted by probe Sval428 (Fig. 1). Sixty-seven clones (19.0%) hybridized with the probe specific for SRB first isolated from the same habitat. ARDRA of this fraction resulted in eight different restriction patterns, subsequently referred to as phylotypes (Fig. 2D). Sequence analysis revealed that the dominant phylotypes were almost all closely related (95 to 100%) to SRB isolates from the same cruise (strains with the prefix "LSv," *Desulfotalea* [23, 48]). The most abundant pattern was found in 27 clones (e.g., Sva1036 and Sva1037), their sequences showing 95% similarity to the 16S rDNA sequence of LSv20 and *Desulforhopalus vacuolatus* (Fig. 3). The sequences of the second-most-dominant pattern, represented by 17 clones, were affiliated with *Desulfotalea* sp. (Sva0999) and LSv23 or LSv53 (Sva0632). Twelve of these clones were identical to the 16S rDNA of LSv53, a psychrophilic SRB isolated by Knoblauch et al. (23) during the same cruise but from a different sampling station (Storfjord). A third dominant phylotype in the Sval428-positive fraction (12 clones, e.g., Sva0010) was phylogenetically related to *Desulfocapsa* sp. (93% similarity) (Fig. 3), while a fourth phylotype (6 clones, e.g., Sva0113) was affiliated with LSv23 or LSv53 (97% similarity). Remaining patterns were represented by only one or two clones.

The second-largest fraction (13.0%) of SRB-related 16S rDNA clone sequences was targeted by probe 687, specific for *Desulfovibrio* and some species of the *Geobacteraceae* (Fig. 1). Diversity in this group was very low (Fig. 2A). Of the seven different ARDRA patterns, one was represented by 39 clones and the remaining six patterns were each represented by one or two clones only. Phylogenetic analysis revealed that the major group (e.g., Sva1033 and Sva0566) was related to *Desulfuromonas* sp. (Fig. 3). The highest similarity was 93.7% (to *Desulfuromonas palmitatis*). Sequencing of clones representing the other patterns also placed them with *Desulfuromonas* sp.

Relatively few 16S rDNA clones were affiliated with *Desulfobulbus* sp. by probe 660 (2.5%) or with *Desulfobacter* sp., *Desulfobacterium* sp., or *Desulfobotulus* sp. by probe 804 (2.3%) (Fig. 1). The diversity in these two groups was also low (Fig. 2B and C). Only three different ARDRA patterns per group were found. The three different clusters that were detected with probe 660 were all, as expected, phylogenetically related to *Desulfobulbus* sp. (e.g., Sva0436 and Sva0631). Five of the clones targeted by probe 804 (e.g., Sva0081 and Sva0863) were affiliated with *Desulfosarcina* sp. (Fig. 3). The two other groups were represented by two clones (e.g., Sva0605) and one clone (Sva0405) only. Although clone Sva0605 hybridized with probe 804, its 16S rDNA sequence was most closely related to *Desulfobacula toluolica* (93.1% similarity).

γ-Proteobacteria. In addition to the SRB, there was a second dominant group in the clone library represented by 16S rDNA clone sequences which fell in the γ subclass of *Proteobacteria*. They were only distantly related to known bacteria (between 85.6 and 92.1%), being related most closely to sulfur-oxidizing bacteria. This group was detected by dot blot hybridization with probe Gamma598. Sixty-four clones (18.0%) hybridized with this new probe developed on the basis of preliminary screening of 30 clones (see above). After restriction endonuclease digestion, 22 different ARDRA patterns became evident (Fig. 1 and 2E). Phylogenetically, the clones formed three distinct clusters (Fig. 4). Clones Sva0071 and Sva0864 belonged to a cluster that was affiliated with sulfur-oxidizing endosymbiotic bacteria such as the gill symbionts *Solemya velum* (92.0%) and *Codakia costata* (92.1%). The second cluster, containing clones Sva1046, Sva0115, and Sva0120, was most closely related to other clone sequences published by Kato and Li (21), derived from deep-sea sediments (97.9% highest similarity). The third cluster (containing, e.g., Sva0091, Sva0854, and Sva0318) could not be assigned stably. Different tree reconstructions affiliated the sequences with sulfur-oxidizing en-

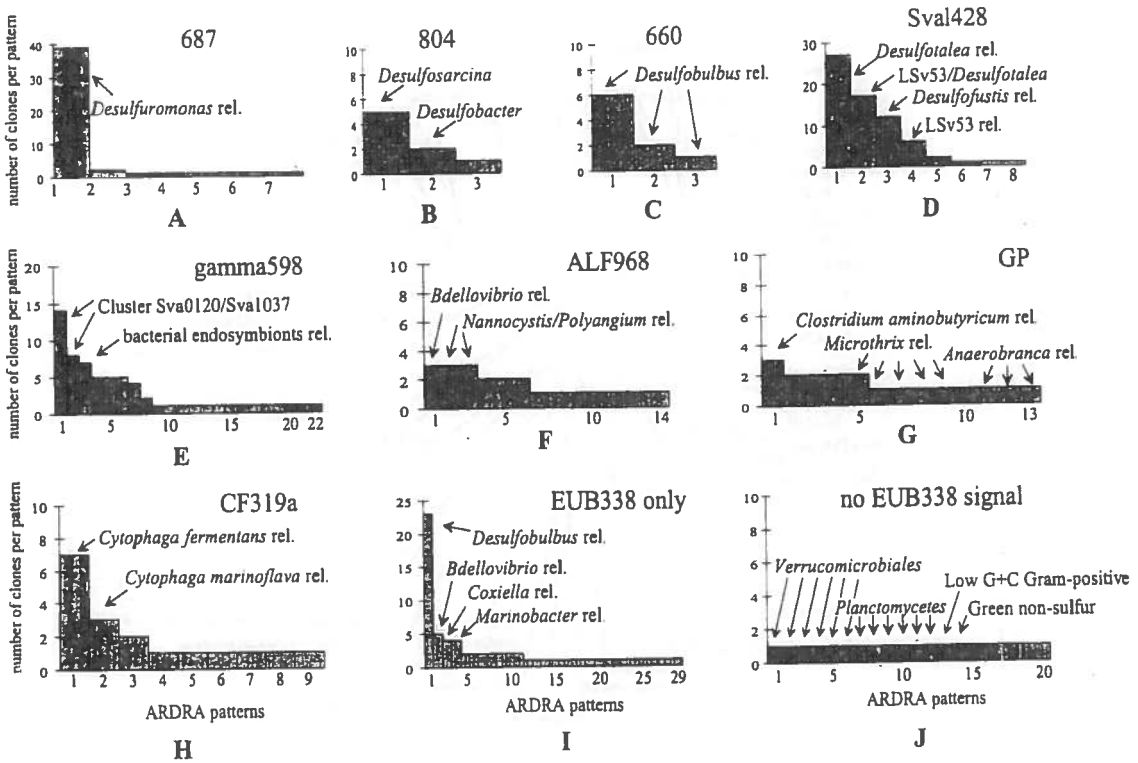

FIG. 2. Distribution of 16S rDNA clone sequences in different ARDRA patterns. The profiles are based on ARDRA and sequence analysis. The closest cultivated relatives (rel.) for the individual ARDRA groups are indicated.

dosymbionts, the *Beggiatoa-Thioploca* group, or with separate groups. To no members of the above-named groups did they show more than 89.5% 16S rDNA similarity. The phylogenetic position in Fig. 4 was consequently indicated by a multifurcation.

Other probe target groups. Probe ALF968 was designed to target the α subclass of *Proteobacteria*. This probe is known to also target some members of the δ subclass of *Proteobacteria* (35). In our study, 59 of the 82 clones hybridizing with probe ALF968 could be assigned to SRB by hybridization with probe Sval428 and sequencing. Consequently, we investigated only the diversity of the remaining 23 clones, which displayed 14 different ARDRA patterns (Fig. 1). The three most abundant patterns were each represented by three clones. The sequences were most closely related to *Bdellovibrio* and *Nannocystis* or *Polyangium* sp., i.e., genera of the δ subclass of *Proteobacteria* (Fig. 2F). The remaining patterns were represented by one or two clones only. Sequencing of 11 of 14 phylotypes hybridizing with ALF968 showed that only one phylotype, represented by two clones, was indeed affiliated with the α subclass of *Proteobacteria* (*Rhodobacter* spp.).

Probe GP, specific for gram-positive bacteria, hybridized with 19 clones (5.4%). Diversity within this group was very high since it contained 13 different patterns (Fig. 2G). The sequences were fairly distantly related to *Clostridium* sp. (89.8%), *Microthrix parvicella* (85.6%), and *Anaerobranca* sp. (86%).

Eighteen clones were assigned to the *Cytophaga-Flavobacterium* cluster by probe CF319a (Fig. 1). We found nine different phylotypes by ARDRA. The most abundant phylotype was represented by seven clones which were most similar (89.1%) to *Cytophaga fermentans* (Fig. 2H).

A large number of clones (37 clones) hybridized only with probe EUB338. We found 29 patterns in this fraction. Only four of these patterns were represented by more than two clones (Fig. 2I). The most dominant pattern (23 clones) was represented by sequences (e.g., Sva0103 and Sva1041) that were most closely related to *Desulfobulbus* sp. relatives (90.1%) (Fig. 3). These sequences had three nucleotide differences from probe 660, specific for *Desulfobulbus* sp., and were, therefore, not detected by this probe. Other phylotypes that were represented by more than two clones were related to *Bdellovibrio* sp. or *Nitrospina* sp. (82.7%; δ-*Proteobacteria*), *Coxiella* sp., and *Marinobacter* sp. (91.5 and 89.8%; both γ-*Proteobacteria*). The less frequent patterns had sequences related to the γ subclass of *Proteobacteria* (e.g., *Marinobacter*, *Methylophaga*, and *Coxiella* relatives), to the δ subclass of *Proteobacteria* (*Desulfobacula*, *Desulfosarcina*, *Nitrospina*, and *Bdellovibrio* relatives), and to the newly described phylum *Holophaga-Acidobacterium* (27).

Rarefaction analysis. We applied rarefaction analysis to evaluate whether screening of 353 clones was sufficient to estimate diversity within the clone library. The expected number of different ARDRA patterns was plotted versus the number of 16S rDNA clones in the clone library. ARDRA of 353 clones resulted in 140 different patterns. The calculated rarefaction curves did not reach a clear saturation, indicating that

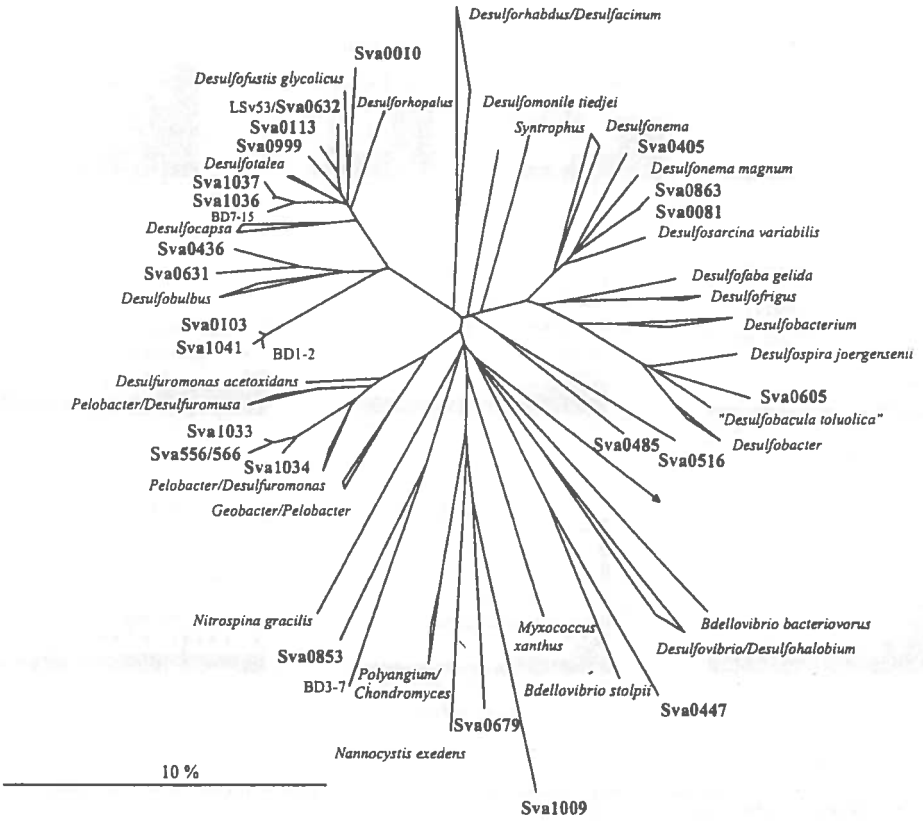

FIG. 3. Phylogenetic tree showing the affiliations of 16S rDNA clone sequences to selected reference sequences of the δ subclass of *Proteobacteria*. The tree was calculated by neighbor-joining analysis and corrected with filters which considered only 50% conserved regions of the 16S rRNA of δ-*Proteobacteria*. 16S rDNA clone sequences are in boldface type. The bar represents 10% estimated sequence divergence.

analysis of an increasing number of clones would have revealed further diversity (Fig. 5).

We did the same rarefaction analysis with the fraction of clones representing 16S rDNAs of SRB (including *Desulfobulbus* relatives detected by EUB338 only). Twenty-two different ARDRA patterns were represented by 155 clones. The calculated rarefaction curve approached saturation, indicating that the diversity of SRB in the clone library was almost covered.

DISCUSSION

Diversity. The sediments investigated in this study are never exposed to temperatures higher than 3°C and might, therefore, be regarded as extreme environments. Studies of the bacterial communities of extreme environments such as a saltern (31) and a low-pH hydrothermal vent system (33) have indicated low bacterial diversity in these habitats. By applying rarefaction analysis to restriction fragment length polymorphism patterns of their 16S rDNA clone library, Moyer et al. (33) could demonstrate that screening of 48 bacterial clones was enough to detect the majority of taxa in the clone library of a hydrothermal vent microbial mat. One aim of our study was to assess bacterial diversity, which was expected to be limited considering the extremely low environmental temperatures; however, rarefaction analysis revealed that by screening 353 clones, the actual diversity in our clone library was only partially covered. It is unlikely that new major groups will be discovered by analyzing additional clone sequences, since the major groups were the same after 30 and 353 clones were screened. Total phylotype richness, i.e., the number of phylotypes present, on the other hand, might reflect the potential within a microbial community to respond to changes in environmental conditions. At a different time point, those phylotypes not detected or represented by only one clone might play an important role in this habitat.

Another approach that has been used to assess completeness of a clone library analysis is to calculate coverage. In our case, coverage was 71.95%, indicating that almost three-quarters of total diversity in the clone library was detected; however, since coverage is based only on the number of unique clones relative to total richness, not taking evenness into account, it should be regarded only as a rough estimate of diversity within a sample of infinite size.

The 16S rDNA inserts of the clones were digested with one tetrameric restriction enzyme. Use of a second enzyme resulted in an increased number of patterns (data not shown). In a study of sulfate-reducing isolates, Rooney-Varga et al. (44) demonstrated that use of four enzymes was necessary to differentiate between sequences having more than 95% similarity. However, since our study was aimed towards an overview of diversity, we concentrated on the differences revealed by

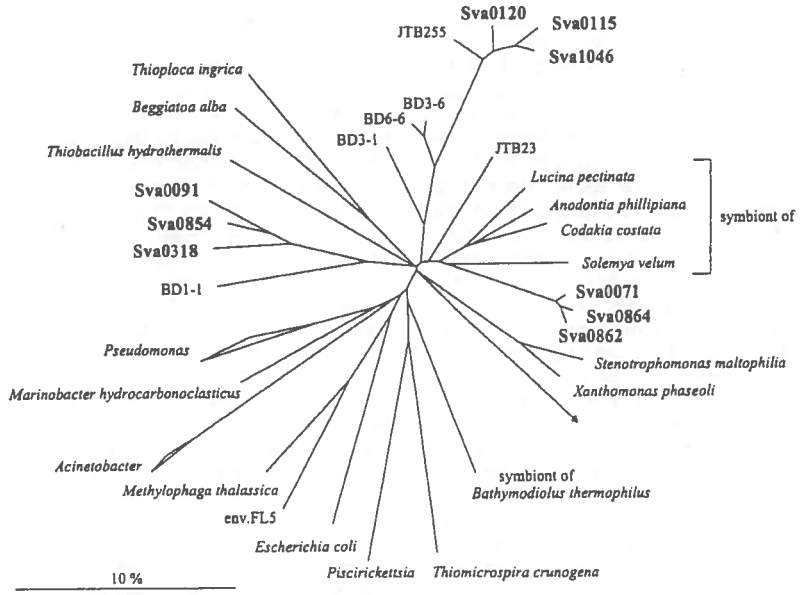

FIG. 4. Phylogenetic tree showing the affiliations of 16S rDNA clone sequences with selected reference sequences of the γ subclass of *Proteobacteria*. The tree was calculated by neighbor-joining analysis and corrected with filters which considered only 50% conserved regions of the 16S rRNAs of γ-*Proteobacteria*. Sva0862 and Sva0854 are not full-length sequences (1,000 bp) and have therefore been added to the existing tree, by a special algorithm included in the ARB software, without allowing for changes of the tree topology based on almost complete sequences. 16S rDNA clone sequences are in boldface type. The bar represents 10% estimated sequence divergence.

one enzyme. The results presented here should therefore be regarded as indicating minimal diversity.

As can be seen in Fig. 2, diversity within each probe target group varied greatly. In particular, group 687 showed very little evenness (distribution of the number of clones per pattern), with one phylotype making up 85% of the 687 positive clones; however, since different probe target groups represent different phylogenetic depths (probe 660, e.g., is specific for one genus and probe 804 is specific for a group of different genera), we refrain from comparing levels of diversity among the different groups. The data might, however, serve as a basis in future analyses for comparing levels of diversity of the same target group in different environmental samples.

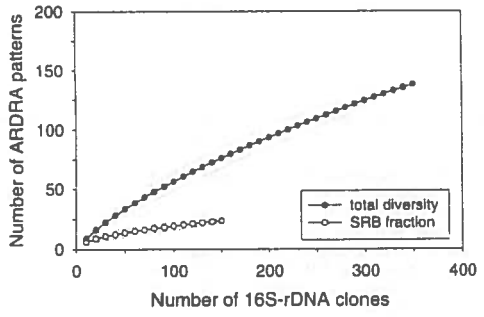

FIG. 5. Rarefaction curves for the different ARDRA patterns of 16S rDNA clones. Rarefaction curves were calculated by using the analytical approximation algorithm described by Hurlbert (18) and 95% confidence intervals estimated as described by Heck et al. (16). The number of different ARDRA patterns in the clone library was determined after digestion with one restriction endonuclease. The expected number of ARDRA patterns (●) is plotted versus the number of clones. Rarefaction curves were also calculated for the fraction of SRB (○). The dotted lines represent 95% confidence intervals.

Methodological considerations. Clone libraries of 16S rDNAs have been widely used to investigate in a cultivation-independent approach the microbial communities of different, mainly pelagic or terrestrial habitats (4, 7, 9, 11, 33, 37, 38, 49). They have helped to elucidate common features within the microbial communities of specific habitats such as marine pelagic environments (9, 38) and have provided additional sequence information for the design and evaluation of probes. However, this experimental approach suffers from specific limitations that potentially confer selectivity via differential cell lysis, variable nucleic acid extraction efficiencies, or biased amplification in the PCR. The high lysis efficiency (93% ± 3.4%) and the high overall diversity in the clone library presented here suggest that our analysis was based on a substantial fraction of the bacterial community from Hornsund sediments; however, it is difficult to assess the potential bias introduced during amplification of the 16S rDNA. These biases are due to primer selectivity or erroneous product ratios caused by product saturation in the later cycles of amplification (52). Furthermore, oligonucleotides specific for a very general phylogenetic group, such as the bacterial 16S rDNA primers we used in PCR, are ultimately bound to miss some members of the community, which, in turn, leads to an underestimation of diversity.

Despite the caveats that clone abundance in the library does not necessarily reflect bacterial abundance at the site and that diversity might not be fully covered, the correlations between results of the clone library and results of completely different approaches such as 16S rRNA quantification analysis (see the accompanying paper [48]) and most probable number counts (23) are encouraging. The largest group of clones (19%) was detected by a probe designed especially for SRB isolated on

the same cruise (23). While some of these isolates came from the same sampling site on the west coast of Spitsbergen (Hornsund), others were obtained from sediments sampled off the east coast (Storfjord). The clones were closely related to these isolates, with one phylotype even showing 100% sequence identity to strain LSv53, which was isolated from the east coast station (Fig. 3). This phylotype was represented by 12 of 353 clones. The same phylotype was also detected in a denaturing gradient gel electrophoresis-Southern blot analysis described in the accompanying paper; however, as expected, quantitative representation of the phylotypes in the clone library corresponds only weakly to the results from 16S rRNA slot blot hybridization (see the accompanying paper [48]).

Phylogenetic composition of the clone library. The clone library was dominated by sequences related to δ-*Proteobacteria*. Even within the clones targeted by the general EUB338 probe only, we could detect one additional phylotype affiliated with the δ subclass of *Proteobacteria*, loosely related to *Desulfobulbus*. Twenty-three clones (6.5%) belonged to this phylotype not targeted by any of the specific SRB probes. Phylogenetic affiliation makes it likely that they are also sulfate reducers. The design and application of a new probe specific for this group and its employment in quantitative rRNA slot blot and in situ hybridizations will show the extent to which it contributes to the bacterial community.

Detailed analysis of the clones targeted by probe 687 showed that all 16S rDNA inserts were affiliated with *Desulfuromonas palmitatis*, whereas no *Desulfovibrio* was detected. *Desulfuromonas palmitatis* is known to reduce sulfur or thiosulfate and iron or to employ a fermentative metabolism; however, the phylogenetic distance between *Desulfuromonas* and the clones is so large (6.3%) that we cannot determine whether these clones represent sulfur or sulfate reducers. All clones had one nucleotide that was different from the probe sequence but gave a clearly positive signal in dot blot hybridization. A one-mismatch control also included in the hybridization analysis did show a distinguishable weaker signal. This example serves as a reminder that discrimination by one nucleotide might not always be possible.

A second dominant group of 16S rDNA clones was distantly related to sulfur-oxidizing symbiotic or free-living bacteria of the γ subclass of *Proteobacteria*, with a similarity value of 92 or 86%. Since no pure culture representatives for this group have been isolated, we can only speculate that they might indeed be involved in the oxidative part of the sulfur cycle. Selective cultivation of sulfur oxidizers from the same habitat is under way.

When investigating Wadden Sea sediments by fluorescence in situ hybridization, Llobet-Brossa et al. (26) found members of the *Cytophaga-Flavobacterium* cluster to be even more abundant than δ-*Proteobacteria*. This cluster has also been found in marine aggregates (4, 40); *Cytophagales*, in general, are known for their ability to associate and glide on surfaces and to degrade a wide variety of polymeric substances (42). They were also a significant constituent of our clone library (5.1%), indicating that *Cytophagales* might be a common member of marine sediment microbial communities.

Since sedimentation regularly brings in organic matter from the water column, we expected to find evidence of allochthonous input in the sediment. Groups that are commonly found in planktonic communities, like some genera of α-*Proteobacteria* (13, 34), were not abundant in the clone library; only 2 of 353 clones belonged to the α-*Proteobacteria*. Furthermore, we did not detect any cyanobacterial sequence and detected only one plastid sequence; however, the presence of allochthonous microorganisms is probably dependent on the time of sampling, with higher abundances expected after a phytoplankton bloom.

Comparison with other clone libraries. Open ocean and coastal planktonic communities are well-studied ecosystems with regard to clone libraries (10, 11, 34, 38, 49). Although the bacterial communities of these habitats are phylogenetically diverse, distinct phylogenetic clusters are repeatedly detected. These results are in line with the idea that in similar climate zones, a limited number of phylotypes account for a substantial fraction of the bacterioplankton at certain times (34). It is still an open question whether the same applies to benthic environments, since only limited data are available on marine sediments. Devereux and Mundfrom (6) established a clone library from a sandy marine sediment, selectively amplifying partial 16S rDNAs of SRB. Gray and Herwig (15) set up a general 16S rDNA clone library, examining 22 clones. Kato and Li (21) investigated clones from deep-sea sediments off Japan. A comparison is difficult, in particular because in many cases only partial sequences are available, but some trends are noteworthy. Sequences related to the *Desulfotalea-Desulforhopalus* cluster were frequently recovered (Fig. 3). The highest similarity values among clones with almost complete sequences were between 99.4 and 97.2%. Furthermore, all these clone libraries also contained sequences related to *Myxobacteria* and *Bdellovibrio* (Fig. 3; see below). *Myxobacteria* have been known mainly as terrestrial organisms (41); their isolation from coastal marine sediments has been attributed to resting cells because of their low salt tolerance. *Bdellovibrio*, on the other hand, has been repeatedly isolated from marine sediments (45). Considering the fact that related known pure cultures are almost all micropredators (41), they might play a role in the control of bacterial abundance. Within the sequences of the γ-*Proteobacteria*, similar congruencies occurred. Both Kato and Li (21) and Gray and Herwig (15) found sequences from the symbiont cluster (Fig. 4) (highest similarities among clones, between 98 and 92%).

More data on the prokaryotic diversity of marine benthic habitats are needed to identify common benthic features. Furthermore, the actual abundance of these conspicuous groups has to be determined via in situ and rRNA slot blot hybridization to evaluate their roles in the bacterial community of marine sediments.

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Community Structure, Cellular rRNA Content, and Activity of Sulfate-Reducing Bacteria in Marine Arctic Sediments

Katrin Ravenschlag, Kerstin Sahm, Christian Knoblauch,
Bo Barker Jørgensen, and Rudolf Amann

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Community Structure, Cellular rRNA Content, and Activity of Sulfate-Reducing Bacteria in Marine Arctic Sediments

KATRIN RAVENSCHLAG,[1] KERSTIN SAHM,[1]* CHRISTIAN KNOBLAUCH,[2] BO B. JØRGENSEN,[2] AND RUDOLF AMANN[1]

Molecular Ecology Group[1] and Department of Biogeochemistry,[2] Max Planck Institute for Marine Microbiology, 28359 Bremen, Germany

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The community structure of sulfate-reducing bacteria (SRB) of a marine Arctic sediment (Smeerenburgfjorden, Svalbard) was characterized by both fluorescence in situ hybridization (FISH) and rRNA slot blot hybridization by using group- and genus-specific 16S rRNA-targeted oligonucleotide probes. The SRB community was dominated by members of the *Desulfosarcina-Desulfococcus* group. This group accounted for up to 73% of the SRB detected and up to 70% of the SRB rRNA detected. The predominance was shown to be a common feature for different stations along the coast of Svalbard. In a top-to-bottom approach we aimed to further resolve the composition of this large group of SRB by using probes for cultivated genera. While this approach failed, directed cloning of probe-targeted genes encoding 16S rRNA was successful and resulted in sequences which were all affiliated with the *Desulfosarcina-Desulfococcus* group. A group of clone sequences (group SVAL1) most closely related to *Desulfosarcina variabilis* (91.2% sequence similarity) was dominant and was shown to be most abundant in situ, accounting for up to 54.8% of the total SRB detected. A comparison of the two methods used for quantification showed that FISH and rRNA slot blot hybridization gave comparable results. Furthermore, a combination of the two methods allowed us to calculate specific cellular rRNA contents with respect to localization in the sediment profile. The rRNA contents of *Desulfosarcina-Desulfococcus* cells were highest in the first 5 mm of the sediment (0.9 and 1.4 fg, respectively) and decreased steeply with depth, indicating that maximal metabolic activity occurred close to the surface. Based on SRB cell numbers, cellular sulfate reduction rates were calculated. The rates were highest in the surface layer (0.14 fmol cell^{-1} day^{-1}), decreased by a factor of 3 within the first 2 cm, and were relatively constant in deeper layers.

Marine sediments play a significant role in the global cycling of carbon and nutrients. Organic matter from primary production settles to the sea floor, where a major part is remineralized by microorganisms that colonize the sediments (50). Steep redox potentials provide niches for a wide variety of metabolically diverse microorganisms. Sulfate reduction is the major bacterial process in marine sediments, accounting for up to 50% of the total organic carbon remineralization (3, 17). The sulfate-reducing bacteria (SRB) make up a complex physiological group of organisms that can use a variety of volatile or long-chain fatty acids, alcohols, or aromatic compounds as carbon and energy sources but cannot use polysaccharides or other polymeric substrates. Some SRB are not completely dependent on sulfate; they can also use alternative electron acceptors, such as Fe(III) (4, 24) and nitrate (49), can disproportionate inorganic sulfur compounds (15, 21), or can grow under fermentative conditions (48). Although some SRB have been shown to survive in the presence of oxygen, no growth has been observed under these conditions (5, 9, 22). SRB, therefore, must combine two divergent needs for survival and growth in the sediment. While an input of organic matter is usually provided by sedimentation from the water column, the optimal redox conditions are most likely deeper in the sediment. Studying the occurrence and distribution of SRB along the depth profile combined with measuring metabolic activity can help elucidate the way that SRB cope with these specific challenges.

* Corresponding author. Present address: TU Hamburg-Harburg, Technical Microbiology, Denickestr. 15, 21071 Hamburg, Germany. Phone: 49-40-428783964. Fax: 49-40-428782909. E-mail: sahm@tu-harburg.de.

The study described here was part of an ongoing research project to investigate microbial communities and microbial physiology at permanently low temperatures. During a previous cruise to Svalbard (Arctic Ocean) in 1995, several studies were performed, including sulfate reduction measurements (35), isolation of psychrophilic sulfate reducers (20), determination of prokaryotic abundance, and vertical profiling of SRB rRNA with selected oligonucleotide probes (37). The SRB community was shown to be highly diverse in terms of species richness as determined by cloning of genes encoding 16S rRNA (rDNA) (32).

With the valuable set of data obtained previously in hand, we went back to Svalbard in 1998 and 1999 with several open questions. Could we back up the relevance of psychrophilic sulfate-reducing isolates and prominent clone groups from the previous cruise? And what was the physiological status of these groups with respect to localization along the sediment profile? To answer these questions, we combined fluorescence in situ hybridization (FISH) and rRNA slot blot hybridization to study SRB obtained from Smeerenburgfjorden. To do this, we used previously described and newly developed oligonucleotide probes for different groups of SRB belonging to the delta subgroup of *Proteobacteria*.

Despite their power, both FISH and rRNA slot blot hybridization have their limitations (for a review see reference 2). FISH may fail due to a low cellular rRNA content of the target organisms, autofluorescence of samples, impermeability of cell walls, and limited accessibility of probe target sites (12). Quantitative slot blot hybridization targets an rRNA pool that depends on both the number of target cells and the rRNA content per cell. Cell numbers cannot be directly inferred from the data, cell morphology and exact localization remain unclear,

TABLE 1. Oligonucleotide probes used in this study

Probe	Specificity	Sequence (5′→3′)	Position[a]	FISH FA concn (%, vol/vol)[b]	Slot blot T_d (°C)[c]	Reference
Uni1390	Universal (all organisms)	GACGGGCGGTGTGTACAA	1390–1407		44[d]	51
EUB338	*Bacteria*	GCTGCCTCCCGTAGGAGT	338–355	10	54[d]	1
NON338		ACTCCTACGGGAGGCAGC	338–355	10		47
DSR651	*Desulforhopalus* spp.	CCCCCTCCAGTACTCAAG	651–668	35	62	25
DSS658	*Desulfosarcina* spp., *Desulfofaba* sp., *Desulfococcus* spp., *Desulfofrigus* spp.	TCCACTTCCCTCTCCCAT	658–685	60	58	25
DSV698	*Desulfovibrio* spp.	GTTCCTCCAGATATCTACGG	698–717	35	58	25
DSV214	*Desulfomicrobium* spp.	CATCCTCGGACGAATGC	214–230	10		25
DSV407	*Desulfovibrio* spp.	CCGAAGGCCTTCTTCCCT	407–424	50		25
DSV1292	*Desulfovibrio* spp.	CAATCCGGACTGGGACGC	1292–1309	35		25
DSD131	*Desulfovibrio* sp.	CCCGATCGTCTGGGCAGG	131–148	20		25
DSMA488	*Desulfarculus* sp., *Desulfomonile* sp., *Syntrophus* spp.	GCCGGTGCTTCCTTTGGCGG	488–507	60		25
Sval428	*Desulfotalea* spp., *Desulfofusiis* sp.	CCATCTGACAGGATTTTAC	428–446	25	52[d]	37
660	*Desulfobulbus* spp.	GAATTCCACTTTCCCCTCTG	660–679	60	59[d]	6
221	*Desulfobacterium* spp.	TGCGCGGACTCATCTTCAAA	221–240	35	57[d]	6
DSB985	*Desulfobacter* spp., *Desulfobacula* spp.	CACAGGATGTCAAACCCAG	985–1003	20	53	25
DRM432	*Desulfuromonas* spp., *Pelobacter* spp.	CTTCCCCTCTGACAGAGC	432–449	40	62	This study
DSF672	*Desulfofrigus* spp., *Desulfofaba* sp.	CCTCTACACCTGGAATTCC	672–690	45		This study
DSC193	*Desulfosarcina* spp.	AGGCCACCCTTGATCCAA	193–210	35		This study
DCC209	*Desulfococcus* spp.	CCCAAACGGTAGCTTCCT	209–226	25		This study
DSS225	Svalbard clone group SVAL1	TGGTACGCGGGCTCATCT	225–242	40		This study
cl81-644	Svalbard clones Sva0081 and Sva0863	CCCATACTCAAGTCCCTT	644–661	25		This study

[a] Position in the 16S rRNA of *E. coli*.
[b] Formamide (FA) concentration in the hybridization buffer.
[c] T_d, dissociation temperature.
[d] Washing buffer containing 1× SSC, and 1% SDS was used.

and rRNA recovery may be influenced by species-dependent differences in the efficiency of cell lysis. In this study we used both methods for quantification of SRB for two reasons. First, many studies have been conducted with one of these two methods (8, 13, 23, 29, 31, 38, 39), but it is still not clear to what degree the limitations of the methods influence the comparability of the data. Second, in order to better understand an organism's role in a given ecosystem, it is important not only to determine the composition of the microbial community but also to combine this information with a measure of the metabolic status. A first step is the calculation of specific rRNA contents for individual groups, which correlates with growth rates under certain circumstances (for a review see reference 26).

MATERIALS AND METHODS

Study site and sampling. Sediment samples were collected on 28 July 1998 from Smeerenburgfjorden, Svalbard, Arctic Ocean (79°42′815″N, 11°05′189″E, station J). The sediment temperature was 0°C, the surface water temperature was 5°C, and the water depth was 218 m. Sediment samples were obtained with a Haps corer, subsampled, and kept at the in situ temperature during transport. Two parallel cores were sliced. One-half of each slice was frozen in liquid nitrogen for RNA extraction (stored at −80°C), and the other half was fixed for 2 to 3 h with 3% (final concentration) formaldehyde, washed twice with 1× phosphate-buffered saline (PBS) (10 mM sodium phosphate [pH 7.2], 130 mM NaCl), and then stored in 1× PBS–ethanol (1:1) at −20°C. The sediment was characterized by a soft brown silty oxidized surface (upper 2 cm) overlaying a transition zone consisting of darker, black-streaked clayey mud. Below the transition zone (2 to 6 cm) there was a black sulfidic zone. Worm tubes were present in the sediment, as were small shells (diameter, 2 to 3 mm) to depths below 10 cm. In addition to the samples obtained at the main study site at Smeerenburgfjorden, sediment samples were collected at the following three stations off the coast of Svalbard: Magdalenefjorden (station I; 79°34′052″N, 11°03′59″E; depth, 125 m; temperature, −0.5°C; samples collected on 28 July 1998), Raudfjorden (station K; 79°46′150″N, 12°04.375″E; depth, 154 m; temperature, −1°C; samples collected on 29 July 1998), and Hornsund (76°59′415″N, 15°53′517″E; depth, 176 m; temperature, 1.0°C; samples collected on 16 July 1999).

RNA extraction and slot blot hybridization. RNA was extracted from 1.5 ml of wet sediment (per layer) by bead beating, phenol extraction, and isopropanol precipitation as described previously (36). The quality of the RNA was checked by polyacrylamide gel electrophoresis. Approximately 50 ng of RNA was blotted onto nylon membranes (Magna Charge; Micron Separations, Westborough, Mass.) in triplicate and hybridized with radioactively labeled oligonucleotide probes as described by Stahl et al. (42). The membranes were washed at different temperatures depending on the dissociation temperature of the probe. The probes used and their dissociation temperatures are shown in Table 1. The dissociation temperatures of the probes were determined as described by Raskin et al. (30), with slight modifications. For dissociation temperature determinations and hybridizations we used washing buffer with a lower sodium dodecyl sulfate (SDS) concentration (1× SSC [150 mM NaCl, 15 mM sodium citrate; pH 7.0]–0.1% SDS). However, for hybridizations with probes Uni1390, EUB338, Sval428, 660, and 221 we used washing buffer containing 1% SDS.

Quantification. Hybridization signal intensity was measured with a PhosphorImager (Molecular Dynamics, Sunnyvale, Calif.) and was quantified as described previously (38). Reference rRNAs isolated from pure cultures of strain LSv23 (= DSM 13040) (19), strain LSv22 (= DSM 13039) (19), *Desulfococcus multivorans* DSM 2059, *Desulfobulbus elongatus* DSM 2908, *Desulfobacterium vacuolatum* DSM 3385, *Desulfovibrio salexigens* DSM2638, *Desulfobacter latus* DSM 3381, *Desulfuromonas acetexigens* DSM 1397, and *Escherichia coli* (purchased from Boehringer, Mannheim, Germany) were used as standards for hybridization with the probes shown in Table 1.

FISH. Samples stored in PBS-ethanol were diluted and treated by mild sonication with a type MS73 probe (Sonopuls HD70; Bandelin, Berlin, Germany) at a setting of 20 s, an amplitude of 42 μm, and <10 W. A 10-μl aliquot of a 1:40 dilution was filtered onto a 0.2-μm-pore-size type GTTP polycarbonate filter (Millipore, Eschborn, Germany). Hybridization and microscopic counting of hybridized and 4′,6′-diamidino-2-phenylindole (DAPI)-stained cells were performed as described previously (39). Means were calculated by using 10 to 20 randomly chosen fields for each filter section, which corresponded to 800 to 1,000 DAPI-stained cells. Counting results were always corrected by subtracting signals observed with probe NON338. The formamide concentrations used are shown in Table 1.

Oligonucleotides. Oligonucleotides were purchased from Interactiva (Ulm, Germany). For FISH, oligonucleotide probes were synthesized with Cy3 fluorochrome at the 5′ end.

Quantification of cell fluorescence. To verify the calculated trend observed for the cellular rRNA content of the *Desulfosarcina-Desulfococcus* group (probe DSS658) along the sediment profile with an independent method, we quantified

TABLE 2. Quantification of SRB in Smeerenburgfjorden sediments by FISH and rRNA slot blot hybridization

Depth (cm)	Absolute cell no. (10^9) ml^{-1}	*Bacteria* (probe EUB338): % DAPI	Concn of prokaryotic RNA (EUB338 + Arch915) (ng ml^{-1})	*Desulfovibrio* spp. (DSV698)			*Desulforhopalus* spp. (DSR651)		
				% DAPI	RNA concn (ng ml^{-1})	% of prokaryotic RNA	% DAPI	RNA concn (ng ml^{-1})	% of prokaryotic RNA
0.25	2.9/2.1[a]	57.9/72.9	9,865/8,977	0.9/0.8	220/338	2.2/3.8	1.1/0.9	257/207	2.6/2.3
0.75	3.5/3.3	55.9/73.6	10,036/13,846	1.1/1.6	264/521	2.6/3.8	1.5/1.1	146/547	1.5/4.0
1.25	3.1/3.4	56.1/65.4	7,321/10,597	0/0.8	178/349	2.4/3.3	2.1/1.6	284/571	3.9/5.4
1.75	4.2/4.2	53.1/57.7	6,879/8,325	0.6/0	152/266	2.2/3.2	2.3/1.3	195/202	2.8/2.4
2.25	4.1/3.5	57.5/48.4	7,161/9,605	0.4/0	171/329	2.4/3.4	2.2/1.5	91/502	1.3/5.2
2.75	3.7/3.3	47.9/51.2	5,037/5,866	0	119/176	2.4/3.0	3.2/2.3	94/96	1.9/1.6
3.25	2.7/3.2	43.7/46.6	4,724/6,417	0	118/177	2.5/2.8	3.0/2.4	138/129	3.0/2.0
3.75	3.1/3.7	42.8/50.0	5,212/6,671	0	128/206	2.5/3.1	2.9/2.3	145/151	2.8/2.3
4.25	3.5/3.4	43.5/47.3	3,613/5,070	0	94/133	2.6/2.6	2.7/1.7	86/86	2.4/1.7
4.75	3.4/3.6	28.4/42.2	3,605/4,427	0	107/113	3.0/2.5	2.7/1.7	132/75	3.7/1.7
5.5	3.5/4.6	26.0/36.0	2,616/4,046	0	77/115	3.0/2.9	1.8/1.8	48/120	1.8/3.0
6.5	3.7/4.7	24.1/27.4	2,984/4,494	0	90/117	3.0/2.6	1.9/1.3	67/91	2.2/2.0
7.5	4.7/3.9	23.6/23.6	2,662/3,520	0	77/98	2.9/2.8	1.5/0.8	65/69	2.5/2.0
8.5	2.9/3.1	28.9/22.6	2,869/3,246	0	82/92	2.9/2.8	1.5/1.3	76/78	2.6/2.4
9.5	3.7/3.9	22/15.7	2,308/2,898	0	64/83	2.8/2.8	1.6/1.2	89/58	3.9/2.0

[a] Core A value/core B value.

the mean FISH fluorescence at the single-cell level by confocal laser scanning microscopy. To ensure that the hybridization conditions were the same, sediment samples were hybridized on filters in 20-ml scintillation vials containing 1 ml of hybridization buffer with 1.25 ng of probe ml^{-1} as described above. The hybridized filters were mounted immediately before microscopy and were analyzed with a confocal laser scanning microscope (Zeiss model LSM510) by using the following settings: pinhole diameter, 176 μm; optical slice thickness, <0.9 μm; and detector gain 822 with an HeNe laser (excitation wavelength, 543 nm; 0.5 mW) and an argon laser (excitation wavelength, 514 nm; 12 mW). Pictures of 20 to 30 randomly selected fields containing a total of approximately 60 probe-targeted cells were used for quantification with the MetaMorph software (version 3.51; Universal Imaging Corp., West Chester, Pa.). Cells were selected manually to determine average cellular gray values and to quantify fluorescence. A mean cell fluorescence value was calculated for each depth; the lowest mean cell fluorescence value was defined as 1, and the mean fluorescence value for cells in each of the other layers was expressed relative to this value.

DNA extraction, PCR amplification, and clone library construction. Total community DNA was directly extracted from the sediment (Smeerenburgfjorden, station J) according to Zhou et al. (52), with slight modifications as described previously (32). Aliquots (1.5 g) of wet sediment from different sections (depth, 2.25 to 3.75 cm) were used for DNA extraction. Extracted DNAs were then combined. The crude DNA was purified by dialysis. Sterile water (1 ml) was added to a six-well microtiter plate, and a 0.025-μm-pore-size nitrocellulose membrane (Millipore) was placed on the water surface. Approximately 30 μl of the crude DNA was dropped onto the membrane and incubated for 3 h at room temperature, and the purified DNA was removed with a pipette. The volume increased during incubation to roughly 400 μl (a 10- to 15-fold increase).

DNAs that were targeted by probe DSS658 were amplified by a specific PCR. One universal bacterial primer, EUB008 (14), and probe DSS658, as a specific second primer, were used for specific amplification of the target 16S rDNAs from the chromosomal DNA pool. A PCR was performed with a Mastercycler Gradient (Eppendorf, Hamburg, Germany) as follows. A mixture containing 50 pmol of each primer, 2.5 μmol of each deoxyribonucleoside triphosphate, 300 μg of bovine serum albumin, 1× reaction buffer, 1× TaqMaster PCR enhancer, and 1 U of MasterTaq DNA polymerase (Eppendorf) was adjusted to a final volume of 100 μl with sterile water. Template DNA was added to the reaction mixture (preheated to 70°C) to avoid nonspecific annealing of the primers to nontarget DNA. The following cycling conditions were used: one cycle at 70°C for 1 min; 38 cycles at 95°C for 1 min, 52°C for 1 min, and 72°C for 3 min; and one cycle at 72°C for 10 min. The annealing temperature was optimized with a temperature gradient in order to use the highest stringency possible. Control DNAs with one, two, or three mismatches with primer DSS658 were used to determine the stringency of amplification. DNA with more than one mismatch could be discriminated completely, but it was not possible to discriminate DNA with only one mismatch without losing the PCR product of the target DNA. The PCR products were cloned in the vector pGEM-T (Promega, Madison, Wis.), and a clone library was constructed as described previously (32). Forty clones were selected for further analysis. Amplified rDNA restriction analysis (ARDRA) was performed in order to identify clones with different inserts. Digestion with two restriction enzymes (*Hae*III and *Rsa*I; Promega) was used to screen the clones as described previously (32).

Sequencing and phylogenetic analysis. Representatives of most ARDRA pattern groups were used for sequencing. PCR products obtained from selected 16S rDNA clones were sequenced by *Taq* cycle sequencing performed with vector primers and a model ABI377 sequencer (Applied Biosystems, Inc.). Sequence data were analyzed with the ARB software package (43). Phylogenetic trees were calculated by performing parsimony, neighbor-joining, and maximum-likelihood analyses with different sets of filters. For tree calculation, only full-length sequences were considered. The 650-nucleotide clone sequences were added to the tree after tree reconstruction. The organisms shown in the tree and the accession numbers of their sequences are as follows: *Desulfobacterium vacuolatum*, M34408; *Desulfobacterium autotrophicum*, M34409; *Desulfobacter postgatei*, M26633; *Desulfobacula toluolica*, X70953; *Desulfofaba gelida*, AF099063; *Desulfofrigus oceanense*, AF099064; *Desulfofrigus fragile*, AF099065; *Desulfobacterium indolicum*, AJ237607; *Desulfonema ishimotoei*, U45992; *Desulfonema limicola*, U45990; *Desulfococcus multivorans*, M34405; *Desulfosarcina cetonicum*, AJ237603; *Desulfosarcina variabilis*, M34407; *Desulfobulbus rhabdoformis*, U12253; *Desulfobulbus elongatus*, X95180; *Desulfotalea psychrophila*, AF099062; *Desulfotalea arctica*, AF099061; *Desulforhopalus vacuolatus*, LA2613; *Desulfofustis glycolicus*, X99707; *Desulfocapsa sulfoexigens*, Y13672; *Desulfocapsa thiozymogenes*, X95181; *Desulfuromonas acetoxidans*, M26634; *Desulfuromonas acetexigens*, U23140; *Desulfovibrio gigas*, M34400; *Desulfovibrio longus*, Z24450; *Desulfovibrio desulfuricans*, M34113; LSv53, AF099058; vadinH60, U81720; Sva0863, AJ240977; Sva0081, AJ240975; S2551, AF177428; str. MMP1991, L06457; AK-01, AF141328; ACE-32, AF142807; CLEAR-29, AF146251, A34, U08389; A52, U08394; RFLP25, AF058007; A01, U85480; DGGE-BS3, AJ011668; and SB-29, AF029047.

SRR measurement. Sulfate reduction rates (SRRs) were measured in whole sediment cores by the radiotracer method (16, 41). Undisturbed sediment cores were injected with 500 kBq of ^{35}S tracer at 1-cm intervals and incubated for 12 h at the in situ temperature in the dark. To stop the reaction, the sediment cores were cut into 1-cm-thick slices that were thoroughly mixed with 20 ml of 20% (wt/vol) zinc acetate and then deep frozen for transport. All samples were distilled with 6 M HCl and chromium(II) chloride in a single-step distillation process to convert reducible sulfur compounds into H_2S (11). SRRs were calculated from the ratio of added ^{35}S-sulfate to produced ^{35}S-sulfide.

Nucleotide sequence accession numbers. The nucleotide sequence data reported in this paper have been deposited in the EMBL, GenBank, and DDBJ nucleotide sequence databases under accession no. AF233491 to AF233500.

RESULTS

FISH detection rates. In the Smeerenburgfjorden sediment a large fraction of the bacteria living in the top 5 cm could be detected by FISH (Table 2). Up to 73.6% (core A, 57.9%) of the total DAPI cell counts hybridized to eubacterial probe EUB338. Below 10 cm the detection rate with probe EUB338 became too low (<20% of the total DAPI cell counts) for further FISH analysis.

SRB community structure. The emphasis of this study was on the SRB community structure in Smeerenburgfjorden sediment. SRB were quantified by both FISH and rRNA slot blot

TABLE 2—*Continued*

Desulfobulbus spp. (660)			*Desulfotalea* spp (Sval430)			*Desulfobacter* spp. (DSB985)			*Desulfobacterium* spp. (221)	
% DAPI	RNA concn (ng ml^{-1})	% of prokaryotic RNA	% DAPI	RNA concn (ng ml^{-1})	% of prokaryotic RNA	% DAPI	RNA concn (ng ml^{-1})	% of prokaryotic RNA	% DAPI	RNA concn (ng ml^{-1})
0	19/31	0.2/0.4	0	113/35	1.2/0.4	0	45/57	0.5/0.7	0	0
0	19/38	0.2/0.3	0	229/71	2.3/0.5	0	67/67	0.7/0.5	0	0
0	22/30	0.3/0.3	0/0.8	229/59	3.1/0.6	0	76/71	1.0/0.7	0	0
0	19/31	0.3/0.4	1.3/1.2	180/25	2.6/0.3	0	28/128	0.4/1.5	0	0
0	20/22	0.3/0.2	1.1/1.0	256/32	3.6/0.3	0	49/60	0.7/0.6	0.5/0	0
0	15/19	0.3/0.3	0.8/1.0	167/46	3.3/0.8	0	15/45	0.3/0.8	2.2/0	0
0	9/23	0.2/0.4	0.7/1.4	173/71	3.7/1.1	0	5/0	0.1/0	1.7/0.6	0
0	10/28	0.2/0.4	1.2/1.3	293/230	5.6/3.4	0	0	0	0.7/0	0
0	7/19	0.2/0.4	1.0/1.8	114/41	3.2/0.8	0	0	0	0	0
0	7/15	0.2/0.4	0.6/1.6	114/31	3.2/0.7	0	0	0	0	0
0	4/13	0.2/0.3	1.4/1.5	64/35	2.5/0.9	0	0	0	0	0
0	6/10	0.2/0.2	0.4/1.4	61/38	2.0/0.9	0	0	0	0	0
0	5/7	0.2/0.2	0.6/1.5	42/39	1.6/1.1	0	0	0	0	0
0	5/8	0.2/0.2	1.3/1.0	32/29	1.1/0.9	0	0	0	0	0
0	5/6	0.2/0.2	0.5/0	25/21	1.1/0.7	0	0	0	0	0

hybridization. The profiles of the individual groups of SRB in duplicate cores revealed comparable trends and abundances, indicating that there was horizontal homogeneity within the sediment at the level of our experimental resolution. The SRB community was dominated by complete oxidizers: the monophyletic group of *Desulfosarcina* spp., *Desulfococcus* spp., *Desulfofrigus* spp., *Desulfofaba* sp., and related clone sequences. This group is targeted by probe DSS658 and is referred to as the *Desulfosarcina-Desulfococcus* group below. Almost 12% of the DAPI cell counts were detected with this probe by FISH (Table 2). The highest abundance occurred at a depth of 2.25 cm, where 3.7×10^8 cells ml of sediment^{-1} accounted for 73% of the total SRB detected. Typically, the DSS658-positive cells had sarcinalike cell morphology (Fig. 1). Approximately 80% of the cells were irregularly shaped cocci that were about 1 μm in diameter and occurred in sarcinalike tetrads, in large clusters consisting of 10 or more cells, or (very often) as diplococci. About 20% of the cells detected were rods (0.5 by 1 to 3 μm).

Similar quantitative results were obtained when we used rRNA hybridization, which also showed that DSS658 rRNA was the most abundant rRNA. The vertical profile had a broad peak at depths between 3.75 and 9 cm, with 14 to 15% of prokaryotic rRNA (Table 2), while the absolute rRNA yields were highest at depths between 1.25 and 3.75 cm, where the mean maximum concentration was 1,000 ng of rRNA ml of wet sediment^{-1}. At depths below 3.75 cm the rRNA yield decreased with depth by a factor of approximately 1.5 to 2.0. The second dominant group, which was present at much lower abundance (one-third the level of the *Desulfosarcina-Desulfococcus* group), was *Desulforhopalus* spp. (Table 2). Probe DSR651 detected a maximum of 3.2% of all cells when FISH was used (1.2×10^8 cells ml^{-1}) and 5.4% of the prokaryotic rRNA. In general, the rRNA yield decreased with depth (Table 2). Members of the genus *Desulfotalea*, a newly described genus of psychrophilic SRB (20), could be detected in numbers of up to 6.9×10^7 cells ml of sediment^{-1} (1.0 to 1.8% of the DAPI cell count) in the depth profile. There was no clear maximum visible by FISH. In vertical profiles for *Desulfotalea* sp. rRNA the values were constant at almost all depths, with a clear maximum (4.4% of the prokaryotic rRNA, mean of two cores) at 3.75 cm.

For quantification of members of the frequently cultivated genus *Desulfovibrio* we used several probes designed by Manz et al. (25). Only with the most general probe, probe DSV698, could cells be detected, and they were detected only in the upper layers (surface layer to a depth of 2.25 cm); the maximum value obtained was only 1.6% of the DAPI cell count (5.2×10^7 cells ml^{-1}). The cell morphology was not vibriolike as expected for most *Desulfovibrio* species; the cells were short or long thin rods, and a few cells were coccoidal. This could have been due to a lack of probe specificity, but there are also rod-shaped *Desulfovibrio* species (e.g., *Desulfovibrio piger* and *Desulfovibrio carbinolicus*) (48). When rRNA hybridization was used, we detected a constant level of *Desulfovibrio* sp. rRNA (approximately 3% of the prokaryotic rRNA) throughout the vertical profile. The recovered rRNA yield decreased with depth.

Other probe target groups, like *Desulfomicrobium* spp. (probe DSV214) and *Desulfarculus* spp.-*Desulfomonile* spp.-*Syntrophus* spp. (probe DSMA488), were below the detection limit. Members of the genus *Desulfobacterium* (probe 221), which are completely oxidizing bacteria, were detected only at depths between 2.25 and 3.75 cm (up to 2.2% of the DAPI cell count). The level of RNA of this group was below the detection limit for slot blot hybridization. *Desulfobulbus* spp. (probe 660) and *Desulfobacter* spp.-*Desulfobacula* spp. (probe DSB985) were not detected by FISH, but small amounts of rRNAs of these organisms were found. Constant small amounts of *Desulfobulbus* sp. rRNAs were detected (0.2 to 0.4% of the prokaryotic rRNA throughout the vertical profile). *Desulfobacter* sp. rRNA was recovered down to a depth of 3.25 cm, and the maximum value was 1.5% of the prokaryotic RNA.

Sulfur-reducing and fermenting bacteria. Members of *Desulfuromonas* spp., which are sulfur-reducing bacteria, and of *Pelobacter* spp., which are strictly anaerobic fermenting bacteria, have been shown to constitute a dominant group in our Svalbard sediment clone library (32). Therefore, we investigated the abundance of these organisms to assess their potential contribution to the sulfur cycle in the sediments studied. This group was targeted by probe DRM432 (Table 2). Members of this group accounted for up to 2.2% of the DAPI cell counts and up to 6.4% of the total prokaryotic RNA, and thus this group's contribution to the sulfur cycle may be important and deserves further attention.

TABLE 2—*Continued*

Desulfosarcina spp. and *Desulfococcus* sp. (probe DSS658)			Sum for SRB detected			DSS clones (probe DSS225): % DAPI	Clones Sva0081 and Sva0863 (probe cl81-644): % DAPI	*Desulfuromonas* spp. (probe DRM432)		
% DAPI	RNA concn (ng ml^{-1})	% of prokaryotic RNA	% DAPI	RNA concn (ng ml^{-1})	% of prokaryotic RNA			% DAPI	RNA concn (ng ml^{-1})	% of prokaryotic RNA
1.7/1.8	465/555	4.7/6.2	3.7/3.5	1,120/1,224	11.4/13.6	1.2/0.7	0.7/0.7	0.7/0.7	201/214	2.0/2.4
3.1/3.1	562/1,180	5.6/8.5	5.7/5.7	1,286/2,423	12.8/17.5	2.3/1.8	1.0/1.4	1.5/1.1	182/490	1.8/3.5
4.7/4.5	648/1,311	8.9/12.4	6.8/7.7	1,437/2,391	19.6/22.6	3.3/2.8	1.6/1.8	1.9/1.5	239/535	3.3/5.1
6.5/6.7	906/848	13.2/10.2	10.6/9.3	1,481/1,500	21.5/18.0	4.0/3.9	1.6/2.6	1.4/1.5	283/349	4.1/4.2
8.9/9.5	595/1,066	8.3/11.1	13.2/12.1	1,181/2,011	15.5/20.9	6.3/5.6	2.4/2.7	1.9/2.2	150/434	2.1/4.5
9.3/11.0	743/498	14.8/8.5	15.6/14.3	1,152/880	22.9/15.0	6.6/7.0	2.6/2.9	1.7/1.7	212/236	4.2/4.0
10.0/11.7	727/522	15.4/8.1	15.4/16.1	1,169/922	24.8/14.4	7.4/6.1	2.3/1.7	1.3/1.4	235/247	5.0/3.8
8.8/7.4	929/836	17.8/12.5	13.6/10.9	1,504/1,449	28.9/21.7	5.4/4.6	1.2/2.3	1.2/0.7	291/351	5.6/5.3
8.8/8.1	608/450	16.8/8.9	12.5/11.6	910/729	25.2/14.4	6.4/3.9	2.5/2.7	0.8/0.5	205/149	5.7/2.9
6.0/6.2	559/590	15.5/13.3	9.2/9.5	918/823	25.5/18.6	3.8/4.1	1.6/0.6	1.3/1.0	229/191	6.4/4.3
6.6/5.2	305/651	11.7/16.1	9.8/8.5	499/933	19.1/23.1	3.7/3.6	0.9/3.1	1.3/1.0	104/246	4.0/6.1
7.1/6.5	378/725	12.7/16.1	9.4/9.1	601/981	20.1/21.8	5.2/3.4	1.9/1.8	0.9/1.0	86/191	2.9/4.3
5.2/5.6	378/519	14.2/14.7	7.3/7.9	567/732	21.3/20.8	3.3/2.8	1.8/1.3	1.1/0.7	68/129	2.6/3.7
4.4/4.2	442/492	15.4/15.2	7.3/6.4	637/698	22.2/21.5	2.4/2.6	0.5/0.7	0.8/0.6	91/126	3.2/3.9
3.0/3.6	393/350	17.0/12.1	5.1/4.7	575/519	24.9/17.9	1.9/1.0	1.0/1.5	0.5/0.2	93/136	4.0/4.7

Predominance of the *Desulfosarcina-Desulfococcus* group at various stations along the coast of Svalbard. Sediment samples from three other sampling sites off the coast of Svalbard and from Smeerenburgfjorden, which was sampled again 1999, were investigated with FISH to determine if the predominance of the *Desulfosarcina-Desulfococcus* group is a common feature at these stations. The vertical profile for DSS658-targeted cells from 1999 Smeerenburgfjorden sediment was almost identical to the profile obtained with samples collected in 1998 (Fig. 2). The highest percentage of cells detected in 1999 was 12.6% at a depth of 2.75 cm (11.7% at a depth of 3.25 cm depth in 1998). Members of the *Desulfosarcina-Desulfococcus* group were also found in high abundance in sediment samples obtained from Raudfjorden (station K) and from Hornsund (Fig. 2); both profiles exhibited a maximum at a depth of 2.75 cm depth (7.5 and 8.5% of the DAPI cell counts, respectively). The sediment profile for station K had another maximum (10.4% of the DAPI cell counts) in a deeper layer (depth, 6.5 cm). Only at Magdalenefjorden (station I) was this group detected in lower numbers (4 to 5% of the DAPI cell counts). This lower abundance was not due to a lower rate of detection of eubacterial cells at this station. DSS658-targeted cells accounted for 13% of the EUB338-detected cells at station I, compared to up to 30% at the other stations.

In-depth analysis of the *Desulfosarcina-Desulfococcus* group. The existence of a prominent group of SRB at several stations prompted us to analyze this group in greater detail. We developed new specific probes for the different genera targeted by DSS658 on the basis of the available 16S rDNA data set (Table 1). Probe DSC193 was specific for *Desulfosarcina* spp., probe DCC209 was specific for *Desulfococcus* spp., probe DSF672 was specific for *Desulfofaba* sp. and *Desulfofrigus* spp., and probe cl81-644 was specific for 16S rDNA clones from Hornsund sediment (32). All of the probes could also be used for FISH. The hybridization conditions were adjusted by using several reference strains. When we used these probes for

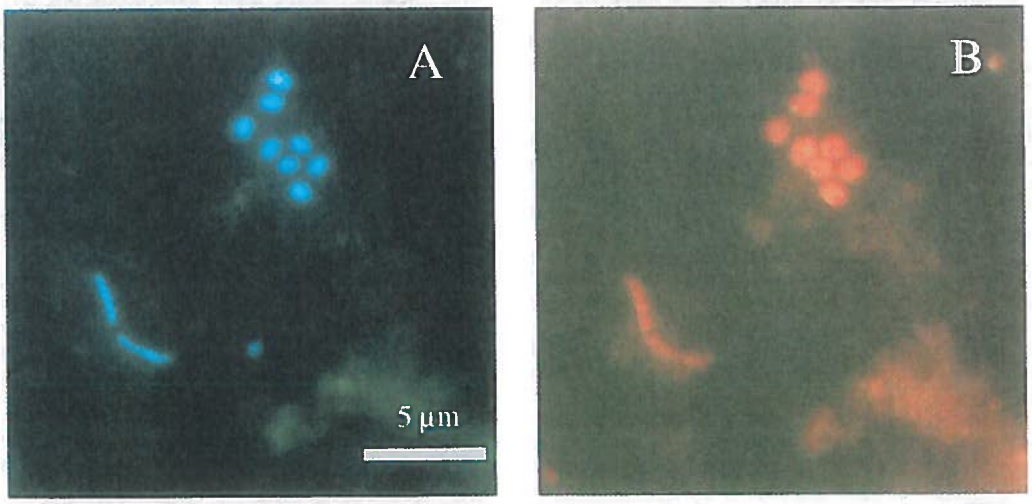

FIG. 1. Epifluorescence micrographs of bacteria in sediment samples from Smeerenburgfjorden, Svalbard, Arctic Ocean. (A) DAPI staining. (B) FISH performed with probe DSS658 specific for the *Desulfosarcina-Desulfococcus* group (same microscopic field).

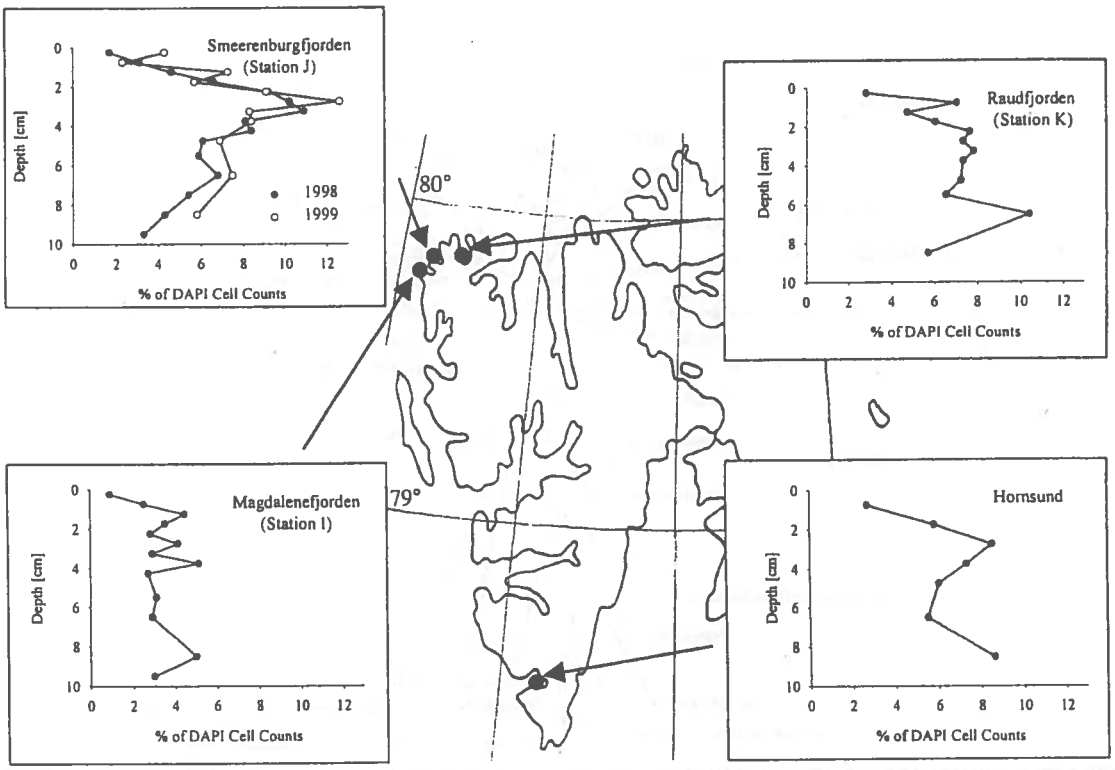

FIG. 2. Detection and quantification of the *Desulfosarcina-Desulfococcus* group at various stations along the coast of Svalbard by FISH (probe DSS658).

known and cultivated genera with Smeerenburgfjorden sediment samples collected in 1998, we did not detect any cells. However, when we used probe cl81-644, which was specific for Svalbard clones Sva0081 and Sva0863, almost 3% of the DAPI-stained cell counts were detected (Fig. 3). The targeted cells had a rod-shaped morphology (0.5 by 1 to 3 μm). Nevertheless, the very abundant sarcinalike cells could not be affiliated with cultivated genera or 16S rDNA clone sequences in the databases.

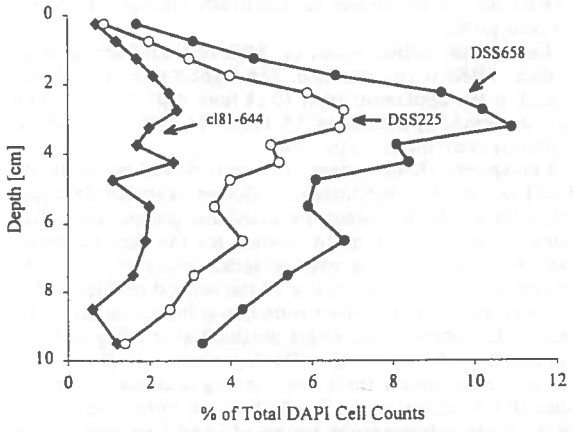

FIG. 3. Depth profile for subgroups of the *Desulfosarcina-Desulfococcus* group as detected by FISH (Smeerenburgfjorden sediment). Symbols: ●, probe DSS658 targeting the *Desulfosarcina-Desulfococcus* group; ○, probe DSS225 targeting group SVAL1; ◆, probe cl81-644 targeting previously cloned 16S rDNA sequences from the same habitat.

Search for the identity of sarcinalike cells. A new strategy was needed to further identify the sarcinalike cells in the DSS658 target group. We used probe DSS658 as a specific primer in combination with a universal eubacterial primer for specific amplification of the DSS658 target 16S rDNAs. To verify that the amplification was specific, we performed parallel PCR with reference DNAs with one to three mismatches with DSS658. Targets with more than one mismatch could be distinguished. A clone library was set up, and 40 clones with an insert of the correct size, 650 bp, were screened by ARDRA. Fourteen different patterns were found after digestion with two restriction enzymes. Sequence analysis of representatives of all of the patterns showed that all of the clone sequences except two gamma-proteobacterial sequences fell in the *Desulfosarcina-Desulfococcus* group (Fig. 4). The highest sequence similarity was 96.9% between clone DSS7 and DGGE BS3, a sequence retrieved from Black Sea sediment (34). *Desulfosarcina variabilis* was the closest relative of the most frequent clone group (19 of 40 clones), which was designated the SVAL1 group (91.2%). On the basis of this new sequence data we developed a probe (DSS225) for SVAL1. *Desulfosarcina variabilis* and *Desulfofaba gelida* exhibited one weak central mismatch with the probe (G = U) and therefore could not be discriminated. The inclusion of *Desulfosarcina* spp. and *Desulfofaba* sp. was not relevant for this study because no members

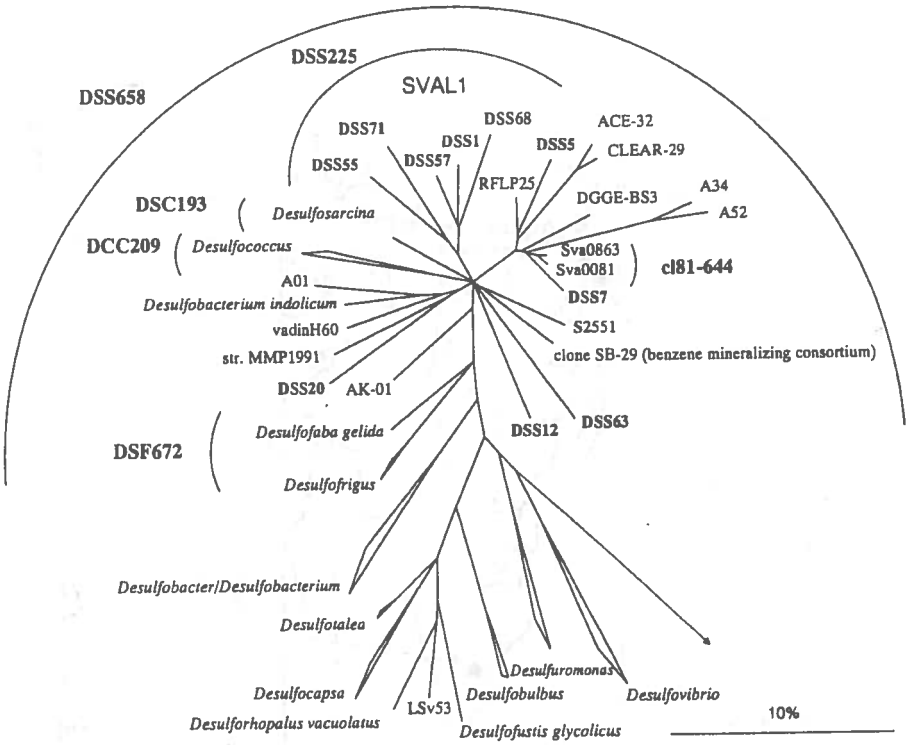

FIG. 4. Phylogenetic tree showing the affiliations of 16S rDNA clone sequences with selected reference sequences of members of the delta subclass of the *Proteobacteria*. The tree was calculated by using maximum-likelihood analysis and was corrected with filters which considered only 50% conserved regions of the 16S rRNAs of members of the delta subclass of the *Proteobacteria*. The DSS clones, as well as clone sequences A01, SB-29, RFLP25, ACE-32, CLEAR-29, A52, A34, and DGGE-BS3, are not full-length sequences (length, 650 to 900 bp) and therefore were added to the existing tree by using a special algorithm included in the ARB software without allowing changes in the tree topology based on almost complete sequences. Different calculations of phylogenetic trees did not result in a stable branching order for some subgroups. Consequently, the phylogenetic affiliations of these subgroups are shown as multifurcations. New cloned 16S rDNA sequences are indicated by boldface type. The group consisting of clone sequences DSS1, DSS5, DSS55, DSS71, and DSS68 was designated SVAL1. Bar = 10% estimated phylogenetic divergence.

of the genera *Desulfosarcina* and *Desulfofaba* were detected in our samples. Using new probe DSS225, we detected very high numbers of cells with sarcinalike morphology (Fig. 3) and very few rods. The distributions of DSS225- and DSS658-targeted cells were almost identical, and maxima occurred at the same depth. Probe DSS658 detected both DSS225 and cl81-644 target cells. By adding the detection rates for the individual more specific probes we could recover roughly 100% of the DSS658-detected cells along the vertical profile.

Applying probe DSS225 on selected layers of sediment samples obtained from the other stations (stations I and K and Hornsund), very high cell numbers and relative proportions of DSS658-targeted cells were detected (data not shown). Up to 94% of DSS658-detected cells were targeted by specific probe DSS225 in Hornsund sediment (station K, 80%; station I, 33%).

Total SRB and SRRs. Adding up the number of cells from the individual groups of SRB, as well as the rRNA recovered from these groups, gave an overview of the detectable SRB population along the depth profile (Fig. 5). Up to 5.2×10^8 SRB ml^{-1} (15% of DAPI cell counts) and up to 25.3% prokaryotic rRNA were detected at depths of 2.25 and 2.75 cm, respectively. The highest SRB rRNA yield, however, was obtained at a 1.25 cm depth with (1,914 ng ml of $sediment^{-1}$). Due to the high standard deviation the sulfate reduction rates (SRR) had to be considered essentially constant along the vertical profile.

Cell-specific SRRs. Based on SRB cell numbers, average cellular SRRs were calculated. The highest rate per cell was found in the uppermost layer (0.14 fmol $cell^{-1}$ day^{-1}). This rate decreased by a factor of 3.5 within the first 3 cm and was relatively constant in deeper layers.

Cell-specific rRNA content. A combination of two methods, FISH and slot blot hybridization, allowed us to calculate specific cellular rRNA contents for individual groups. The calculated average cellular rRNA content for the *Desulfosarcina-Desulfococcus* group, the most abundant group in this study, exhibited a trend similar to that of the vertical profile of SRB cell-specific SRRs. The RNA content was highest in the first 5 mm of the sediment; the values obtained were 0.9 fg of RNA per cell (core A) and 1.4 fg of RNA per cell (core B) (Fig. 6). In the vertical profile there was a strong decrease in the cellular rRNA content in the first 1.75 cm; in cores A and B the rRNA content decreased by factors of 3 and 6, respectively. In deeper layers the cellular rRNA content remained low. To verify these findings with an independent method, we quantified the average cell fluorescence at the single-cell level with confocal laser scanning microscopy (Fig. 6). We found that the

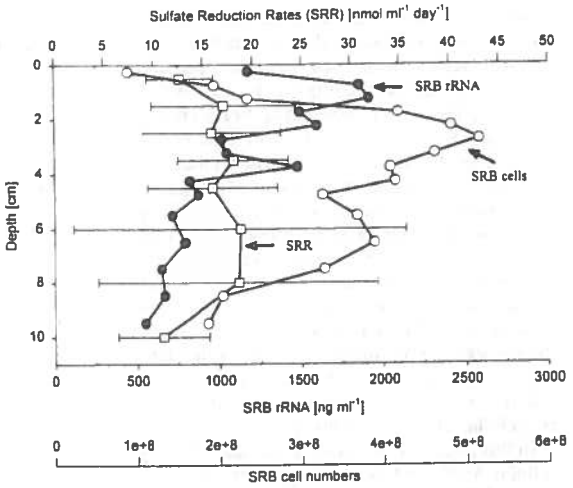

FIG. 5. Depth profiles for SRB abundance, SRB rRNA concentrations, and SRRs. Numbers of SRB cells (○) and rRNA concentrations (●) were determined by adding the values for the groups targeted by probes DSS658 (*Desulfosarcina-Desulfococcus* group), DSR651 (*Desulforhopalus* spp.), DSV698 (*Desulfovibrio* spp.), Sval428 (*Desulfotalea* spp.), DSB985 (*Desulfobacter* spp.), 221 (*Desulfobacterium* spp), and 660 (*Desulfobulbus* spp.); means based on the values for two cores are shown. The SRRs (□) are mean values based on the values for three cores.

cells in the uppermost layer had an approximately twofold brighter signal with probe DSS658 than the cells in deeper layers. This finding supports the calculated trend since a brighter FISH signal is related to a higher cellular rRNA content.

DISCUSSION

SRB community structure. The major group of SRB identified was the *Desulfosarcina-Desulfococcus* group. Between 49 and 73% of FISH-detected SRB and between 44 and 70% of total SRB rRNA belonged to this group of completely oxidizing sulfate reducers. The predominance of the *Desulfosarcina-Desulfococcus* group in the Smeerenburgfjorden sediment was confirmed with sediment samples taken 1 year later. This group was also found to be the dominant group at three other sampling sites off the coast of Svalbard. Thus, members of the *Desulfosarcina-Desulfococcus* group seem to be able to survive under various conditions. This conclusion is supported by the fact that high abundances of members of the *Desulfosarcina-Desulfococcus* group have been shown previously in different habitats. Sahm et al. (38) found between 71.7 and 85% SRB rRNA in a coastal sediment, Rooney-Varga et al. (33) detected up to 15.5% of bacterial rRNA with probes specific for 16S rDNA clones affiliated with *Desulfosarcina variabilis* and *Desulfococcus multivorans*, and Edgcomb et al. (10) estimated high cell numbers based on probe-detected rDNAs in salt marsh sediments. Furthermore, Mußmann and Llobet Brossa found high levels of members of the *Desulfosarcina-Desulfococcus* group in Wadden Sea sediment (personal communication). *Desulfosarcina* spp. and *Desulfococcus* spp. are known to be nutritionally versatile with respect to potential electron donors and are capable of complete oxidation of organic carbon to CO_2 (48). Some strains and 16S rDNA clone sequences in this group (Fig. 6) have been isolated from contaminated sites; strain S2552 (accession no. AF177428) was isolated from an oil reservoir, clone RFLP25 (accession no. AF058007) was derived from a polychlorinated biphenyl-dechlorinating culture (27), and strain AK-01 (accession no. AF141328) was isolated from an estuarine sediment with a history of chronic petroleum contamination (40).

The predominance of members of the *Desulfosarcina-Desulfococcus* group may reflect the availability of a variety of complex organic matter rather than the input of one specific substrate as an electron donor. This seems only reasonable in a natural habitat, where a diverse community of prokaryotes might produce a wide range of carbon sources in the food chain. Using bag incubations of sediment slurries, Purdy et al. (28) demonstrated that the availability of a single substrate potentially favors other groups; e.g., propionate supported the growth of *Desulfobulbus* spp. The nutritional versatility of the *Desulfosarcina-Desulfococcus* group could also be advantageous in case of competition for limited carbon sources in this extreme habitat. Other species, like *Desulfovibrio* spp., can use only a few simple organic acids, hydrogen, and (in some cases) ethanol as an electron donor. They do not grow well in the presence of low substrate concentrations but were found to be favored by higher substrate concentrations (44, 45, 48).

In previous studies on the microbial community of Svalbard sediments, Sahm and coworkers quantified selected groups of SRB by slot blot hybridization and found that rRNA of members of the *Desulfovibrionaceae* was dominant (37). However, due to results obtained with a clone library established by using the same sediment samples (32), they assumed that the rRNA detected might have come from organisms belonging to the *Geobacteraceae* group because a significant portion of the clone sequences in the Svalbard sediment clone library (32) gave positive signals with the same probe (probe 687). All

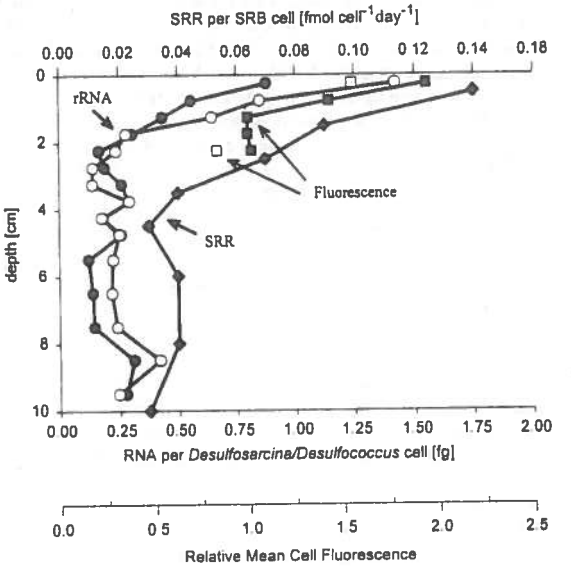

FIG. 6. Depth profiles for specific rRNA contents and mean cell fluorescence for DSS658 (*Desulfosarcina-Desulfococcus* group)-targeted cells and SRRs per SRB cell. The average cellular rRNA contents were determined by combining FISH and rRNA hybridization data (●, core A; ○, core B). The mean fluorescence of hybridized cells was quantified by laser scanning microscopy in the following way. For each depth a mean cell fluorescence was calculated; the lowest mean cell fluorescence value was defined as 1, and the mean fluorescence values for cells in the other layers were expressed relative to this value (■, core A; □, core B). SRRs per SRB cell (◆) were based on SRB abundance.

sequences were sequences of members of the family *Geobacteraceae* and were most closely related to the *Desulfuromonas palmitatis* sequence. The fact that we detected high levels of *Desulfuromonas* rRNA in the present study supports this conclusion. The newly isolated genus *Desulfotalea* was the second most abundant group of SRB in the studies of Sahm et al.; the relative abundance was 0.6 to 4.4% of the prokaryotic rRNA at the relevant depth. In the present study we obtained similar results; *Desulfotalea* spp. accounted for 0.3 to 5.6% of the prokaryotic rRNA. However, detection of additional groups of SRB (e.g., the *Desulfosarcina-Desulfococcus* group) showed that there are other groups that are present at even higher abundance. Sahm et al. did not find significant amounts of *Desulfosarcina-Desulfococcus* rRNA (37). A possible explanation for the failure to detect *Desulfococcus-Desulfosarcina* rRNA is that these organisms were not targeted by the probe used (probe 804) (6). We cannot resolve this discrepancy yet, since the sequence data for the dominant subgroup of uncultured sarcinalike cells which we describe in this paper does not contain the target position for probe 804.

Resolution of the *Desulfosarcina-Desulfococcus* group. Although only a few strains of the DSS658 target group have been cultivated so far, perhaps due to the use of substrates that are too simple (such as lactate or propionate), molecular biological studies have revealed a very high diversity in this group. In the last few years the diversity of the *Desulfosarcina-Desulfococcus* group has been greatly extended by 16S rDNA cloning (7, 27, 33, 46), denaturing gradient gel electrophoresis analysis (34), and cultivation with complex substrates (40). The sequences often exhibit only 90% sequence similarity to their closest relative or to a cultivated strain. Cultivation of this major group of marine SRB should be a goal for future studies.

In our study none of the *Desulfosarcina-Desulfococcus* cells detected could be affiliated with known genera. The closest relative of the most abundant clone group, group SVAL1, was *Desulfosarcina variabilis*, with 92% sequence similarity. The newly designed probe DSS225, which is specific for group SVAL1, detected up to three-quarters of the DSS658-targeted cells and produced an almost identical vertical profile. However, the phylogenetic distance between *Desulfosarcina* spp. and the clone sequences is so large (8%) that we can only speculate on the physiological properties of the organisms. The ability to oxidize substrates completely to CO_2 and nutritional versatility are features that are common to almost all species belonging to the *Desulfosarcina-Desulfococcus* group; thus, we assume that the bacteria detected also have these physiological characteristics. Attempts to perform directed cultivation of SRB from the same habitat are under way. Additional studies on the presence and abundance of the new SVAL1 group in other habitats, like antarctic sediments or temperate environments, should show whether the dominance and ecological significance of this group are restricted to Svalbard sediments.

Specific cellular rRNA content and specific SRRs. In this study we combined FISH and rRNA hybridization data to calculate average cellular rRNA contents of *Desulfosarcina-Desulfococcus* cells. The calculated average cellular rRNA content of these cells was greatest in the upper 5 mm of sediment and decreased steeply within the first 2 cm. The ribosome content and with that the rRNA content are directly connected to the growth rate in steady-state cultures (for a review see reference 26). Molin and Givskow, however, have cautioned to use cellular rRNA measurements on cells growing in a complex environment under changing nutritional conditions to address cellular growth activities. To translate the measured cellular rRNA contents into absolute growth rates, pure-culture experiments performed with specific strains are needed. Even then, in a natural habitat different biological and nonbiological factors interfere with each other and might activate different global-transcriptional control networks in the cell, thereby influencing the direct correlation between growth and rRNA synthesis. We would like to add that species heterogeneity within a probe target group might further complicate the picture. In our study, however, the major target group of SRB was dominated by one group of closely related organisms throughout the whole depth profile, and the cellular rRNA content was consistent with independently determined cellular SRRs. This makes us confident that we obtained useful information about the physiological state of the SRB detected. These results suggest that although growth rates might be generally low in the natural habitat, they change along the depth profile. Closeness to the sediment surface guarantees the availability of different substrates and could therefore explain why the highest cellular rRNA contents are in the first layer.

In this paper we describe, to our knowledge for the first time, cellular SRRs and the corresponding vertical profile obtained for total SRB cells, as quantified by a cultivation-independent method. The cellular SRRs, which were calculated from numbers of SRB cells as detected by FISH, were highest at the sediment surface, where they were 0.14 fmol of SO_4 per day, and decreased steeply with depth to 0.02 fmol of SO_4 per day. They were lower, by factors of 5 to 50, than the specific SRRs of mesophilic SRB that were grown in pure cultures at 4°C (19). For *Desulfosarcina variabilis* and *Desulfococcus niacini* SRRs of 0.7 ± 0.4 and 1.2 ± 0.05 fmol of SO_4 cell^{-1} day^{-1}, respectively, were obtained. Nevertheless, our calculated rates seem to be in a reasonable range for natural, substrate-limited environments. The general finding that the cellular SRRs were much higher in the first 5 mm than in the suboxic or anoxic zones might even be more pronounced since SRRs probably are underestimated rather than overestimated in oxidized layers (18).

Combination of FISH and rRNA slot blot hybridization for quantification of bacteria—methodological considerations. Quantification of SRB by FISH and quantification of SRB by slot blot hybridization gave comparable results. This comparability is encouraging. A comparison of studies based on FISH and studies based on slot blot hybridization is possible almost without reservation, although the two methods have different drawbacks (2). Despite the different methodological constraints, only detection of groups at levels just above the detection limit resulted in minor discrepancies in this study. For example, *Desulfobacter* sp. rRNA could be detected in some layers, but no cells were detected by FISH, suggesting that the rRNA detected was distributed over a relatively large fraction of probably less active cells with low cellular rRNA contents.

A combination of the two methods allowed us to calculate the specific cellular rRNA contents. In this study we found a good correlation between the cellular SRR and the cellular rRNA content of SRB. It would be rewarding to investigate other natural systems for this correlation between cellular activity and rRNA content.

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3

Quantitative Molecular Analysis of the Microbial Community in Marine Arctic Sediments (Svalbard)

Katrin Ravenschlag, Kerstin Sahm, and Rudolf Amann

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Quantitative Molecular Analysis of the Microbial Community in Marine Arctic Sediments (Svalbard)

KATRIN RAVENSCHLAG, KERSTIN SAHM, and RUDOLF AMANN

Molecular Ecology Group; Max Planck Institute for Marine Microbiology; 28359 Bremen; Germany

Fluorescence in situ hybridization (FISH) and rRNA slot-blot hybridization with 16S rRNA-targeted oligonucleotide probes were used to investigate the phylogenetic composition of a marine Arctic sediment (Svalbard). FISH resulted in the detection of a large fraction of microbes living in the top 5 cm of the sediment. Up to 65.4 ± 7.5% of total DAPI cell counts hybridized to the bacterial probe EUB338 and up to 4.9 ± 1.5% to the archaeal probe ARCH915. Besides δ-proteobacterial sulfate-reducing bacteria [up to 16%, (55)] members of the *Cytophaga/Flavobacterium*-cluster were the most abundant group detected in this sediment, accounting for up to 12.8% of total DAPI cell counts and up to 6.1% of prokaryotic rRNA. Furthermore, members of the order *Planctomycetales* accounted for up to 3.9% of total cell counts. In accordance with previous studies, these findings support the hypothesis that these bacterial groups are not simply setting with organic matter from the pelagic zone but are indigenous to the anoxic zones of marine sediments. Members of the γ-proteobacteria also constituted a significant fraction in this sediment (6.1 ± 2.5% of total cell counts, 14.4% ± 3.6% of prokaryotic rRNA). A new probe (GAM660) specific for sequences affiliated with free-living or endosymbiotic sulfur-oxidizing bacteria was developed. A significant number of cells was detected by this probe (2.1 ± 0.7% of total DAPI cell counts, 13.2 ± 4.6% of prokaryotic rRNA), showing no clear zonation along the vertical profile. Gram-positive bacteria as well as and the β-proteobacteria were near the detection limit in all sediments.

Knowledge of the microbial diversity of marine pelagic and benthic communities has been greatly extended recently by molecular studies based on the analysis of 16S rDNA

(see e.g. 11, 12, 22, 46, 47, 52, 54, 56, 72). Numerous new 16S rDNA sequences have been retrieved both from marine sediments and from the water column, indicating that the vast majority of species has not been cultivated yet. Several studies using the cultivation-independent approach of 16S rDNA cloning have helped to elucidate common features within the microbial communities of specific habitats such as marine benthic environments (12, 37, 38, 56, 72). Furthermore, they have provided additional sequence information for the design and evaluation of nucleic acid probes for the identification and quantification of distinct bacterial populations.

While microbial diversity can be readily studied by PCR-based 16S rDNA cloning, community structure can not be deduced from cloning studies (3) due to potential biases introduced during DNA retrieval and amplification (19, 57, 69). For reliable characterization of community structure, quantitative methods such as fluorescence in situ hybridization (FISH) or rRNA slot-blot hybridization are more suitable (3). To date, a number of studies have been performed using either of these two methods to quantify different groups in marine sediments (17, 39, 40, 55, 61-63, 73). Most of these studies, however, focussed on specific microbial groups such as sulfate-reducing bacteria (55, 62, 63) or *Archaea* (40, 73).

Here, we describe the community composition of a marine Arctic sediment (Smeerenburgfjorden, Svalbard) using both FISH and rRNA slot-blot hybridization for quantification. The sulfate-reducing community of Smeerenburgfjorden sediment has recently been described in detail (55); sulfate reducers accounted for up to 16% of total cell numbers and up to 29% of prokaryotic rRNA. In this study, we report the contribution of other major phylogenetic groups, such as the β- and γ-proteobacteria, the *Cytophaga/Flavobacterium*-cluster, *Planctomycetales*, and Gram-positive bacteria, to the total microbial community along vertical gradients. We did not screen for α-proteobacteria because the available probes, ALF1b (43) and ALF968 (48), also target a wide variety of δ-proteobacterial sequences including sulfate-reducing bacteria and members of the genera *Pelobacter*, *Geobacter*, *Desulfuromonas*, *Synthrophus*, and *Polyangium/Chondromyces*. In this sediment, δ-proteobacteria contributed up to 34.5% of prokaryotic rRNA and up to 17.5% of total cell counts (55), and therefore have greatly affected the detection of α-proteobacteria.

To the best of our knowledge, there is only a single previous study which has described the quantitative importance of these groups in marine sediments (39). Llobet-Brossa and colleagues used FISH for quantification. They found high abundances of members of the *Cytophaga/Flavobacterium* cluster and the order *Planctomycetales*, two groups usually regarded as including mainly pelagic, aerobic bacteria (5, 10, 21), in Wadden Sea sediments

(39). This study reports the first rRNA profiles of these major phylogenetic groups in marine sediments.

In addition to quantification of these major phylogenetic groups, a new probe specific for a cluster of 16S rDNA clone sequences affiliated to free-living and endosymbiotic sulfur-oxidizing bacteria of invertebrates was developed and applied. Sequences of this group were abundant in a Svalbard sediment clone library (56) and also dominant in clone libraries from other marine sediments, e.g. different deep-sea sediments off Japan (37, 38), coastal sediments off Japan (72) and seagrass-colonized sediments from the Bassin d'Archachon (8). The potential ecological importance of this group is discussed with regard to its abundance, the stratification of its distribution, and the possible occurrence of symbiotic and free-living forms.

MATERIAL AND METHODS

Study site and sampling. Sediment samples were collected on 28 July 1998 from Smeerenburgfjorden, Svalbard, Arctic Ocean (79°42'815N, 11°05'189E, "station J"). The sediment temperature was 0°C, surface water 5°C and water depth 218 m. Sediment was sampled with a Haps-corer, subsampled and kept at in situ temperature during transport (72 h). The sediment was characterized by a soft brown silty oxidized surface (upper 2 cm) overlaying a transition zone of darker, black-streaked clayey mud. Below the transition zone (2-6 cm) a black sulfidic zone followed. Worm tubes as well as small shells (2-3 mm) were present in the sediment to a depth below 10 cm. Two parallel cores were sliced: one half of each slice was frozen in liquid nitrogen for RNA extraction (stored at -80°C), the other half was fixed for 2-3 hours at a final concentration of 3% formaldehyde, washed twice with 1xPBS (10 mM sodium phosphate pH 7.2; 130 mM NaCl) and was finally stored in 1xPBS/EtOH (1:1) at -20°C.

RNA extraction and slot-blot hybridization. RNA was extracted from 1.5 ml wet sediment (per layer) by bead-beating, phenol extraction and isopropanol precipitation as described previously (61). The quality of the RNA was checked by polyacrylamide gel electrophoresis. Approximately 50 ng RNA was blotted on nylon membranes (Magna Charge, Micron Separations, Westborough, MA) in triplicate and hybridized with radioactively labeled oligonucleotide probes as described by Stahl et al. (67). Membranes were washed at different temperatures depending on the dissociation temperature (Td) of the probe. Probes used and dissociation temperatures are given in Table 1. The dissociation temperatures of the probes were determined as described by Raskin (53) with slight modifications.

TABLE 1. Oligonucleotide probes used in this study

Probe	Specificity	Sequence (5'-3')	target	Position [a]	FISH [FA] [b]	Slot-Blot T_d [°C]	Ref.
UNI1390	Universal (all organisms)	GACGGGCGGTGTGTACAA	16S/ 18S	1390-1407	not used	44*	(77)
ARCH915	*Archaea*	GTGCTCCCCCGCCAATTCCT	16S	915-935	35	56*	(2)
EUK1379	*Eucarya*	TACAAAGGGCAGGGAC	18S	1379-1394	not used	42*	(30)
EUB338	*Bacteria*	GCTGCCTCCCGTAGGAGT	16S	338-355	10	54*	(1)
NON-EUB	negative control	ACTCCTACGGGAGGCAGC	16S	338-355	10	not used	(74)
GP1199	Most Gram-positive bacteria	AAGGGGCATGATG	16S	1199-1211	--	34*	(41)
GAM42a	γ-proteobacteria	GCCTTCCCACATCGTTT	23S	1027-1043	35	60	(43)
BET42a	β-proteobacteria	GCCTTCCCACTTCGTTT	23S	1027-1043	not used	58	(43)
PLA886	*Planctomycetales*, some *Eucarya*	GCCTTGCGACCATACTCCC	16S	886-904	35	62	(49)
CF319a	*Cytophaga/ Flavobacterium*-cluster	TGGTCCGTGTCTCAGTAC	16S	319-336	35	56	(42)
GAM660	16S-rDNA clone sequences affiliated with endosymbionts and some other species in the γ-proteobacteria.	TCCACTTCCCTCTAC	16S	660-674	35-40	52	This study

[a] Position in the 16S/23S rRNA of *E.coli*
[b] Formamide concentrations in the hybridization buffer in % (v/v)
* Dissociation temperatures (Td) determined for washing buffer containing 1xSSC and 1% SDS

For Td determinations and hybridizations (probe BET42a, GAM42a, GAM660, CF319a, PLA886) washing buffer with a lower sodium dodecylsulfate (SDS) concentration was used (1xSSC (150 mM NaCl, 15 mM sodium citrate; pH 7.0); 0.1% SDS). However, for hybridizations with probe Uni1390, EUB338, EUK1379 and ARCH915 washing buffer with 1% SDS was used.

Quantification. Hybridization signal intensity was measured with a PhosphorImager (Molecular Dynamics, Sunnyvale, Calif.) and quantified as described previously (63). Reference rRNA isolated from pure cultures of *Cytophaga lytica* (DSM 7489), *Pirellula marina* str. 1, *Methanolobus tindarius* (DSM 2278), *Arthrobacter* strain KT1113.15, *Zoogloea* str. Cadagno, *Halothiobacillus kellyi* (DSM 13162) and strain 'Milos AII2' as well as rRNA of *Saccharomyces cerevisiae* and *E. coli* (purchased from Roche, Germany) served as standards for hybridization with the probes given in Tab.1.

Fluorescence in situ hybridization (FISH). PBS/Ethanol stored samples were diluted and treated by mild sonication with an MS73 probe (Sonopuls HD70, Bandelin, Berlin, Germany)

at a setting of 20 s, amplitude 42 μm, and <10 W. An aliquot of 10 μl of a 1:40 dilution was filtered on 0.2 μm GTTP polycarbonate filters (Millipore, Eschborn, Germany). Hybridization and microscopy counts of hybridized and 4',6'-diamidino-2-phenylindole (DAPI)-stained cells were performed as described previously (66). Means were calculated from 10 to 20 randomly chosen fields on each filter section, corresponding to 800-1000 DAPI stained cells. Counting results were always corrected by subtracting signals observed with the probe NON338. Formamide concentrations are given in Table 1.

Oligonucleotides. Oligonucleotides were purchased from Interactiva (Ulm, Germany). For FISH, oligonucleotide probes were synthesized with the fluorescent dye Cy3 at the 5' end.

RESULTS

Total cell counts and domain specific probing. Total cell numbers were determined by DAPI-staining. They were in the range of 2.1-4.7 x 10^9 ml^{-1} wet sediment and showed little variation among two parallel sediment cores. There was no significant decrease of total cell numbers with increasing sediment depth (Fig.1, Table 2), even to 19 cm depth.

Bacteria and *Archaea* were quantified by both FISH and rRNA slot-blot hybridization, with domain-specific probes. FISH resulted in the detection of a large fraction of microbes living in the top 5 cm of the sediment. Up to 65.4 ± 7.5% of total DAPI cell counts hybridized to the eubacterial probe EUB338 and up to 4.9 ± 1.5% to the archaeal probe ARCH915. EUB338-detection rate strongly decreased by factors of 2.6 (core A) and 4.6 (core B) along a vertical profile (Fig.1) from the sediment surface to 10 cm depth. Below 10 cm depth, the detection rate was too low for further FISH analysis (<20% of total DAPI cell counts). Depth profiles of bacterial rRNA were in good accordance with profiles of FISH-detected cells. Recovered rRNA was highest near the surface (up to 13.6 μg ml^{-1} sediment) and decreased with depth to 2.3 μg ml^{-1}.

Archaea mainly occurred only in numbers near the detection limit, set at 1% of DAPI-stained cells (Tab.2). Only in the uppermost layer were *Archaea* found in higher numbers, with up to 6.4% of DAPI cell counts and 1.9 x 10^8 cells ml^{-1}. Below the surface layer, the relative contribution of *Archaea* remained relatively constant at approximately 1.0 to 1.5% of total DAPI cell counts along a vertical profile. No increase of *Archaea* cell numbers was detected in sediment layers at depths of 11-15 cm. Quantification of *Archaea* by slot-blot hybridization were in the same range (0.6-1.7% of total rRNA) as determined by FISH.

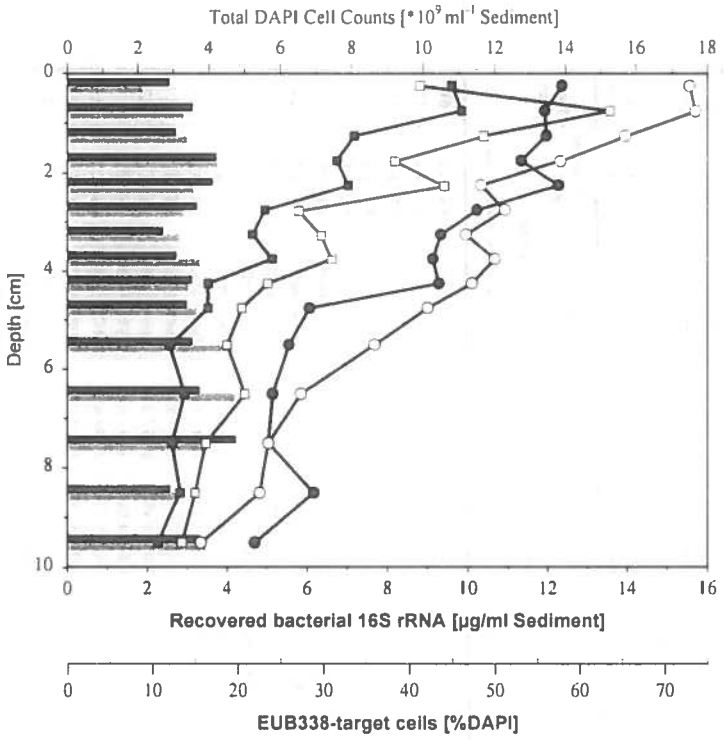

Fig.1: Depth profile of total cell counts, EUB338 counts and recovered bacterial 16S rRNA in duplicate cores.

by slot-blot hybridization were in the same range (0.6-1.7% of total rRNA) as determined by FISH.

Eukaryotic rRNA was quantified using probe EUK1379. Highest percentages were detected in the upper layers (0-3.25 cm depth). The mean in this region was 20.8±5.8% of total rRNA, as compared to 13.2±2.3% in the layers between 3.75 and 9.5 cm depth. rRNA detected by the bacterial, archaeal, and eukaryotic probes were 80-100% of total rRNA as quantified using universal probe UNI1390. Especially in the upper layers, only about 80% of total rRNA was detected with the domain specific probes.

The two parallel cores were quite similar in total cell numbers, FISH detection rates, and recovered rRNA (Fig.1, Tab.2). Therefore, in the following sections mean values of the two cores are discussed.

***Cytophaga/Flavobacterium* cluster.** A large fraction of the microbial community could be affiliated with the *Cytophaga/Flavobacterium* cluster (Table2, Fig.2.) Their relative abundance ranged from 11% (3.5×10^8 cell ml^{-1}) in the uppermost layers to 3% (2.0×10^8 cell ml^{-1}) at 5 cm depth. Below 5 cm, CF319a-target cells were near or below detection limit. *Cytophaga/Flavobacterium* rRNA detection was also highest at the

TABLE 2. Quantification of bacteria by FISH and rRNA slot blot hybridization in Smeerenburgfjorden sediments (Core A / Core B).

Depth [cm]	Absolute prokaryotic cell numbers [$*10^9$ ml^{-1}]	total RNA [Uni1390] ng RNA ml^{-1}	*Bacteria* [EUB338] % of total DAPI cell counts	% of universal RNA	ng RNA ml^{-1}	*Archaea* [Arch915] % of total DAPI cell counts	% of universal RNA	ng RNA ml^{-1}	*Eucarya* [EUK1379] % of universal RNA	ng RNA ml^{-1}	*Cytophaga/Flavobacteria* [CF319a] % of total DAPI cell counts	% of prokaryotic RNA	ng RNA ml^{-1}
0.25	2.9 / 2.1	15658 / 18429	57.9 / 72.9	61.3 / 47.8	9601 / 9845	6.4 / 3.3	1.7 / 0.9	264 / 169	19.2 / 26.3	3010 / 4841	9.6 / 12.8	6.1 / 5.4	606 / 481
0.75	3.5 / 3.3	14750 / 22860	55.9 / 73.6	66.7 / 59.3	9845 / 13564	1.8 / 1.8	1.3 / 1.2	191 / 282	12.4 / 15.9	1829 / 3629	10.3 / 10.4	5.5 / 5.8	554 / 799
1.25	3.1 / 3.4	13943 / 20448	56.1 / 65.4	51.5 / 50.8	7179 / 10391	2.9 / 1.8	1.0 / 1.0	142 / 206	25.1 / 30.4	3500 / 6225	7.4 / 6.8	4.1 / 4.1	299 / 433
1.75	4.2 / 4.2	11449 / 13035	53.1 / 57.7	58.9 / 62.8	6744 / 8183	2.1 / 1.5	1.2 / 1.1	135 / 142	23.9 / 15.2	2734 / 1983	7.2 / 4.8	4.3 / 3.8	297 / 315
2.25	4.1 / 3.5	11276 / 18647	57.5 / 48.4	62.3 / 50.5	7028 / 9417	1.8 / 1.5	1.2 / 1.0	133 / 188	18.0 / 20.8	2026 / 3883	7.6 / 3.0	4.2 / 5.4	299 / 522
2.75	3.7 / 3.3	7795 / 8318	47.9 / 51.2	63.4 / 69.6	4944 / 5791	1.4 / 0.8	1.2 / 0.9	93 / 75	21.4 / 12.0	1666 / 995	8.5 / 7.1	4.3 / 3.6	217 / 213
3.25	2.7 / 3.2	8266 / 9157	43.7 / 46.6	56.1 / 69.3	4638 / 6344	1.4 / 1.7	1.0 / 0.8	86 / 73	25.8 / 12.0	2129 / 1258	7.6 / 3.9	4.1 / 3.3	193 / 215
3.75	3.1 / 3.7	7896 / 9342	42.8 / 50.0	64.9 / 70.8	5125 / 6613	1.4 / 1.7	1.1 / 0.6	87 / 58	12.1 / 13.7	954 / 1181	8.2 / 3.3	3.7 / 3.4	192 / 229
4.25	3.5 / 3.4	5964 / 6494	43.5 / 47.3	59.2 / 77.3	3531 / 5019	1.4 / 0.4	1.4 / 0.8	83 / 52	18.3 / 12.6	1093 / 895	4.3 / 2.8	4.9 / 3.1	177 / 159
4.75	3.4 / 3.6	5559 / 5952	28.4 / 42.2	63.4 / 73.5	3526 / 4373	1.4 / 1.1	1.4 / 0.9	79 / 54	15.3 / 13.8	853 / 811	4.4 / 1.8	4.2 / 3.2	152 / 140
5.5	3.5 / 4.6	3306 / 4956	26.0 / 36.0	77.7 / 80.6	2570 / 3997	n.d.	1.4 / 1.0	46 / 49	8.3 / 15.5	274 / 769	1.8 / 2.0	4.0 / 3.4	106 / 137
6.5	3.7 / 4.7	3345 / 4897	24.1 / 27.4	87.6 / 90.8	2929 / 4448	n.d.	1.6 / 0.9	55 / 46	10.1 / 13.1	339 / 643	1.0 / 1.7	3.7 / 2.7	112 / 122
7.5	4.7 / 3.9	3283 / 3972	23.6 / 23.6	80.1 / 87.5	2631 / 3474	n.d.	0.9 / 1.2	31 / 46	12.4 / 13.5	408 / 536	0 / 0	3.5 / 3.1	93 / 110
8.5	2.9 / 3.1	3599 / 4331	28.9 / 22.6	78.5 / 73.9	2824 / 3200	1.0 / 1.7	1.3 / 1.1	45 / 46	11.7 / 12.9	421 / 559	0 / 0.5	3.4 / 3.5	97 / 112
9.5	3.7 / 3.9	3199 / 3589	22 / 15.7	71.1 / 79.7	2273 / 2861	1.0 / 0.9	1.1 / 1.0	35 / 38	12.6 / 14.9	402 / 534	0 / 0	3.5 / 3.1	81 / 90
11	3.9 / 3.4					1.7 / 0.7							
13	2.7 / 3.4					0.6 / 0.8							
15	2.5 / 3.4					0.6 / 1.2							
17	3.3 / 2.7												
19	2.7 / 2.1												

Depth [cm]	Beta-subclass of Proteobacteria [Bet42a] % of prokaryotic RNA	ng RNA ml^{-1}	Gamma-subclass of Proteobacteria [Gam42a] % of total DAPI cell counts	% of prokaryotic RNA	ng RNA ml^{-1}	clone sequences and symbionts affiliated to the gamma-subclass of *Proteobacteria* [Gam660] % of total DAPI cell counts	% of prokaryotic RNA	ng RNA ml^{-1}	Planctomycetales [Pla886] % of total DAPI cell counts	% of universal RNA	ng RNA ml^{-1}	Gram-positive bacteria [GP] % of prokaryotic RNA	ng RNA ml^{-1}
0.25	1.6 / 1.7	161 / 155	12.1 / 9.0	17.6 / 19.4	1739 / 1744	2.9 / 2.9	11.7 / 6.9	1153 / 621	2.2 / 1.9	30.7 / 17.0	4815 / 3131	0 / 0.2	0 / 17
0.75	1.8 / 1.7	180 / 229	11.6 / 9.2	20.7 / 19.4	2075 / 2813	1.8 / 2.7	9.5 / 10.5	950 / 1449	3.0 / 2.8	23.5 / 16.1	3464 / 3684	0 / 0.4	0 / 52
1.25	1.8 / 1.5	132 / 159	8.9 / 9.5	22.2 / 17.8	1626 / 1888	2.5 / 2.8	11.6 / 10.8	851 / 1142	3.6 / 3.5	53.6 / 19.7	7468 / 4037	0 / 0.5	0 / 49
1.75	1.4 / 1.6	99 / 134	8.0 / 6.9	20.2 / 16.8	1388 / 1402	0.8 / 2.0	17.0 / 8.2	1170 / 682	2.8 / 2.7	26.0 / 20.6	2975 / 2683	0.1 / 1.4	4 / 116
2.25	1.3 / 1.6	96 / 158	7.3 / 6.2	17.5 / 16.2	1254 / 1558	1.6 / 3.1	10.8 / 11.4	772 / 1094	3.7 / 3.8	19.1 / 17.2	2154 / 3204	0.5 / 1.3	35 / 129
2.75	1.1 / 1.2	58 / 72	5.5 / 6.1	14.5 / 13.3	733 / 780	1.2 / 2.1	18.1 / 6.1	910 / 359	2.8 / 3.9	15.8 / 13.1	1229 / 1091	0.7 / 2.2	36 / 129
3.25	1.3 / 1.2	60 / 75	4.7 / 4.7	14.5 / 12.5	686 / 804	2.6 / 2.7	18.4 / 6.7	871 / 431	3.5 / 3.3	19.1 / 14.1	1578 / 1287	0.3 / 2.6	16 / 167
3.75	1.3 / 1.1	70 / 77	8.7 / 6.0	14.7 / 12.6	764 / 841	2.6 / 2.8	20.1 / 9.0	1050 / 599	3.1 / 3.6	19.3 / 13.5	1524 / 1257	0.2 / 2.1	13 / 137
4.25	1.1 / 1.1	40 / 57	4.2 / 4.2	13.7 / 11.8	495 / 596	2.2 / 2.5	17.6 / 7.0	635 / 353	1.7 / 1.9	16.2 / 15.2	964 / 984	0 / 1.2	0 / 63
4.75	1.3 / 1.1	47 / 45	5.1 / 3.6	13.4 / 11.5	483 / 509	2.2 / 2.7	20.4 / 9.5	734 / 423	1.8 / 1.8	26.5 / 14.0	1475 / 831	0 / 0.7	0 / 31
5.5	1.2 / 1.1	31 / 45	4.4 / 5.0	11.9 / 12.0	311 / 486	2.8 / 2.4	14.2 / 14.0	3730/ 565	2.9 / 1.5	21.8 / 16.0	720 / 793	0 / 0.3	0 / 13
6.5	1.2 / 1.2	36 / 56	5.8 / 4.0	11.2 / 11.6	336 / 520	1.8 / 2.1	16.6 / 14.2	496 / 638	2.5 / 3.1	27.8 / 14.3	931 / 701	0 / 0.1	0 / 6
7.5	1.2 / 1.1	31 / 38	6.4 / 3.0	9.3 / 11.8	246 / 415	2.4 / 1.3	18.3 / 11.6	4878 / 408	1.7 / 2.8	31.0 / 14.5	1019 / 575	0 / 0	0 / 0
8.5	1.2 / 0.9	26 / 30	4.2 / 2.9	9.3 / 11.4	268 / 369	0.6 / 1.9	19.4 / 12.0	557 / 391	1.3 / 2.0	31.8 / 15.7	1143 / 681	0 / 0.2	0 / 6
9.5	0.9 / 1.0	20 / 28	4.0 / 3.0	10.0 / 11.7	231 / 338	1.8 / 0.4	21.3 / 12.4	493 / 359	1.9 / 1.2	38.7 / 19.0	1238 / 681	0 / 1.4	0 / 40

sediment surface (5.7% of prokaryotic rRNA) and decreased slightly to 3.3% at 9.5 cm. CF319a-target cells were morphologically highly diverse, and included long and short rods (0.5-1.5 µm in length), filaments (up to 10 µm length) and cocci. About three-quarter of the detected cells were very small (≤ 0.5 µm). Several very thin filaments could barely be detected by DAPI-staining. Some of the *Cytophaga/Flavobacterium* cells were found attached to sediment particles or other organic matrices (Fig.3). These cells were difficult to remove from the particles by sonication.

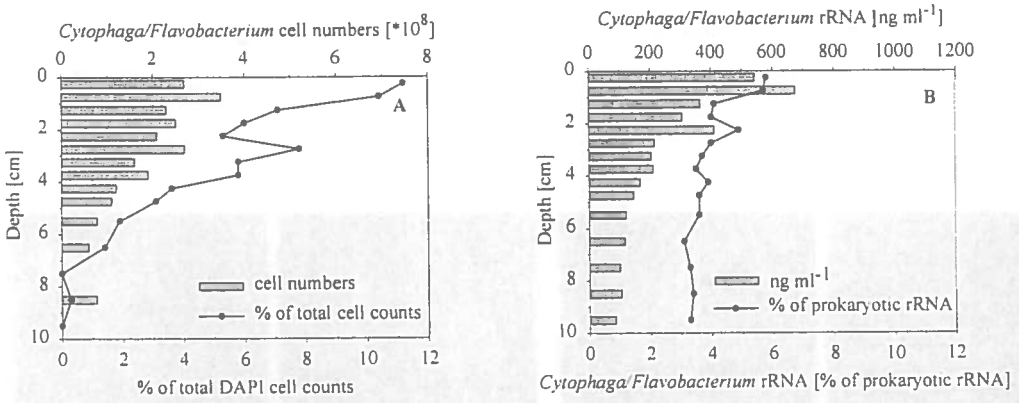

Fig.2: Depth profile of cell numbers (A) and rRNA (B) of members of the *Cytophaga/Flavobacterium*-cluster detected by probe CF319a. Mean of two parallel cores is shown.

Planctomycetales. Probe PLA886 is specific for *Pirellula* spp., *Planctomyces* spp., *Isophaera* spp., and several clone sequences within the order *Planctomycetales*. Furthermore, the probe also binds to a wide variety of eukaryotic 18S rRNAs. For FISH analysis, this lack of specificity is not relevant because, in general, a visual differentiation of *Eucarya* and *Bacteria* is possible. Members of the *Planctomycetales* made up a quantitatively important fraction of the microbial community in Smeerenburgfjorden sediments and ranged between 1.5% and 3.7% of total prokaryotic cell counts. There was no clear maximum visible at any specific depth. The highest detection corresponded to 1.4×10^8 cells ml^{-1} sediment. The cells were usually large cocci, approximately 1 µm in diameter (Fig. 3); occurring as single or rosette-forming cells or in disordered clusters of about ten cells. All target cells showed a bright fluorescence signal. In slot-blot hybridization, the problem of hybridization of PLA886 to eukaryotic rRNA became relevant. Very high values (13.1-38.7% of total rRNA) were detected. A comparison of slot-blot profiles for probes PLA886 and EUK1379 showed similar maxima.

γ-proteobacteria. γ-proteobacteria, as detected by probe GAM42a, comprised a dominant group in Smeerenburgfjorden sediments (Table 2, Fig.4). In the upper layers, this group accounted for up to 10.5% of total DAPI cell counts. Detection by FISH decreased slightly with depth and was lowest at 10 cm depth, with 3.5% of total DAPI cell counts. The morphology of the GAM42a-target cells was quite diverse (Fig. 3). Cell size varied, but a large fraction of detected cells was very small (size ≤ 0.5 µm). The majority of target cells had a very bright FISH signal, indicating a high cellular rRNA content. The γ-proteobacterial rRNA also made up a quantitatively important fraction of the microbial community rRNA, with up to 20.0% of prokaryotic rRNA hybridizing to GAM42a. The relative contribution to the prokaryotic rRNA decreased by a factor of approximately 2 from the surface to 10 cm depth.

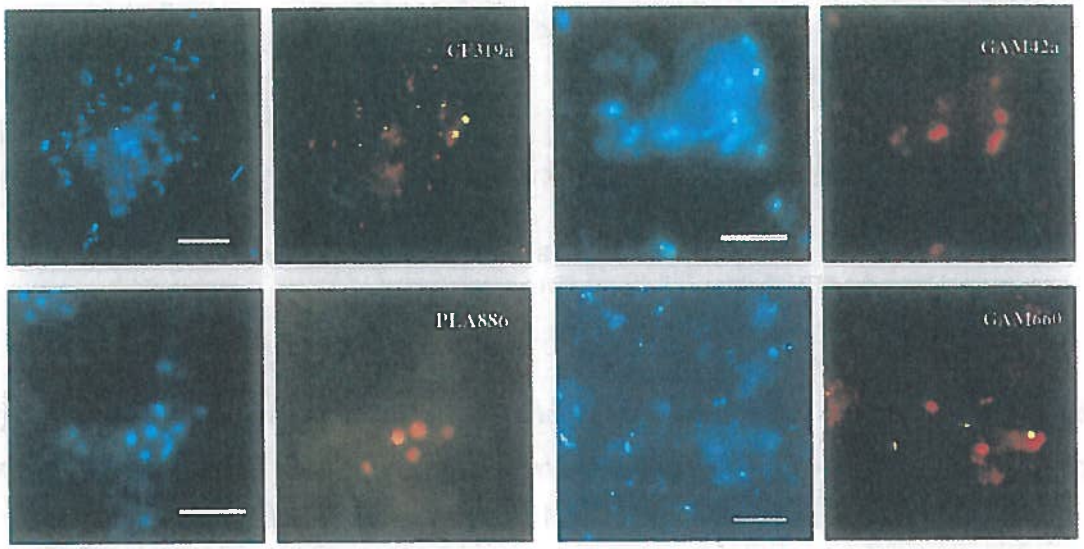

Fig.3: Epifluorescence micrographs of bacteria in sediment samples from Smeerenburgfjorden (Svalbard). Specific hybridizations for the *Cytophaga/Flavobacterium* cluster (CF319a), for *Planctomycetales* (PLA886), the γ-proteobacteria (GAM42a) and a γ-proteobacterial subgroup which is affiliated to free-living and symbiotic sulfur-oxidizing bacteria (GAM660) and corresponding DAPI staining (same microscopic field). Bar, 5 µm (applies to all panels).

Potential sulfur-oxidizing bacteria within the γ-proteobacteria. Probe GAM660 was designed to be specific for clone sequences affiliated with free-living and endosymbiotic sulfur-oxidizing bacteria which were abundant in a Svalbard sediment clone library (56). Because of their phylogenetic affiliation, these sequences could potentially originate from sulfur-oxidizing bacteria. In addition to our clone sequences, probe GAM660 also targets closely related (up to 97.9%) γ-proteobacterial sequences which were retrieved from other marine sediments (8, 37, 38), endosymbionts of *Riftia pachyptila*, other vestimentiferan

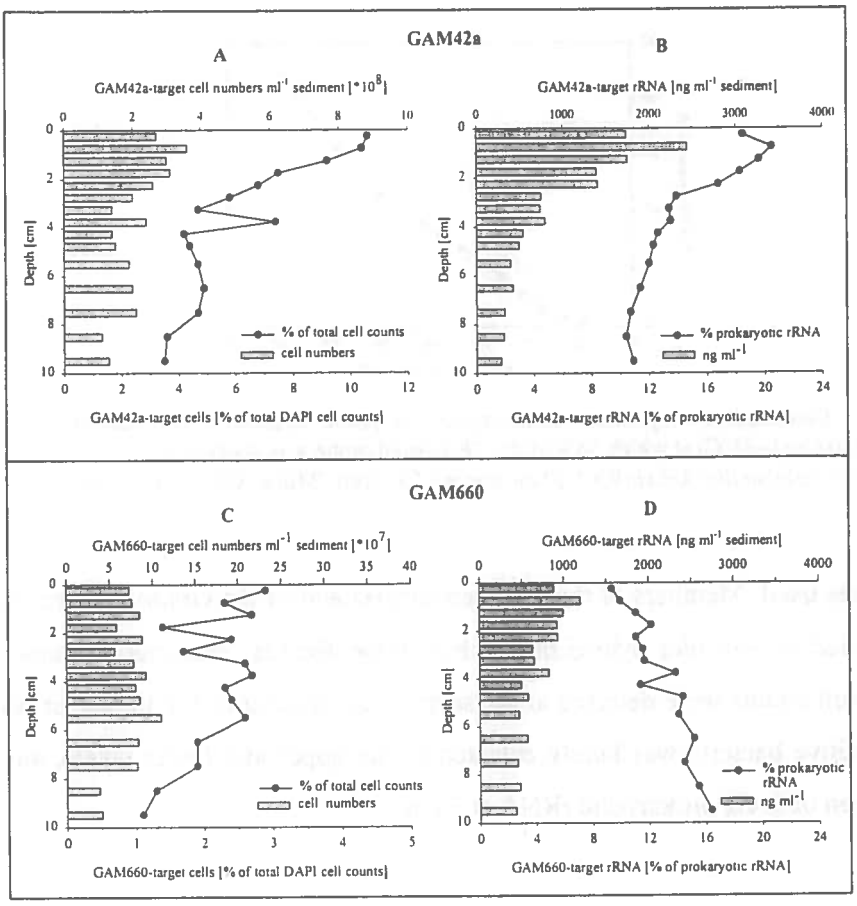

Fig.4: Depth profile of the γ-proteobacteria (GAM42a) and of a subgroup within this group which is affiliated to endosymbiotic or free-living sulfur-oxidizing bacteria (GAM660): cell numbers (A, C) and rRNA (B,D). For GAM42a mean of two cores is shown.

tubeworms and of several bivalves (13-16, 20, 32), *Thiobacillus ferrooxidans*, *Halothiobacillus kellyi* and *Coxiella burnetii* (Table 3). A clear discrimination between target and non-target organisms was possible with FISH as well as with rRNA slot-blot hybridization (Fig. 5). Probe GAM660 hybridized to free-living bacteria in Smeerenburgfjorden sediment samples. Up to 2.9% of total DAPI cell counts (9.4×10^7 cells ml^{-1}) were detected in the surface layer. In deeper layers, the detection rate remained relatively constant and varied between 0.4 and 3.1% of total DAPI cell counts. In general, targeted cells were cocci that very often occurred as diplococci (Fig.3). Due to their small size, it was impossible to investigate targeted cells for the presence of sulfur inclusion bodies. The FISH-detected fraction was relatively small compared with the fraction (13.2±4.2% of prokaryotic RNA) detected by slot-blot hybridization.

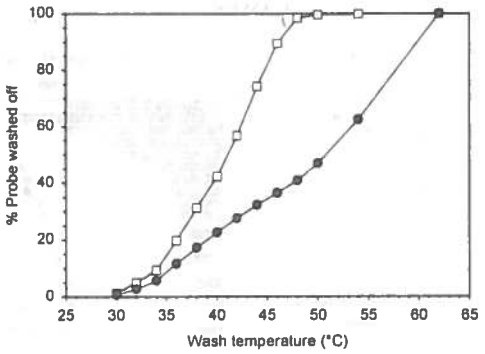

Fig.5: Dissociation temperature determination of probe GAM660. The dashed line shows the temperature (~52°C) at which 50% of the ^{32}P-labeled probe was washed off.
(●, *Thiohalobacillus kellyi* rRNA (0 mismatch); □, strain 'Milos AII2' rRNA (1 mismatch control).

Other probes used. Members of the β-proteobacteria and of the Gram-positive bacteria were only quantified by slot-blot hybridization. For probe Bet42a, maximum values of 1.7% of total DAPI cell counts were detected at the surface, decreasing to 1% in deeper layers. rRNA of Gram-positive bacteria was barely detected in the upper and lower layers, and reached a maximal mean of 1.4% prokaryotic rRNA at 3 cm.

DISCUSSION

Total cell counts in the Smeerenburgfjorden sediments were relatively constant along a vertical profile from the sediment surface to 20 cm depth. The average abundance of $3.4 \pm 0.6 \times 10^9$ ml^{-1} was comparable with previous reports for other marine sediments (e.g. (39, 61, 75)) although in contrast to our results, all other studies including one of four other sampling sites off the coast of Svalbard (61) reported decreasing cell numbers with depth. In Svalbard sediments, Sahm et al. reported cell numbers decreased with depth by factors of 3, 7 and 9 within the first 28 cm (61). In Wadden Sea sediments, the total cell numbers decreased by a factor of 2.4 within the first 5 cm of the sediment (39). Wellsbury et al. (75) reported constant cell numbers in the uppermost layers (up to 8 cm depth) of an estuarine sediment. They explained this rather unusual depth profile by a high tidal influence and high sediment porosity. In our case, tidal influence can be excluded. The sediment, however, was characterized by a relative high water content in the first 2-3 cm. Since most sediment bacteria can be found attached to particles, a higher pore water content leads to lower cell numbers per ml and could be one cause of the constant cell numbers throughout the profile.

As in other sediments (61, 63, 65) the recovered rRNA was mainly of bacterial and eukaryotic origin. *Archaea* made up only a minor part of the microbial community with about 1-3% of total cells and of prokaryotic rRNA. Although a relatively large number of cells were not detected by the domain-specific probes in FISH, the lack of detection of significant amounts of archaeal rRNA in slot-blot hybridization suggests that *Archaea* are not a major component of this arctic sediment. To date, probe ARCH915 includes more than 95% of currently-available archaeal sequences in the databases. Low *Archaea* counts are in accordance with previous studies from other marine sediments (39, 61).

The *Cytophaga/Flavobacterium* cluster and the order *Planctomycetales* typically contain aerobic species. *Cytophaga/Flavobacterium* have been shown to be abundant in the marine water column (18, 21). Recently, Llobet-Brossa et al. found significant cell numbers of both groups in Wadden Sea sediments, even in anoxic zones (39). Data from clone libraries derived from several marine sediments (22, 37, 38, 56) and a freshwater sediment (45) supported this finding. Input of complex organic substrates to anaerobic sediments resulted in a strong increase among members of the *Cytophaga/Flavobacterium* cluster (60). These findings indicate a potential ecological relevance of these bacteria as hydrolytic fermentative organisms in a mainly anaerobic habitat. In our study, the *Cytophaga/Flavobacterium* cluster along with the γ-proteobacteria and sulfate reducers was one of the three most abundant groups, with high numbers of more than 1.5×10^8 cells ml^{-1} also in the anoxic layers up to a depth of 4.75 cm. Calculations of cellular rRNA contents of *Cytophaga/Flavobacterium* cells made by combining FISH-detected cell numbers and the detected rRNA revealed relatively constant cellular rRNA contents with depth (range 0.1 to 0.2 fg rRNA cell). *Planctomycetales* made up between 1.2 and 3.9 % of DAPI stained cells down to a depth of 9.5 cm, with a maximum in their proportional contribution at 2.25 cm. These data support the hypothesis that these bacterial groups are multiplying even in anoxic zones in the sediment.

A reliable quantification of *Planctomycetales* rRNA was not possible because of the cross-hybridization of probe PLA886 with a wide variety of *Eucarya*. A comparison of slot-blot profiles for probes PLA886 and EUK1379 showed similar shapes and maxima. Therefore, there was presumably a very high contribution of eukaryotic rRNA to PLA886-target rRNA. Since not all organisms targeted by EUK1379 are also targeted by PLA886, a simple subtraction of the values is not possible.

Sulfur-oxidizing bacteria isolated from marine sediments are often members of the genera *Thiomicrospira* (6, 7, 33) or *Thiobacillus* (33, 58). In addition *Beggiatoa* spp./ *Thioploca* spp. have often been found in sediments and used for ecophysiological studies (25,

31, 44). *Thiomicrospira* spp. and *Thiobacillus* spp. were often retrieved from most probable number (MPN) dilution series for chemolithoautotrophic sulfur-oxidizing bacteria, but only in maximal numbers of 1.4×10^6 cells ml^{-1} sediment (6, 64). In MPN dilution series of Smeerenburgfjorden sediments, growth of chemolithoautotrophic sulfur-oxidizing bacteria was observed to 10^{-3} dilutions. This result contradicts the idea that they might be numerically abundant. Using the new probe GAM660 which is specific for 16S rDNA clone sequences affiliated to free-living or endosymbiotic sulfur-oxidizing bacteria retrieved from several marine sediments (8, 37, 38, 56, 72), an abundance of up to 1.1×10^8 cells ml^{-1} was demonstrated in Smeerenburgfjorden sediment. In Wadden Sea sediments, this group was also detected by FISH and accounted for up to 4.6×10^7 cells ml^{-1} sediment (up to 2.3% of total DAPI cell counts; Kolb & Ravenschlag, unpublished data). Further functional studies of GAM660-target organisms are needed to find out if these abundant bacteria are really sulfur oxidizers. Possible experiments include large-insert DNA libraries (59, 68) of GAM660-target cells for the identification of genes involved in the sulfur oxidation or the combination of microautoradiography with FISH, allowing the assignment of radiotracer uptake to specific phylogenetic groups (9, 35, 50).

In some layers, detection of the subgroup GAM660-target rRNA was even higher than rRNA yield of total γ-proteobacterial rRNA. Due to the stringent washing temperature hybridization with non-target organisms having one mismatch to the probe sequence can be excluded (Fig. 5). However, the discrepancy can not currently be clarified, because GAM42a targets 23S rRNA. GAM660 targets mostly uncultivated organisms for which the 23S rRNA sequences are yet unknown and can not be determined easily.

Relative contribution of GAM660 rRNA was significantly higher than for FISH-detected cells (2.4 to 32.3 fold). GAM660 also targets chemoautotrophic symbionts from several bivalve molluscs and tubeworms. Thus, the high relative percentage of GAM660-rRNA could mean a contribution of rRNA derived from endosymbiotic bacteria of bivalves or other eukaryotic hosts. Such bacteria would not have been counted in FISH due to exclusion during pipetting or sedimentation in dilution steps. The rRNA of these organisms and their hosts, however, might be included in the extracted rRNA used for slot-blot hybridization. Chemoautotrophic symbionts have not yet been cultured from their hosts, nor has a free-living stage of the symbionts been isolated from the environment. There is evidence that some hosts obtain their symbionts via environmental transmission (23, 24, 34), which involves the reinfection of the new host generation from an environmental stock of free-living symbiont forms as done by, for example, *Codakia orbicularis* (23). GAM660-target cells could

potentially represent such a free-living symbiont form. The vertical profiles of GAM660-detected rRNA and GAM660-target cells showed no stratification as might be expected for aerobic chemoautotrophic organisms. However, nitrate respiration has been demonstrated in several endosymbionts, for example from *Solemya reidi* (76), *Riftia pachyptila* (29), and *Lucinoma aequizonata* (28), as well as in the ectosymbionts of nematodes (27). For the endosymbiontic bacteria, motility of the hosts might be another explanation for the lack of zonation.

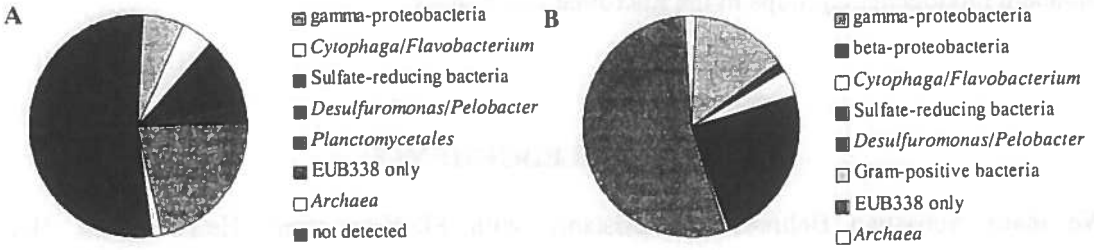

Fig. 6: Overview of the microbial community structure of Smeerenburgfjorden sediments as revealed by FISH (A) and quantitative rRNA slot-blot hybridization (B). For the individual phylogenetic groups mean of the abundance along the vertical profile was calculated. Fractions shown indicate the percentage of total DAPI-stained cells (FISH) and the relative percentage of prokaryotic rRNA (slot-blot), respectively. Since probe PLA886 targeting *Planctomycetales* is also targeting a wide variety of Eukaryotes (see text) the results are not included in part B. Due to the FISH results it can be expected, that a fraction of the unidentified EUB338-target rRNA is formed by *Planctomycetales*

Adding up the mean detection rates along a vertical profile for the different bacterial groups (including the large fraction of sulfate-reducing bacteria (55)), 57.8 ± 12.7% of total detectable bacterial cells (23.9 ± 7.5% of total DAPI cell counts) and 44.9 ± 5.5% of bacterial rRNA could be assigned to specific phylogenetic groups (Fig.6). One explanation for the relatively large "black box" could be the limited coverage with the current probe set, which has been shown by the rapid growth of the 16S rRNA sequence database to be rather incomplete. Furthermore, there are certainly other bacterial groups which make up a quantitatively important fraction in Smeerenburgfjorden sediments. For example, 16S rDNA sequences affiliated to the order *Verrucomicrobiales* (26) were repeatedly found in clone libraries from marine sediments (56, 72) or marine snow (54) and sequences related to *Arcobacter* spp. or other ε-proteobacteria were repeatedly retrieved from marine sediments (4, 8, 36-38, 51, 72). Furthermore, the genus *Arcobacter* accounted for up to 1.6% of total cell counts in Wadden Sea sediments (39). An ability to carry out nitrate reduction and sulfide-oxidation has been reported for *Arcobacter* spp. (70, 71). Further studies will be needed to

investigate the quantitative contribution of *Verrucomicrobium* spp., *Arcobacter* spp. and as yet unknown phylogenetic groups to microbial communities of marine sediments.

This study reports the first rRNA profiles for major phylogenetic groups in marine sediments and compares these data with abundances determined by FISH. More combined quantitative studies of microbial community structures in marine sediments are needed to identify common benthic features. Furthermore, studies are needed to identify the organisms contributing to the large "black box". A major goal for future work will be to combine of these data with measurements of microbial activities to address the functional role of abundant phylogenetic groups in the microbial community.

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4

Isolation of a Psychrophilic Strain of *Thiomicrospira* spp. from Marine Arctic Sediments and Description of *Thiomicrospira arctica* sp. nov.

Katrin Ravenschlag, Jan Kuever, Ruth Meinke,
Rudolf Amann, and Thorsten Brinkhoff

In preparation

Isolation of a Psychrophilic strain of *Thiomicrospira* spp. from marine Arctic sediments and Description of *Thiomicrospira arctica* sp. nov.,

KATRIN RAVENSCHLAG[1], JAN KUEVER[2], RUTH MEINKE[2], RUDOLF AMANN[1], and THORSTEN BRINKHOFF[3]

Molecular Ecology Group[1] and Department of Microbiology[2], Max-Planck-Institute for Marine Microbiology, 28359 Bremen, Germany, and Institute for Chemistry and Biology of the Marine Environment, Carl von Ossietzky Universität, 26111 Oldenburg, Germany[3]

A psychrophilic chemolithoautotrophic sulfur-oxidizing bacterium was isolated from marine Arctic sediments taken off the coast of Svalbard with thiosulfate as the electron donor and CO_2 as carbon source. Comparative analysis of 16S rDNA sequences suggested that the new strain SVAL-E was a member of the genus *Thiomicrospira*. Further genotypic (DNA-DNA-similarity, DNA GC content) and phenotypic characterization revealed that strain SVAL-E (ATCC 700955, DSM 13458) likely represents a member of a new species, *Thiomicrospira arctica*.

SVAL-E was obligately autotrophic and strictly aerobic. Nitrate was not used as an electron acceptor. Chemolithoautotrophic growth was observed with thiosulfate, tetrathionate and sulfur. The temperature limit for growth was between -2°C and 20.8°C, with an optimum growth temperature of 11.5-13.2°C, which is about 13-15°C lower than the optima of all other *Thiomicrospira* spp. described so far.

The average maximum growth rate on thiosulfate was 0.07 h^{-1}. Cells are slightly bent rods like the cells of their closest relatives. To our knowledge the new strain is the first described psychrophilic chemolithoautotrophic sulfur-oxidizing bacterium.

Thiomicrospira species are obligately chemolithoautotrophic sulfur-oxidizing bacteria which have been detected in different habitats all over the world. They were found in several marine sediments, e.g. in intertidal mud flats and a continental shelf sediment, in hydrothermal vent

systems, but also in hypersaline ponds, a saline spring and a freshwater pond (e.g., 2, 8, 10, 13, 21). As indicated by molecular biological or microbiological techniques, members of this genus appear to be ecologically significant at hydrothermal vent sites (6, 11), while in an intertidal mud flat habitat *Thiomicrospira* spp. were found in much lower densities than other sulfur-oxidizing bacteria (5).

In general, very little is known about psychrophilic sulfur-oxidizing bacteria. Mats of *Beggiatoa* species were described for cold seeps (1), but it is unknown whether these species are psychrophilic or not. In a recent study Teske et al. (18) reported that sulfur-oxidizing bacteria from cold deep-sea sediments and from hydrothermal vent systems show habitat-related differences in growth temperature. The temperature optima of these isolates, however, were not determined. In this study we aimed to obtain psychrophilic *Thiomicrospira* isolates from permanently cold marine Arctic sediments.

MATERIALS AND METHODS

Sources of organisms. Marine Arctic sediments were sampled off the coast of Svalbard in July 1998. Strain SVAL-E originated from Jonsfjorden sediment (78°32.616N/12°18.075E; water depth 168 m; station E). The *in situ* temperature was around 0°C.

Enrichment, isolation and cultivation. Strain SVAL-E was obtained from a enrichment culture inoculated with a mud sample of the upper sediment layer (0-0.5 cm depth). The medium (TP) used and the isolation procedure were the same as described previously (4), with the exception that the cultures were incubated at 4°C.

Growth experiments. Batch cultures were grown at 4°C in 2 l flasks containing 500 ml medium on a rotary shaker (100 rpm) in the dark. Routine cultivation of the isolates and utilization of different substrates were investigated in 15 ml tubes containing 5 ml TP medium or in 50 ml tubes containing 20 ml medium. Large-scale cultivation was done in 3 l and 20 l glass carboys supplied with 40 mM thiosulfate, in which the pH was monitored by a sterilized pH electrode (Ingold, Germany) and readjusted by titration with Na_2CO_3 (1 M) through a personal computer program controlling a peristaltic pump. The program was developed by Volker Meyer at the Max-Planck-Institute for Marine Microbiology. The fermenter was aerated with sterile pressurized air through sparkling devices.

The maximum growth rate in TP medium was determined at 4°C by total 4′, 6-diamidino-2-phenylindole (DAPI) cell counts (7), and increase in optical density. For DAPI

cell counts means were calculated from 10-20 randomly chosen fields on each filter section, corresponding to 800-1000 DAPI stained cells.

An estimate of the optimal pH value and the lowest and highest values tolerated by the isolates was obtained by using TP medium adjusted to different initial pH values (in steps of 0.5 pH values) and supplied with pH indicators covering different pH ranges (bromocresol green, 3.8 - 5.4; bromocresol purple, 5.2 - 6.8; bromothymol blue, 6.0 - 7.5; phenol red, 6.8 - 8.4; phenolphtalein, 8.2 - 9.8). The pH-range for growth was determined by screening for acidification on the basis of colour change of the pH indicator. The optimum pH at 4°C was also determined in a fermenter by measuring the oxygen turnover rates at different pH values between pH 6 and 9 in steps of 0.5 pH units. The experiment was started after the fermenter reached equilibrium. After the substrate supply was stopped the medium was saturated with oxygen. Then aeration of the fermenter was stopped and the first pH value adjusted. Substrate supply was switched on and the decrease of oxygen was measured. After oxygen concentration reached 0%, the substrate supply was switched off, the fermenter was aerated and the next pH value was adjusted. The decrease of oxygen at different pH values was plotted against time and the steepness of the straight line in the range between 20 and 80% oxygen saturation was determined by linear regression. The determined steepness was plotted against the pH values and the 2nd order polynom was regressed. From the obtained curve the maximum pH optimum was determined.

The optimal growth temperature was determined in a thermally insulated aluminium block, which was heated electrically to 32°C at one end and cooled to -3°C with a refrigerated circulation thermostat at the other end. The block contained 30 rows of four holes, so that samples could be incubated simultaneously at temperature intervalls of 0.5°C with maximum of four replicates. The temperature limits of growth were established by screening for acidification for 30 d. The optimal growth temperature was determined within 36-48 h after inoculation.

The Na^+ requirement was determined as described previously (4).

Utilization of inorganic and organic electron donors and anaerobic growth. The utilization of inorganic and organic electron donors, including growth on hydrogen, and tests for anaerobic growth were carried out as described by Brinkhoff and co-workers (4).

DNA base composition and DNA-DNA hybridization. The G+C content and the DNA-DNA hybridizations were done at the DSMZ, Braunschweig, Germany, and performed as described previously (4).

PCR amplification of 16S rDNA fragments. Two primers, GM3F and GM4R (11) were used to amplify the almost complete 16S rRNA gene. PCR amplification was performed as described by Muyzer and co-workers (11). PCR products were purified by using a QiaQuick PCR Purification Kit (Qiagen Inc., Chatsworth, Calif.).

Sequencing and phylogenetic analysis. PCR products were sequenced by *Taq* Cycle Sequencing with a model ABI377 (Applied Biosystems, Inc.) sequencer. Sequence data were analyzed with the ARB software package (17). Phylogenetic trees were calculated by parsimony, neighbor-joining, and maximum-likelihood analysis with different sets of filters. For tree calculation, only full length sequences were considered.

The organisms shown in the tree and the accession numbers of their sequences are as follows: *Anodontia philippina* gill symbiont, L25711; *Bathymodiolus thermophilius* gill symbiont, M99445; *Calyptogena magnifica* symbiont, M99446; *Codakia costata* gill symbiont, L25712; *Cycloclasticus pugetii*, U12624; *Francisella tularensis*, Z21931; *Hydrogenovibrio marinus*, D86374; *Methylophaga marina*, X95459; *Piscirickettsia salmonis*, U36941; *Rhizobium leguminusarum*, D12782; str. clone FL5, L10936; symbiont of *Solemya velum*, M90415; symbiont of *Vesicomya chordata* gill, L25713, L25714; *Thioalcalomicrobium aerophilum*, AF126548; *Thioalcalomicrobium sibericum*, AF126549; *Thiomicrospira chilensis* str. Ch-1, AF013975; *Thiomicrospira crunogena* str. MA-3, AF069959; *Thiomicrospira crunogena* str. L12, L01576; *Thiomicrospira frisia* str. JB-A2, AF013974; *Thiomicrospira kuenenii* str. JB-A1, AF013978; *Thiomicrospira pelophila*, L40809; *Thiomicrospira* sp. str. Art-3, AF013973; *Thiomicrospira* sp. str. JB-A1F, AF013976; *Thiomicrospira* sp. str. JB-B2, AF013972; *Thiomicrospira* sp. str. Milos-T1, AJ237757; *Thiomicrospira* sp. str. Milos-T2, AJ237758; *Thiomicrospira* sp. str. SL-1, AF013971; *Thiomicrospira* sp. str. MA2-6, L40811; *Thiomicrospira thyasirae*, AF016046; *Thiomicrospira* sp. str. SVAL-D, AJ404732; *Thiotrix nivea*, L40993; *Thyasira flexuosa* gill symbiont, L01575; *Wolbachia persica*, M21292.

Nucleotide sequence accession numbers. The 16S rDNA sequence will appear in the EMBL, Genbank, and DDBJ nucleotide sequence database under the accession no. AJ404731 (str. SVAL-E).

RESULTS

Isolation of strain SVAL-E. Subsequent to enrichment, a pure cultures was obtained by subculturing single colonies. Sequencing of the 16S rRNA genes of strain SVAL-E showed a phylogenetic affiliation to the genus *Thiomicrospira*. Highest similarities to yet described *Thiomicrospira* spp. was 96.1% to *Thiomicrospira chilensis* (see Table 1).

A PCR using specific primers (2) for the selective amplification of 16S rDNA from *Thiomicrospira* spp. failed.

Morphology. Cells of strain SVAL-E appear as single motile rods of 0.5-0.6 x 1.2-1.5 µm (Fig. 1b). They showed reduced levels of motility. Cells were Gram-negative and spore formation was absent.

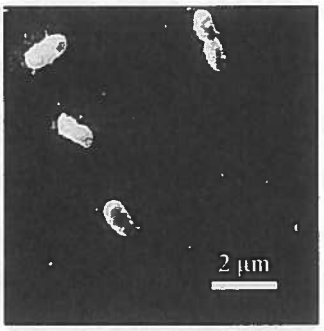

Fig. 1. Electron micrographs of strain SVAL-E, showing the typical rod-shaped cell morphology.

Growth conditions (Table 1). Strain SVAL-E was strictly aerobic and grew autotrophically on thiosulfate, tetrathionate and sulfur, but not on sulfite, thiocyanate and formiate. Growth of strain SVAL-E on thiosulfate lowered the pH to 5.1. Intermediate formation of elemental sulfur was observed on solid media and in liquid media. No growth occurred in TP medium supplemented with any of the organic substrates tested. The oxidation of thiosulfate was not inhibited by any of the organic substrates, except by acetate. Addition of vitamin B12 enhanced growth, but was not essential. Growth of strain SVAL-E was observed between pH 6.5 and 9.0 with an optimum pH of 7.5-8.0.

The temperature limit for growth was between -2.0 and 20.8°C with an optimum growth temperature of 11.5 - 13.2°C. Cells of strain SVAL-E, which were preincubated for two month at 10, 14 and 20°C, respectively, grew in a broader temperature range (-2.0 to 24°C) and showed a higher optimum growth temperature (14.5-17.3°C, 15.3-17.6°C, and 15.7-17.3°C after preincubation at 10°C, 14°C, and 20°C, respectively).

Strain SVAL-E was able to grow at a Na^+ concentrations between 40 and 1240 mM. A Na^+ concentration at 250 mM resulted in best growth.

DNA base ratio. The DNA G+C content of strain SVAL-E is 43.5 ± 0.7 mol%.

Phylogenetic analysis and DNA-DNA hybridizations. The similarity matrix values and the results of the DNA-DNA hybridization are shown in Tables 2 and 3, respectively.

Table 1: Morphological and physiological characteristics among *Thiomicrospira* spp.*

	Organism			
	T. pelophila	*T. frisia*	*T. chilensis*	SVAL-E
Shape	vibrio	rod	rod	rod
Width (μm)	0.2-0.3	0.3-0.5	0.3-0.5	0.5-0.6
Length (μm)	1-2	1-2.7	0.8-2	1.2-1.5
Motility	+	+	+	+ $
G+C content (mol%)	45.7	39.6	49.9	43.5
Ubiquinone	Q-8	Q-8	Q-8	n.d.
Maximum growth rate (h^{-1})	0.3	0.45	0.4	0.07
optimal pH	7.0	6.5	7.0	7.5-8.0
pH range	5.6-9.0	4.2-8.5	5.3-8.5	6.5-9.0
optimal temperature (°C)	28-30	32-35	32-37	11.5-13.2
temperature range (°C)	3.5-42	3.5-39	3.5-42	-2.0-20.8
optimal Na+ concentration (mM)	470	470	470	250
Na+ concentration range (mM)	40-1240	100-1240	100-1240	40-1240
Vitamin B12-dependent	+	-	-	-
Ribulose 1,5-bisphosphate carboxylase	+	+	+	n.d.
Formation of sulfur from thiosulfate at pH 7.0 in liquid medium	+	-	+	+

*: from (3, 4, 10) and own data; $: only few cells showed motility; n.d.: not determined

Table 2. 16S rDNA sequence similarity values (%) between strain SVAL-E and related Thiomicrospira spp.

		1	2	3	4	5	6	7	8
1	*Thiomicrospira pelophila*								
2	*Thiomicrospira crunogena* str. MA-3	92.6							
3	*Thiomicrospira kuenenii* str. JB-A1	92.2	95.6						
4	*Thiomicrospira frisia* str. JB-A2	91.4	94.7	93.9					
5	*Thiomicrospira* sp. str. Art-3	91.3	94.6	93.7	99.5				
6	*Thiomicrospira* str. Milos-T2	90.8	94.9	94.6	97.3	97.4			
7	*Thiomicrospira chilensis* str. Ch-1	91.7	94.6	93.2	95.3	95.1	95.8		
8	*Thiomicrospira* sp. str. SVAL-D	92.4	95.5	93.8	96.0	96.1	96.7	96.9	
9	***Thiomicrospira* sp. str. SVAL-E**	92.4	95.4	93.7	96.0	96.1	95.5	96.1	99.2

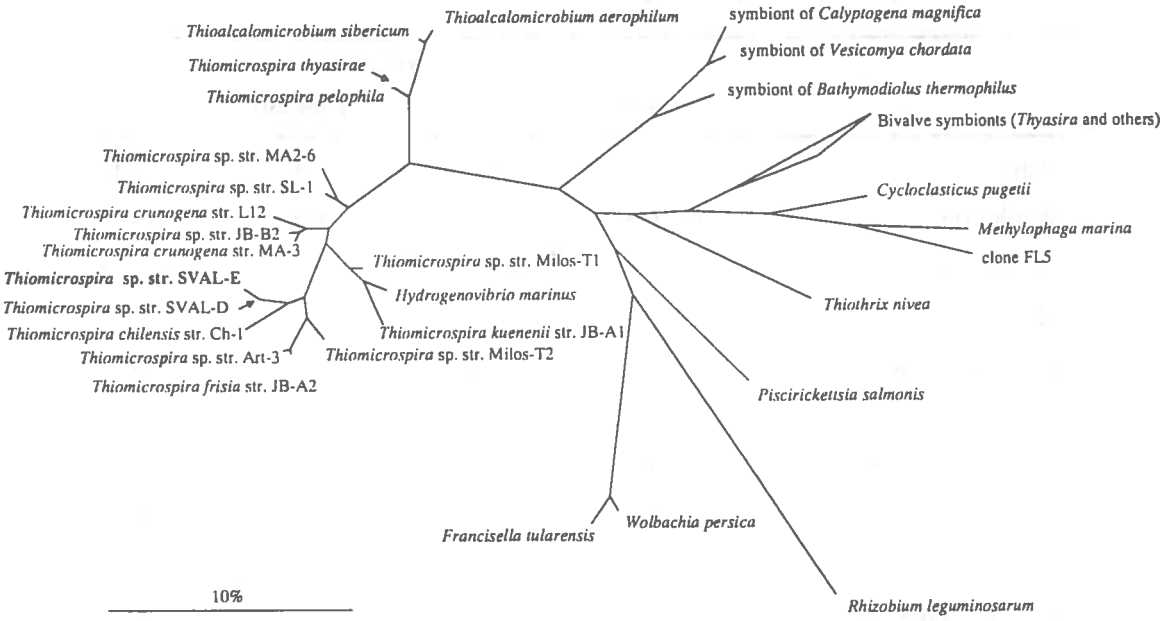

Fig.2: 16S rDNA phylogenetic tree showing the affiliation of the new psychrophilic isolate, strain SVAL-E, to other *Thiomicrospira* spp. and selected reference sequences of the gamma-group of Proteobacteria. The tree was calculated by maximum-likelihood analysis and based on nearly complete 16S rDNA sequences. Bar gives 10% estimated sequence divergence.

Comparison of the nearly complete 16S rRNA genes showed that strain SVAL-E has at least a 3.9% sequence divergence to all other described *Thiomicrospira* spp. The phylogenetic analysis (Fig. 2) places SVAL-E in a subbranch together with *Thiomicrospira frisia*, *Thiomicrospira chilensis* and *Thiomicrospira* sp. strain SVAL-D, which was isolated from Isfjorden sediment (Svalbard, 78°10.907N/14°34.124E).

The level of DNA-DNA hybridization between the different *Thiomicrospira* strains gives values of less than 56% for strain SVAL-E (Tab.3).

Table 3. Levels of DNA-DNA similarity for *Thiomicrospira* spp.

	% DNA-DNA similarity	
Organism	*T. chilensis*	*T. frisia*
T. chilensis	100	
T. frisia	18.0	100
str. SVAL-E	55.6	28.6

DISCUSSION

Recent investigations of Arctic sediments from the same habitat demonstrated a great diversity of bacteria involved in the sulfur-cycle (12, 16). Several new genera of sulfate-reducing bacteria could be isolated from this habitat and were identified as psychrophilic during further characterization (9). Psychrophilic chemolithoautotrophic sulfur-oxidizing bacteria, however, have to our knowledge not been described yet, even though wide areas of the worlds oceans constantly have temperatures below 4°C.

It is long known that *Thiomicrospira* species are present in the deep-sea, but all isolates characterized so far were obtained from hydrothermal vent habitats and not from cold environments and showed temperature optima between 25 and 32°C (8, 13, 20). A widely accepted definition for psychrophilic bacteria is that these have a temperature optimum for growth at about 15°C, a maximum temperature at about 20°C, and a minimum temperature at 0°C or lower (15). In contrast not only to the hydrothermal vent isolates but to all other so far described *Thiomicrospira* species the new strain SVAL-E fits these conditions.

The broader temperature range and the higher optimum growth temperature of cells of strain SVAL-E, which were preincubated for two month at 10, 14 and 20°C, respectively, indicates an adaptation to the changed growth conditions. It is known that the ability of psychrophiles and psychrotrophs to grow at low, but not moderate, temperatures depends on adaptive changes in cellular proteins and lipids. Changes in proteins are genotypic, and are related to the properties of enzymes and translation systems, whereas changes in lipids can be genotypic or phenotypic and are important in regulating membrane fluidity and permeability. The upper growth temperature limit can result from the inactivation of a single enzyme type or system, including protein synthesis or energy generation (14).

With the present strain the habitat range of the worldwide distributed genus *Thiomicrospira* is also extended to cold habitats. *Thiomicrospira* species appear to be adaptable to different environmental conditions and the main condition for their occurrence seems to be the presence of reduced sulfur compounds.

A PCR with primers specific for the genus *Thiomicrospira* (2) gave no product. Comparison of the 16S rRNA sequences showed several mismatches for the primers. Therefore, it is necessary to design new primers/probes to include all members of the genus.

The new isolate, strain SVAL-E, is a chemolithoautotrophic sulfur-oxidizing bacterium, clearly belonging to the genus *Thiomicrospira*. Genotypic and phenotypic characteristics, especially the low temperature optima, separate strain SVAL-E from all so far described

Thiomicrospira species and revealed that strain SVAL-E likely represents a member of a new species. As shown in Table 2 the level of DNA-DNA hybridization for this isolate is far below the critical value of 70 (19). The isolate SVAL-E is considered as a new species and given the name *Thiomicrospira arctica*.

Description of *Thiomicrospira arctica* sp. nov.

Thiomicrospira arctica (arc´ti.ca L. adj. *arcticus, -a, -um*). Cells are Gram-negative, motile and bent-rod shaped (0.5-0.6 x 1.2-1.5 μm). *T. arctica* is strictly aerobic and grows autotrophically on thiosulfate, tetrathionate and sulfur, but not on sulfite and thiocyanate. The organism does not grow heterotrophically. When thiosulfate is used as the primary energy source small amounts of sulfur are produced. During the growth on reduced sulfur compounds the pH decreases from neutrality to around 5.1. Autotrophic growth on thiosulfate occurs between pH 6.5 and 9.2 and at a temperature of -2.0 and 20.8°C; optimum growth occurs at pH 7.5-8.0 and at 11.5-13.2°C. The optimal Na^+ concentration for growth is 250 mM; growth is possible between a Na^+ concentration of 40 and 1240 mM. On thiosulfate agar, cells produce yellow, smooth, entire colonies (average diameter on 1% (w/v) agar after 3-6 weeks is 1 mm), in which sulfur is deposited and acid produced. The G+C content of the DNA is 43.5 ± 0.7 mol%. As determined by 16S rRNA gene sequence analysis, *T. arctica* belongs to the gamma subclass of the *Proteobacteria* and is closely related to previously described members of the genus *Thiomicrospira*. The type strain of the species is SVAL-E (ATCC 700955, DSM 13458). The accession number in the EMBL, Genbank, and DDBJ nucleotide sequence database is AJ404731.

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A Marine Microbial Consortium Apparently Mediating Anaerobic Oxidation of Methane

Antje Boetius, Katrin Ravenschlag, Carsten Schubert, Dirk Rickert,
Friedrich Widdel, Armin Gieseke, Rudolf Amann,
Bo Barker Jørgensen, Ursula Witte, and Olaf Pfannkuche

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A Marine Microbial Consortium Apparently Mediating Anaerobic Oxidation of Methane

ANTJE BOETIUS*, KATRIN RAVENSCHLAG*, CARSTEN J. SCHUBERT*, DIRK RICKERT† FRIEDRICH WIDDEL*, ARMIN GIESEKE*, RUDOLF AMANN*, BO BARKER JØRGENSEN*, URSULA WITTE*, OLAF PFANNKUCHE†

**Max Planck Institute for Marine Microbiology, 28359 Bremen, Germany;*
†GEOMAR Research Center for Marine Geosciences, 24148 Kiel, Germany

A large fraction of the globally produced methane is coverted to CO_2 via anaerobic oxidation in marine sediments[1]. Strong geochemical evidence for net methane consumption in anoxic sediments is based on methane profiles[2], radiotracer experiments[3], and stable carbon isotope data[4]. However, the elusive microorganisms mediating this reaction have not yet been isolated, and the pathway of anaerobic oxidation of methane is insufficiently understood. Recent data suggest that certain archaea reverse the process of methanogenesis by interaction with sulfate-reducing bacteria (SRB)[5-7]. Here we provide the first microscopic evidence for a structured consortium of archaea and SRB that was identified by fluorescence *in situ* hybridization using specific 16S rRNA-targeted oligonucleotide probes. In this novel example of a structured archaeal-bacterial symbiosis, the archaea grow in dense aggregates of ca. 100 cells and are surrounded by sulfate-reducing bacteria. These aggregates were abundant in gas hydrate-rich sediments with extremely high rates of methane-based sulfate reduction and apparently mediate anaerobic oxidation of methane.

At the Cascadia convergent margin off the coast of Oregon, discrete methane hydrate layers are exposed at the seafloor, at a water depth of 600-800 m corresponding to the hydrate stability limit[8]. These hydrate layers are formed from gaseous methane which continuously ascends along faults generated by accretionary tectonics. The crest of the southern Hydrate Ridge (44°34'N, 125°09'W, 780 m water depth) is populated by large communities of clams of the genus *Calyptogena,* and by thick bacterial mats of the sulfide-oxidizing *Beggiatoa,*

both of which indicate areas of active gas seeping[9]. Undisturbed sediment cores with *Beggiatoa* mats were obtained using a video-guided multiple corer during RV SONNE Cruise SO143-2 in August 1999[10]. These samples often released gas bubbles due to decompression during recovery. Sulfate reduction rates (SRR) were extremely high in sediments covered by *Beggiatoa* mats, reaching more than 5 μmol cm^{-3} d^{-1} in the surface sediments (Fig. 1). Integrated over the upper 15 cm, the resulting SRR is 140 mmol m^{-2} d^{-1} and represents the highest value ever measured in cold marine sediments. At a nearby reference station without gas hydrates and vent colonization, SRR were below detection limit (<1 nmol cm^{-3} d^{-1}). Thus, at the Hydrate Ridge, sulfate reduction is clearly fuelled by high methane fluxes from below whereas organic deposition from surface waters is not a significant substrate source for sulfate-reducing bacteria (SRB). A similar phenomenon was observed at gas seeps in the Gulf of Mexico with 600-fold higher SRR at methane seeps (up to 2.5 μmol cm^{-3} d^{-1}, calculated from sulfate concentration profiles) compared to reference stations[11]. The restriction of such high SRR to sediments rich in methane is evidence for a direct link between the processes of methane and sulfate turnover. It was proposed that sulfate is the terminal electron acceptor in the zone of anaerobic oxidation of methane[3], according to

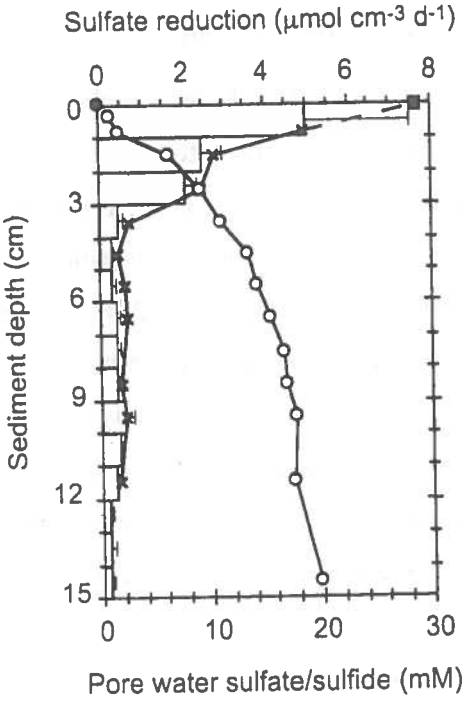

Figure 1. Depth profiles of sulfate reduction rates, pore water sulfate (x) and dissolved sulfide (o) concentrations from methane-rich sediments. Full symbols indicate the respective concentrations in the overlying bottom water. Average SRR values are shown as columns. The multiple corer SO143/173-1B-TVMC was positioned on a *Beggiatoa* mat[10]. One core was taken for pore water chemistry and three replicate subcores were obtained for SRR. Error bars indicate the standard deviation between the subcores.

$$CH_4 + SO_4^{2-} \rightarrow HCO_3^- + HS^- + H_2O \quad (1)$$

Assuming this stoichiometry, the turnover of methane can exceed 5 mM d^{-1} in the sediments of the Hydrate Ridge, where a solubility of methane of 80 mM is reached above decomposing

gas hydrates at *in situ* temperature (4°C) and hydrostatic pressure (80 bar) . As one product of anaerobic oxidation of methane, sulfide accumulates to concentrations almost equimolar with those of sulfate depletion (Fig. 1). Intense sulfide production explains the occurrence of sulfide-based *Beggiatoa/Calyptogena* communities at the Hydrate Ridge. The methane-derived bicarbonate precipitates as calcium carbonate and forms large structures exposed at the crest of the Hydrate Ridge[8].

In Hydrate Ridge sediments, the archaeal isoprenoids crocetane and pentamethylicosane were found to be highly depleted in ^{13}C ($\delta^{13}C$: -124‰ vs. PDB)[12]. Additionally, in *Beggiatoa*-covered sediments of 0-10 cm depth, we found archaeol and *sn*-2-hydroxyarchaeol at high concentrations (8 μg per g sediment dry weight) and similarly depleted in ^{13}C (-114‰ and -133‰, respectively). These lipids are common in archaea and are particularly prominent in methanogens[13]. Such highly ^{13}C depleted lipid biomarkers are due to consumption of methane with a $\delta^{13}C$ of -62 to -72 ‰[8] and subsequent fractionation. Very light iso- and anteiso-C_{15} fatty acids (-63 and -75‰, respectively) which occur abundantly in SRB were also detected at high concentrations (10 μg per g sediment dry weight). These values are similar to or even lower than values reported from methane-rich environments such as a gas hydrate bearing seep[7], a Miocene limestone from an ancient vent system[14], and an active mud volcano[15].

In the *Beggiatoa*-covered sediments of the Hydrate Ridge, abundant cell aggregates were detected by fluorescence *in situ* hybridization (FISH) specific for the domain Archaea[16]. These cell aggregates were not found at the reference station without methane seepage. The archaeal cells in the aggregates were detected with probe EelMS932 targeting clone sequences which were retrieved from a similar methane-rich environment (Eel River Basin, California)[7] and which are phylogenetically affiliated with the order Methanosarcinales. The aggregated archaea were poorly stained with DAPI (Fig. 2a,c,e) and were recognized as such only by the probe signal (Fig. 2b,d,f). Specific FISH analysis of the outer layer of DAPI-stained cells revealed that these are members of the domain Bacteria[17], and belong to the SRB of the delta-proteobacteria. The SRB surrounding the archaeal aggregates were targeted with probe DSS658 (Fig. 2g) specific for the branch *Desulfosarcina/Desulfococcus*[18], and with probe DSS225 which is highly specific for a new subgroup of that branch[19]. The closest cultivated relative of this subgroup is *Desulfosarcina variabilis* with up to 91.2% 16S rDNA sequence similarity.

An average archaea/SRB-consortium consisted of an inner sphere of 2.3±1.3 µm diameter containing ca. 100 coccoid archaeal cells, each 0.5 µm in diameter. These were partially or fully surrounded by ca. 200 cells of SRB (0.3-0.5 µm in diameter), which formed an outer shell of mostly 1-2 cell layers. The size spectrum of 100 archaea/SRB-consortia ranged from 1 to 11 µm in diameter with an average of 3.2±1.5 µm. The smallest aggregates consisted of only 1-3 archaeal cells and 1-3 cells of SRB, and may represent early stages of the consortium development, while the largest contained ca. 10,000 cells. The consortia were highly abundant in surface sediments at sulfide concentrations <10 mM (Fig.1), with a maximum of $7 \cdot 10^7$ aggregates cm^{-3} at 1-2 cm depth (Fig. 3). The average number in the upper 5 cm was $3 \cdot 10^7$ aggregates cm^{-3} which is equivalent to ca. $3 \cdot 10^9$ archaeal cells cm^{-3} and $6 \cdot 10^9$ cells of SRB cm^{-3}. Hence, the consortia comprised 94% of all archaea detected with domain-specific probe ARCH915[16] and 96% of all SRB detected

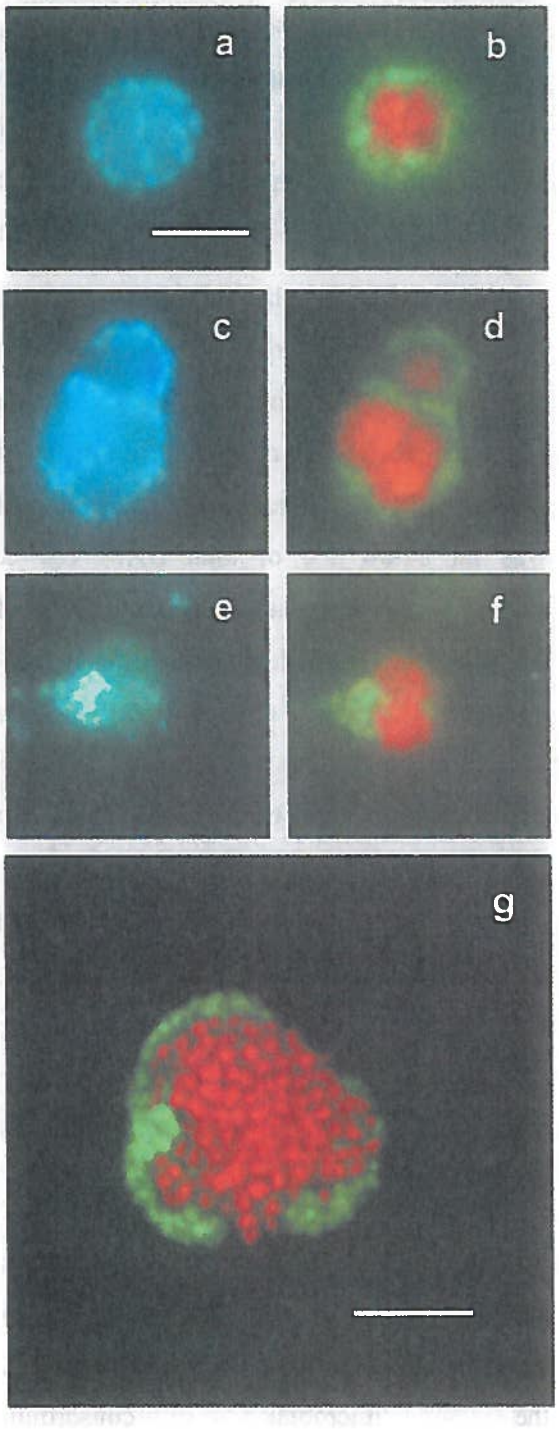

Figure 2. *In situ* identification of Archaea-SRB aggregates with fluorescently labeled rRNA-targeted oligonucleotide probes. The archaea are displayed in red, and the SRB in green. The scale bar is equivalent to 5 µm. The aggregates were visualized using filter sets specific for DAPI, CY3 and FLUOS for identical microscopic fields. Single xy-images of the same optical section were combined. **a/b**, **c/d**, **e/f** Epifluorescence micrographs of different aggregates stained with DAPI (left panel) and hybridized with the CY3-labeled probe EelMS932 (5'-AGCTCCACCCGTTGTAGT-3' and the FLUOS-labeled probe DSS658 (5'-TCCACTTCCCTCTCCCAT-3')[19] (right panel). After testing for their specificity at ≥60% v/v formamide both probes were hybridized at 40% formamide to give optimal brightness. **g** Confocal laser scanning micrograph of the hybridization with the CY3-labeled probe EelMS932 (archaea) and the FLUOS-labeled probe DSS658 (SRB).

with 8 different genus-specific probes[18,20,21]. From the abundance and biovolume of cells in the consortia, a biomass of SRB of 0.12 mg cell dry mass per cm^3 is calculated. Hence, the SRR of 5 μmol cm^{-3} d^{-1} would yield a specific rate of 42 μmol·(mg cell dry mass)$^{-1}$ d^{-1}. This value is within the range of specific rates in cultures of SRB grown under optimal conditions in the laboratory (15-470 μmol·(mg cell dry mass)$^{-1}$ d^{-1})[22].

To identify other organisms potentially consuming methane in the Hydrate Ridge sediments, specific probes for FISH of other archaea and methylotrophic bacteria were developed. Four different probes for the newly described phylogenetic ANME-1 cluster, suspected to consume methane anaerobically in Eel River Basin sediments[7], did not hybridize with the aggregated archaea. Hybridization of non-aggregated archaea with ANME-1 probes was far below 1% of total DAPI cell counts at lowest stringency (0% formamide). Furthermore, no other methanogenic archaea of the orders Methanosarcinales and Methanobacteriales were detected by FISH using specific 16S rRNA probes tested with appropriate reference organisms. FISH counts of aerobic methylotrophs of the alpha- and gamma-proteobacteria were also below detection limits at all stations. Thus, aerobic methane oxidation by relatives of known methanotrophs does not appear to be an important process in the methane-rich sediments of the Hydrate Ridge. Rather, the abundant, strongly $\delta^{13}C$–depleted consortia of methanogenic archaea and SRB are mediating the anaerobic oxidation of methane. This process is assumed to be a reversal of methane formation involving methanogens and a sulfate reducing partner which effectively scavenges intermediates such as H_2 and acetate[5,6,26].

So far, only few examples of prokaryotic symbioses based on metabolic interaction in direct cell contact have been identified, e.g. the microbial consortium "Chlorochromatium"[23] and the cluster of the nitrifying bacteria *Nitrosomonas* and *Nitrobacter*[24]. The advantage of the archaea/SRB consortium compared to free-living cells would be a highly efficient transfer of intermediates by molecular

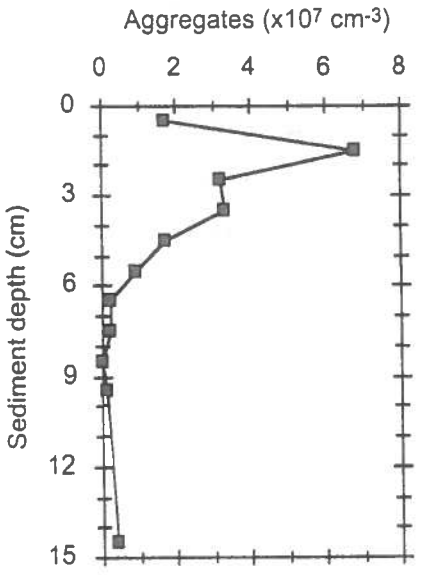

Figure 3. Abundance of archaea/SRB-consortia in a sediment core from a *Beggiatoa* mat

diffusion[25]. Members of the Methanosarcinales, as observed in the consortia, encompass metabolically diverse methanogens and may form methane not only from CO_2 and H_2, but also from simple methyl group-containing compounds such as acetate, methanol or methylamines and methyl sulfide (but not from formate which is used by different methanogens). Members of the *Desulfosarcina/Desulfococcus* branch include nutritionally versatile SRB that oxidize organic compounds including acetate completely to CO_2, and several species can grow autotrophically with CO_2, H_2 and sulfate.

The low ^{13}C signature of SRB lipids is best explained if reverse methanogenesis leads to an organic intermediate that serves not only as electron donor for sulfate reduction, but also as a cellular carbon source. A hypothesized bimolecular reaction of methane, in which both carbon atoms of acetate are derived from methane coupled to simultaneous H_2 formation[26], would offer the most favorable explanation for a strong ^{13}C-depletion in SRB. With any organic intermediate, the cellular carbon of SRB would still be partly derived from CO_2 via some biosynthetic reactions. However, at the high methane oxidation rate estimated from our SRB measurements, the densely packed consortia may maintain a methane-derived light CO_2/HCO_3^- pool (Eq. 1) that is not fully equilibrated with the surrounding heavier porewater pool. Furthermore, autotrophic growth of SRB using the carbon monoxide dehydrogenase pathway (present in members of the *Desulfosarcina* branch) for CO_2 fixation is associated with pronounced ^{13}C discrimination[27]. Hence, reverse methanogenesis via CO_2 and H_2 may also remain relevant as a model.

The free energy change by anaerobic oxidation of methane (Eq. 1) at the pressure (approx. 8 MPa) in the zone with the highest numbers of aggregates is ΔG = -40 kJ per mol methane (ΔG = -30 kJ at a methane pressure of 0.1 MPa prevailing after sampling). The intermediate concentrations required to keep both reverse methanogenesis and sulfate reduction energetically feasible are 10^{-10} to 10^{-9} M (0.01 to 0.1 Pa) for hydrogen and $3 \cdot 10^{-12}$ to $3 \cdot 10^{-8}$ M for acetate. This requirement cannot easily be reconciled with the observed high SRR. Due to the lack of reliable K_M values of SRB and mass transfer rates within densely packed consortia, kinetic considerations considerations based on extremely low intermediate concentrations remain uncertain. Most likely, the maintenance of a microenvironment strongly depleted of hydrogen, acetate or other possible intermediates is a prerequisite for the process of anaerobic oxidation of methane via reversed methanogenesis in the consortia.

Methods

Sulfate reduction rates. Sediment cores were immediately transferred to a cold room (4°C), and $^{35}SO_4^{2-}$ was injected horizontally into the intact sediment cores at 1-cm depth intervals. The cores were incubated for 24 h at *in situ* temperature before the reaction was stopped by mixing the sediments with 20% zinc acetate. The samples were stored frozen until the single-step acidic destillation of the Cr-II-reduced sulfur compounds was carried out as described[28]. SRR was calculated from the ratio of radioactive sulfide to the total radioactive sulfate added. Measurements of porewater sulfate and sulfide were performed as described previously[8].

Lipid analysis. For the determination of lipids, freeze-dried and gently ground sub-samples were extracted by successive sonication and centrifugation in methanol, methanol:methylene chloride (1:1) and methylene chloride. After saponification (6% KOH), the neutral fraction was extracted with hexane, and derivatized with BSTFA (Sigma) prior to injection onto a HP5 chromatographic column (30 m length, 0.32 mm I.D., 0.17 μm film thickness). Column temperature was programmed from 90 °C to 180 °C at a rate of 10 °C min^{-1} and then at a rate of 6 °C min^{-1} to 320 °C (30 min isothermal). The acid fraction was recovered after adding HCl and fatty acids were transferred to fatty acid methyl esters with BF_3-methanol. Chromatographic conditions were the same as for the neutral fraction. Individual compounds were identified on a Finnigan MAT GCQ Ion Trap. Stable carbon isotopes were determined under the same chromatographic conditions with a HP6890 gas chromatograph coupled to a Finnigan Delta Plus isotope mass spectrometer. Reported δ values are corrected for the introduction of additional carbon atoms by derivatization with either BSTFA or BF_3-MeOH.

FISH. Sediment cores from methane-rich sites and from a reference site not enriched in methane were sliced into 1 cm intervals. Samples were fixed for 2-3 h with 4% formaldehyde, washed twice with 1xPBS (10 mM sodium phosphate; 130 mM NaCl) and finally stored in 1xPBS/EtOH (1:1) at -20°C. Stored samples were diluted and treated by mild sonication for 20 s with a MS73 probe (Sonopuls HD70, Bandelin, Germany) at an amplitude of 42 μm <10 W. An aliquot was filtered on 0.2 μm GTTP polycarbonate filters (Millipore). Hybridization and microscopy counts of hybridized and 4',6'-diamidino-2-phenylindole (DAPI)-stained cells were performed as described previously[29]. CY3- and carboxyfluorescein- (FLUOS) labeled oligonucleotides were purchased from Interactiva (Germany).

Calculation of biomass and rates. The biovolume and biomass of SRB were calculated assuming a spherical cell shape with a diameter of 0.5 μm. Consequently, $6 \cdot 10^9$ cells would

have a biovolume of 0.39 mm^3. With a wet mass/volume ratio of approximately 1 mg mm^{-3} and a conversion coefficient of 0.3 mg dry mass (mg wet mass)$^{-1}$, the cell dry mass per cm^3 of sediment was 0.12 mg.

Calculation of free energy. Free energy changes (ΔG values) were calculated from $G°_f$ data[30] via $\Delta G°$ values. Calculations were done for the indicated methane pressures, a temperature of 4 °C, a pH of 7.5 (if H^+ ions are involved), and average concentrations of SO_4^{2-}, HCO_3^- and HS^- of $2 \cdot 10^{-2}$, $1 \cdot 10^{-2}$ and $2 \cdot 10^{-3}$ M, respectively (as prevailing at the sediment depth with the highest number of aggregates). For SO_4^{2-}, HCO_3^- and HS^- in seawater, activity coefficients of 0.1, 0.5 and 0.5, respectively, were estimated[30]. The influence of temperature on $\Delta G°$ (e.g., for Eqn. 1: $\Delta G°_{277K}$ = -16.2 kJ vs. $\Delta G°_{298K}$ = -16.6 kJ per mol methane oxidized) was calculated via the integrated Gibbs-Helmholtz equation including enthalpy ($\Delta H°$) values[30]. H_2 pressures and acetate concentrations were calculated that allow a free energy threshold of approximately -10 kJ per mol methane for each partner involved in the overall reaction (eqn 1).

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16S rDNA-Klonsequenzen aus marinen Sedimenten -Übersicht-

Kategorie	Klonnamen	nächster kultivierter Verwandter	Sequenz-ähnlichkeit [%]	Sediment	Referenz
α-Proteobakterien	NKB7 (AB013259), TAYNAYA-12 (AF142958), TAYNAYA-15 (AF142961), B2M72 (AF223255), TAYNAYA-14 (AF142960), TAYNAYA-13 (AF142959), CE58 (AF211286), CE104 (AF211283)	*Roseobacter* species	91-95	Küstensedimente Tiefseesedimente	Li *et al.*, 1999b Bowman *et al.*, 2000 Cifuentes *et al.*, 2000 Tanner *et al.* unveröff.
	B2M58 (AF223254) 93,3% zu *Rhodobium marinum* (D30790); TAYNAYA-8 (AF142954) 95,4% zu *Amaricoccus macauensis* (U88042); BD1-10 (AB015522) 86% zu *Rickettsia honei* (AF060705); BD2-8 (AB015538) 88% zu *Rickettsia canada* (L36104); BD7-3 (AB015579) 88% zu *Rhodovulum euryhalinum* (D16426); BD1-17 (AB015526) 89% zu *Rhodobium orientis* (D30792); BD1-8 (AB015520) 90% zu *Rhodovulum sulfidophilum* (D16422); JTB260 (AB015246) 90% zu *Ochrobactrum intermedium* (AJ242582); n36d (AF194189) 90% zu *Caedibacter caryophila* (AJ238683); B2M68 (AF223301) 81.8% zu *Rhizobium fredii*; BD5-10 (AB015566) 93% zu Methylobacterium mesophilicum (D32225); EH-20 (U43630) und EH-23 (U43631) beide 97% zu *Methylosinus sporium* (Y18946); BD5-9 (AB015565) 99% zu *Sphingomonas echinoides* (AJ012461); B2M25 (AF223253) 95,8% zu *Sphingomonas terrae* (D13727); n28d (AF194188) 91% zu *Rhodothalassium salexigens* (D14431); JTB36 (AB015242) 90% zu *Rhodospirillum rubrum* (X87278); JTB131 (AB015245) 93% zu *Methylocystis parvus* (Y18945); CE104 (AF211283) 95% zu *Ruegeria atlantica* (AF124521)				
β-Proteobakterien	B2M14 (AF223284) 92,2% zu *Methylophilus methylotrophus*; BPC087 (AF154097) 96% zu *Herbaspirillum seropedicae* (Y10146); NKB6 (AB013258) 91% zu *Rhodocyclus tenuis* (D16208); BD3-8 (AB015550) und n4r (AF194185) beide 94% *Nitrospira marina* (L35501); BCP087 (AF154097) 96% zu *Herbaspirillum seropedicae* (Y10146), BD1-33 (AB015530) 96% zu *Burkholderia graminis* (U96941), BD1-33 (AB015530) 95% zu *Burkholderia kururiensis* (AB024310); BD5-11 (AB015567, 99% zu *Comamonas acidovorans* (AF149849),				
γ-Proteobakterien	BD2-13 (AB015541), NKB4 (AB013256)	*Teredinibacter turnerae* (M64338)	90/93	Tiefseesedimente "cold-seeps"	Li *et al.*, 1999a Li *et al.*, 1999b
	CE57 (AF211282), B2M15 (AF223287), JTB35 (AB015250), BPC036 (AF154089), SA51 (AB022612), BD7-8 (AB015583)	*Methylophaga marina X87338)*	85-96	Kustensediment Tiefseesedimente "cold-seeps"	Cifuentes *et al.*, 2000 Li *et al.*, 1999a; Li *et al.*, 1999c, O'Neill *et al.*, unveröff. Tanner *et al.*,unveröff. Urakawa *et al.* 1999
	MT33 (AF211276), TIHP368-52 (AB031651), TIHP302-07 (AB031601), TIHP302-07 (AB031595), EH-10, EH-15, BD1-7, TK99, SB09	*Alcanovorax borkumii* (Y12579)	82-92	Küstensedimente Tiefseesedimente	Li *et al.*, 1999a Urakawa *et al.*, 1999 Gray *et al.*, 1996 Tanner *et al.*,unveröff. Urakawa *et al.*, unveröffentl.
	JTB256, BPC023 (AF154087), n26r (AF194200)	*Coxiella burnetii* (D89799)	90-91	Tiefseesedimente "cold-seeps" Ästuarsedimente	Li *et al.*, 1999c O'Neill *et al.*, unveröffentl. Todorov *et al.*, unveröffentl.

Kategorie	Klonnamen	nächster kultivierter Verwandter	Sequenz-ähnlichkeit [%]	Sediment	Referenz
γ-Proteobakterien	JTB254 (AB015223), B2M54 (AF223298), B2M32 (AF223289), B2M23 (AF223296), TIHP368-55 (AB031653)	Symbiont of *Riftia pachyptila trophosome* (M99451)	91-95	Küstensedimente "cold-seeps"	Cifuentes *et al.*, 2000 Li *et al.*, 1999c Urakawa *et al.*, unveröffentl.
		GamSI			
	BPC022 (AF154086), n25d (AF194199), B2M19 (AF223302), B2M18 (AF223286), EH-5 (U43633), JTB148 (AB015252), BD1-7 (AB015514), JTB23 (AB015248)	*Lamellibrachia columna* endosymbiont (U77481)	89-94	Küstensedimente "cold-seeps" Tiefseesedimente Astuarsedimente	Cifuentes *et al.*, 2000 Gray & Herwig, 1996 Li *et al.*, 1999a Li *et al.*, 1999c
		GamSII	88-95		
	JTB255 (AB015254), n15d (AF194197), EH-3 (U43632), B2M48 (AF223290), B2M61 (AF223200), BD3-6 (AB015548), BD6-6 (AB015576), BD5-16 (AB015571), B2M31 (AF223288), B2M52 (AF223291), BD3-1 (AB015547), SB21 (AB022626), n19 (AF194198)	*Codakia costata* gill symbiont (L25712)	87-94	Küstensedimente "cold-seeps" Tiefseesedimente Ästuarsedimente	Cifuentes *et al.*, 2000 Gray & Herwig, 1996 Li *et al.*, 1999a Li *et al.*, 1999c Todorov *et al.*, unveröffentl. Urakawa *et al.*, 1999
		GamSIII			
	BD6-5 (AB015575)/BD4-3 (AB015557)/ NKB1 (AB013253)/ BD7-5 (AB015581)/ JTB247 (AB015251), BD5-14 (AB015570), BD3-15 (AB015555), NKB2 (AB013254), TIHP302-35b (AB031621), BD6-3 (AB015573), BD3-13 (AB015554), BD4-16 (AB015563), NKB3 (AB013255), JTB12 (AB015249)	*Pseudomonas* species	97-99	Tiefseesedimente	Li *et al.*, 1999a; Li *et al.*, 1999b Li *et al.*, 1999c Urakawa *et al.*, unveröffentl.
	EH-9 (U43634)/EH-26 (U43639) 92% zu *Rhabdochromatium marinum*; NKB5 (AB013257) 88,0% zu *Methylomonas rubra*; BD3-12 (AB015553) 98% zu *Aranicola proteolyticus*; TK75 (AB022638) 91% zu *Escherichia coli*; BD1-9 (AB015521)/BD6-1 (AB015572)/ BD4-11 92% zu *Serratia proteamaculans*; TIHP302-01, -06, -10, -16, -21-27, -31, -34, -37 (u.a. AB031611) 98% *zu Serratia marcescens* (M59160); SA59 (AB022614)/ SA68 (AB022616)/SA16 (AB022609)/ SA41 (AB022610)/SA79 (AB022618) TIHP302-38 (AB031624) u.a. >98% zu verschiedenen *Vibio* species; n6r (AF194186) 90% zu *Ornithodoros moubata symbiote* (AB001521), TIHP302-39 (AB031625)/TIHP302-20a (AB031605)/TIHP368-77 (AB031662) und TIHP302-04 (AB031592) alle 97% zu *Stenotrophomonas maltophilia*, BPC028 (AF154088) 91% zu *Stenotrophomonas maltophilia* (AJ131117), a2/a4r (AF194191/AF104193) 99% zu *Psychrobacter pacificensis*(AB016057), B2M66 (AF223294)/B2M67 (AF223293) 90% zu *Methylophaga thalassica*; B2M71 (AF222392) 92% zu *Azotobacter paspali*; EH-18 (U43638) 87% zu *Halomonas campusalis* (AF054286), CE102 (AF211280) 89% zu *Legionella lyticum* (X97358), BD2-6 (AB015536) 90% zu *Solemya terraeregina* gill symbiont (U62131); CE100 (AF211279) 94% zu *Amoebobacter roseus* (AJ006062), MT58 (AF211277) 95% zu *Rhabdochromatium marinum* (X84316); MT44 (AF211281) zu *Marinobacter*; BD3-7 (AB015549); B2M38 (AF223303) 90% zu *Solemya occidentalis* gill symbiont (U41049); B2M28/B2M60 (AF223297, AF223299) 91/92% zu *Codakia orbicularis* symbiont; SA50 (AB022611) 94% zu *Riftia pachyptila* endosymbiont (U77478); B2M19 (AF223302) 95% zu *Thiocystis gelatinosa* (Y11317)			

Kategorie	Klonnamen	nächster kultivierter Verwandter	Sequenz-ähnlichkeit [%]	Sediment	Referenz
δ-Proteobakterien	TIHP368-21 (AB031641), MT27 (AF211274), MT25 (AF211272), CE105 (AF211258), B2M33 (AF223276), B2M26 (AF223264), CE53 (AF211261), TK02 (AB022634), B2M62 (AF223306), B2M44 (AF223267), B2M57 (AF223271), L29, B2M36 (AF223277), A34 (U08389), A52 (U08394), A1 (U08385), CE101 (AF211263), EH-2 (U43640), B2M56 (AF223270), Dangast A14, B2M16 (AF223261), CE48 (AF211262), ODP8-U3 (AF121085), SB-29 (AF029047), BS3 (AJ011668), 2B14 (U85479), 4D19 (U85479)	*Desulfosarcina variabilis* (M34407) *Desulfococcus multivorans* (M34405) **DelSI**	88-95 86-95	Küstensedimente Salzmarschen	Bidle *et al.*, 1999 Cifuentes *et al.*, 2000 Devereux *et al.*, 1994 Gray & Herwig, 1996 Kolb, 2000 Phelps *et al.*, 1998 Urakawa *et al.*, unveröffentl. Tanner *et al.*,unveröff. Rosselló-Mora *et al.*, 1999
	ODPB-B7 (AF12090), SB-9 (AF029042)/ MT21 (AF211269), MT22 (AF211271), JTB133 (AB015244), ODP8-10 (AF121087)	*Desulfobacter hydrogenophilus* (M34412) *Desulfobacula toluolica* (X70953)	90-94 90-94	Küstensedimente Tiefseesedimente "cold-seeps" (Methanhydrate)	Phelps *et al.*, 1998 Li *et al.*, 1999c Bidle *et al.*, 1999 Tanner *et al.*,unveröff.
	JTB20 (AB015241), CE24 (AF211259), MT67 (AF211270), SA82 (AB022619), MT28 (AF211275)	*Desulfobacterium catecholicum* (AJ237602) ***Desulforhopalus/ Desulfotalea*-Gruppe**	92-98	Küstensedimente Tiefseesedimente	Li *et al.*, 1999b Tanner *et al.*,unveröff Urakawa *et al.*, 1999
	B2M53 (AF223269), L23, B2M1 (AF223259), B2M9 (AF223274), A30 (U08387), A36 (U08391)	*Desulfofustis glycolicus* (X99707) **Desulfofustis-Gruppe**	90-96	Küstensedimente	Cifuentes *et al.*, 2000 Devereux *et al.*, 1994 Kolb, 2000
	ODPB-U4 (AF121082), BPC005 (AF154090), ODPB-B4 (AF121089), MT24 (AF211269)	*Desulfocapsa sulfexigens* (Y13672) **Desulfocapsa-Gruppe**	90-94	"cold-seeps" (Methanhydrate)	Bidle *et al.*, 1999 O'Neill *et al.*, unveröffentl. Tanner *et al.*,unveröff
	BG25 (U85473), NKB13 (AB013265), TIHP368-19 (AB031638), BD1-2 (AB015515), n16d (AF194204)	*Desulfobulbus propionicus* (M34410) **Desulfobulbus-Gruppe**	90-93	Küstensedimente Tiefseesedimente Ästuaren Sedimente Salzmarschen	Li *et al.*, 1999a Li *et al.*, 1999b Urakawa *et al.*, unveröffentl. Todorov *et al.*, unveröffentl.

Kategorie	Klonnamen	nächster kultivierter Verwandter	Sequenz-ähnlichkeit [%]	Sediment	Referenz
δ-Proteobakterien	GCA017 (AF154102), SB-30 (AF029048), EH-14 (U43642), EH-7 (U43641), n20d (AF194205), B2M37 (AF223265)	*Desulfobacterium anilini* (AJ237601) **DelSII**	86-94	Küstensedimente "cold-seeps" Ästuaren Sedimente	Gray & Herwig, 1996 Urakawa *et al.*, 1999 Cifuentes *et al.*, 2000 O'Neill *et al.*, unveröffentl. Todorov *et al.*, unveröffentl.
	CE103 (AF211267), n2d (AF194201), CE49 (AF211260), BD4-10 (AB015560), n34r (AF194208), BPC076 (AF154096), SB-21 (AF029045)	*Nitrospina gracilis* (L35503) **Nitrospina gracilis-Gruppe**	83-92	Tiefseesedimente Küstensedimente Ästuaren Sedimente,	Urakawa *et al.*, 1999 Cifuentes *et al.*, 2000 O'Neill *et al.*, unveröffentl. Todorov *et al.*, unveröffentl.
	EH-24 (U43643), B2M29 (AF223275), B2M27 (AF223279), L60, TIHP302-12 (AB031598)	*Chondromyces apiculatus* (M94274)	79-87	Küstensedimente	Gray & Herwig, 1996 Cifuentes *et al.*, 2000 Kolb, 2000, Urakawa *et al.*, unveröffentl.
	BPC065 (AF154094), GCA047 (AF154103), NKB14, -15, -16 16(AB013266-68), TIHP368-09 (AB031631), JTB38 (AB015243)	*Desulfovibrio desulfuricans* (M37312)	82-87	Küstensedimente Tiefseesedimente "cold-seeps"	Li et al, 1999a Li et al, 1999b Urakawa *et al.*, unveröffentl.
		Geobacter metallireducens	83-85		O'Neill *et al.*, unveröffentl.
	CE46 (AF211265), CE98 (AF211266), ODPB-U9 (AF121083)	*Bdellovibrio stolpii* (M34125)	82-87	Küstensedimente ""cold-seeps" (Methanhydrate)	Bidle *et al.*, 1999 Tanner *et al.*,unveröff.
	A33 (U08388) 97% zu *Desulforhopalus singaporensis* (AF118453), BG74 (U85477) 92% zu *Desulfoarculus baarsii* (M34403), TAYNAYA-10, -11 (AF142956/ -57) 86% zu *Malonomonas rubra* (Y17712), ODP8-U1 (AF121084) 89% zu *Desulfobulbus rhabdoformis* (U12253), B2M47 (AF223268) 91% zu *Desulfuromonas thiophila*, A8 (U08397), n40d (AF194209) 88%, MT63 (AF211303)/MT70 (AF211301) 90% alle zu *Pelobacter acetylenicus* (X87278); CE99 (AF211304) 94% *Syntrophothermus lipocalidus* (AB021305), B2M23 (AF223296) 92% zu *Geobacter arculus* (U96917); B2M24 (AF223263) 92% zu *Desulfuromosa succinoxidans* (X79415); NKB19 (AB013271) 86% zu *Geobacter metallireducens* (L07834); A35 (U08390) 84% zu *Geobacter hydrogenophilus* (U28173); CE59 (AF211306) 92% zu *Pelobacter venetianus* (U41542)				
ε-Proteobakterien	JTB129 (AB015259), JTB360 (AB015259), BD1-29 (AB015529), B2M5 (AF223282), B2M7 (AF223283), B2M13 (AF223281), D1a1 (AJ271654), A3b2 (AJ271655), B4b1 (AJ271653)	*Arcobacter nitrofigilis* (L14627) **Arcobacter-Gruppe**	93-95	Küstensediment Tiefseesedimente "cold-seeps"	Li *et al.*, 1999a Li et al, 1999c Cifuentes *et al.*, 2000 Thamdrup *et al.*,2000
	ODPB-B3 (AF121088), ODBB-B9 (AF121091), ODP8-U6 (AF121086), JTB146 (AB015257), NKB11 (AB013263)	*Thiomicrospira denitrificans* (L40808) **EpSI**	92%	Tiefseesedimente Methanhydrat-reiche Sedimente	Bidle *et al.*, 1999 Li *et al.*, 1999b Li et al, 1999c

Kategorie	Klonnamen	nächster kultivierter Verwandter	Sequenz-ähnlichkeit [%]	Sediment	Referenz
ε-Proteobakterien	n29 (AF194212), n18r (AF194210), n22 (AF194211), NKB8 (AB013260), NKB9 (AB013261), NKB10 (AB013262), NKB12 (AB013264), BD1-4 (AB015517), BD2-1 (AB015531), BD2-5 (AB015535), BD7-6 (AB015582), BD7-9 (AB015584), A7 (U08396), BPC056 (AF154091), GCA014 (AF154101), SB-17 (AF029044), SA63 (AB022615), n38r (AF194213)	*Alvinella pompejana* epibiont **EpSII**	89-91	Küstensedimente Tiefseesedimente "cold-seeps" Ästuarsedimente	Devereux *et al.*, 1994 Phelps *et al.*,1998 Li *et al.*, 1999a Li *et al.*, 1999b O'Neill *et al.*, unveröffentl. Todorov *et al.*, unveröffentl.) Urakawa *et al.*, 1999
	B2M2 (AF223307) 89% zu *Arcobacter skirrowi* (L14625)				
Acidobacteriales	A39, A40, B2M21, B2M12, B2M55, B2M17, EH-6, BPC066 (AF154095), BPC015 (AF154085)	*„Geothrix fermentans"* (U41563)	75-90	Küstensedimente "cold-seeps"	Devereux *et al.*, 1994 Cifuentes *et al.*, 2000 Gray & Herwig, 1996 O'Neill *et al.*, unveröffentl.
Verruco-microbiales	BD2-18 (AB015546), L16; MT29 (AF211331), MT56 (AF211330), BD2-3 (AB015533), CE59 (AF211306)	*Verrucomicrobium spinosum* (X90515)	74-87	Küstensedimente Tiefseesedimente	Li *et al.*, 1999a Kolb, 2000 Tanner *et al.*,unveröff.
Planctomycetales	L7, L34, L35, L36, L51, BPC061 (AF154092), EH-25, EH-8, L1, L30	*Pirellula marina* (X62912)	83-89	Küstensedimente "cold-seeps"	Gray & Herwig, 1996 Kolb *et al.*, 2000 O'Neill *et al.*, unveröffentl.
	BD2-16 (AB015544), BD3-11 (AB015552), BD7-11 (AB015586), n33d (AF193570)	*Pirellula marina* (X62912)	74-79	Tiefseesedimente Ästuarsedimente	Li *et al.*, 1999a, Todorov *et al.*, unveröffentl.
Flavobacteria	TAYNAYA-16 (AF142962) und BD2-17 (AB015545) beide 94% zu *Gelidibacter algens* (AF001367); TAYNAYA-6 (AF142952) 98% zu *Polaribacter franzmannii* (U14586); TAYNAYA-5 (AF142951) zu *Cytophaga uliginosa*; TAYNAYA-4 (AF142950); TAYNAYA-7 (AF142953), L20 95% zu *Cytophaga fuicola* (AJ005973); JTB132 (AB015260) 87% zu *Cytophaga fermentans* (M58766), BD2-17 (AB015545) 92% und JTB244 (AB015262) 97% zu *Psychroserpens burtonensis* (U62913); JTB143 (AB015261) 90% zu *Polaribacter filamentus* (U73726),				
Sphingobacteria	JTB248 (AB015263) 88% zu *Cytophaga diffluens*(M58756); B2M39 (AF223256) 91,7% zu *Microscilla arenaria* (M60455); BD1-27 (AB015528) 89% zu *Microscilla sericea* (M58794),MT60 (AF211288) zu 89% *Microscilla furvescens* (M85792); BD2-2 (AB015532) 86%, SB-5 (AF029041) 88% und JTB250 (AB015264) 86% zu *Microscilla agregans* (M58791), BD7-10 (AB015585) zu *Flexibacter ruber*; CE55 (AF211292), 94% und CE61 (AF211300) 91% zu *Flavobacterium salegens* (M92279); CE22 (AF211290) 94%, CE54 (AF211291) 88,0%, alle zu *Cytophaga fermentans*(D12661); SB-1 (AF029039) 84% zu *Flexibacter tractuosus* (M58789); TAYNAYA-17 (AF142963) 88% zu *Flexibacter canadensis* (M62793), BD1-15 (AB015524) 88%, BD2-15 (AB015543) 88% und BD7-14 (AB015587) 91% zu *Rhodothermus marinus* (X80994), JTB251 (AB015265) 88% zu *Haliscomenobacter hydrossis* (M58790), TAYNAYA-27 (AF142975) 88% zu *Sphingobacterium multivorum* (AB020205);				

Kategorie	Klonnamen	nächster kultivierter Verwandter	Sequenz-ähnlichkeit [%]	Sediment	Referenz
Bacteroides	TAYNAYA-26 (AF142974), B2M45 (AF223258) 93% zu *Bacteroides fragilis*, BD1-16 (AB015525) 90% zu *Bacteroides ovatus* (L16484);				
Spirochaeta	MT10 (AF211318), MT6 (AF211319), MT2 (AF211302) und weitere 10 Klone 86-93% zu *Spriochaeta* spp.				
Actinobacteria	BCP063 (AF154093), BD2-10, JTB31, TIHP302-29 (AB031615), TIHP368-75 (AB031661), EH-4, EH-13, TAYNAYA-20, A21, A55	Isolate Koll (AJ224541)	87-96	Küstensedimente Tiefseesedimente "cold-seeps"	Bowman *et al.*, 2000 Devereux et al, 1994 Gray *et al.*, 1996 Li *et al.*, 1999a O'Neill *et al.*, unveröffentl. Urakawa *et al.*, unveröffentl
		Microthrix parvicella (X89560)	76-84		
	BD2-11 (AB015578),BD7-2 (AB015540), n11d (AF193568), n21d (AF193569)	*Acidothermus cellulolyticus* (X70635)	81-86	Tiefseesedimente Ästuarsedimente	Li *et al.*, 1999a Todorov *et al.*, unveröffentl.
	B2M43 (AF223285) 86% zu *Streptomyces griseocarneum;* SB11 (AB022623)/ TK100 (AB022640)/ SB20 (AB022625)/ SB53 (AB022632)/ SB66 (AB022632)/ SB39 (AB022629)/BD4-12 (AB015562) 92-95% zu *Microbacterium arborescens* (X77443), TK19 (AB022636)/ TK01 (AB022633)/ TK36 (AB022637)/ SB19 (AB022624)/ SB60 (AB022631)/ SB26 (AB022627)/ SB01 (AB022620) 92-95% zu *Clavibacter michiganensis* (X77435); BPC009 (AF154099) 99% zu *Propionibacterium acnes* (AB042288);				
Firmicutes	JTB243 (AB15271), JTB138 (AB015269), SB-15 (AF029043), GCA018 (AF154105), GCA025 (AF154106), SB-45 (AF029050), TAYNAYA-1 (AF142964), TAYNAYA-18 (AF142966), TAYNAYA-2 (AF142965)	*Acetohalobium arabaticum* (Z21975)	84-87	Küstensedimente Tiefseesedimente "cold-seeps"	Li *et al.*, 1999c Phelps *et al.*, 1998 Bowman, 2000 Urakawa *et al.*, 1999 O'Neill *et al.*, unveröffentl.
	TIHP368-34 (AB031649) 87% zu *Moorella glycerini* (MGU82327); n3 (AF193561) 88% zu *Lactosphaera pasteurii* (X87150);BPC060 (AF154081) 97% zu *Bacillus niacini* (AB021109), BPC094 (AF154082) und BPC043 (AF154080) beide 97% zu *Bacillus firmus* (D15268); BD3-7 (AB015549) 86% zu *Bacillus mycoides* (AF155957); BD5-12 (AB015568) 99% zu *Bacillus flavothermus* (Z26932); TIHP368-67 (AB031657) 92% zu *Clostridium papyrosolvens* (X71852); JTB220 (AB015266) 85% zu *Anaeroplasma abactoclasticum;* BPC090 (AF154098) 99% *Microbacterium arabinogalactanolyticum* (Y17228); JTB326 (AB015272) 91% zu *Acetivibrio cellulolyticus*; JTB215 (AB015270) 89% zu *Eubacterium angustum* (L34612); EH-21 (U43650)/EH-19 (U43649) 92% zu *Eubacterium oxidoreducens* (AF202258); BD4-9 (AB015559) 90% zu *Thermoactinomyces vulgaris* (AF138739)				

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