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PHYLOGENETISCHE UND ÖKOLOGISCHE UNTERSUCHUNGEN AN BAKTERIEN DES OXIDATIVEN UND REDUKTIVEN MARINEN SCHWEFELKREISLAUFS MITTELS RIBOSOMALER RNA

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„ I always look back to our boat cruises, and my land journeys, when through unfrequented countries, with a kind of extreme delight, which no scenes of civilisation could have created."

Charles Darwin, Voyage of the Beagle, 1839

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VORWORT

Die vorliegende Arbeit wurde am Max-Planck-Institut für marine Mikrobiologie, Bremen, unter Leitung von Prof. Bo Barker Jørgensen, in der Nachwuchsgruppe Molekulare Ökologie mit Dr. Niels Birger Ramsing (Universität Århus, Dänemark, gegenwärtig Montana State University, USA) als Betreuer angefertigt. Sie ist im Rahmen gemeinsamer Projekte des Max-Planck-Institutes für marine Mikrobiologie mit Prof. Yehuda Cohen, Moshe Shilo Institut für marine Biogeochemie, Hebräische Universität von Jerusalem, Jerusalem, Israel, Prof. Heribert Cypionka, AG Paläomikrobiologie im Institut für Chemie und Biologie des Meeres, Universität Oldenburg, sowie mit Prof. Victor A. Gallardo, Centro EULA-Chile, Universität von Concepcion, Concepcion, Chile, entstanden. Die Finanzierung der Arbeiten erfolgte durch einen Doktorandenvertrag und aus Forschungsmitteln des Max-Planck-Institutes, sowie aus einem durch die "Deutsch-israelische Gesellschaft für wissenschaftliche Forschung und Entwicklung (GIF)" finanzierten Forschungsprojekt "Sulfatreduzierende Bakterien in oxischen marinen Habitaten", für dessen Antragstellung ich Prof. Bo Barker Jørgensen und Prof. Yehuda Cohen herzlich danke.

Die hier vorliegende Dissertationsschrift ist in sechs Teile gegliedert. Teil eins bis drei enthalten die Einführung in das Thema, die Fragestellung und die Erläuterung methodologischer Voraussetzungen und Innovationen aus dem Gebiet der molekularen mikrobiellen Ökologie, die in einer in einem geowissenschaftlichen Fachbereich eingereichten Arbeit ungewöhnlich sind. Im vierten Teil werden die Ergebnisse in Form von acht Zeitschriftenpublikationen dargestellt, die im folgenden angegeben sind. Schwerpunkte bilden die Themen "Sauerstofftolerante sulfatreduzierende Bakterien" und "Sulfidoxidierende marine Bakterien". Im fünften Teil der Arbeit folgen Zusammenfassung und Diskussion der Arbeiten, mit Ausblick auf weiterführende Forschungsvorhaben. Das Literaturverzeichnis bildet den sechsten Teil, abschließend die - zu kurz gefaßten und nicht alle Beteiligten nennenden - Danksagungen.

---------------------- **Sauerstofftolerante Sulfatreduzierende Bakterien** ----------------------

4.1. Molecular identification of bacteria from a co-culture by denaturing gradient gel electrophoresis of 16S ribosomal DNA fragments as a tool for isolation in pure cultures.
Andreas Teske, Pavel Sigalevich, Yehuda Cohen, Gerard Muyzer. Diese Arbeit wurde im Juli 1995 zur Veröffentlichung in der Zeitschrift *Applied and Environmental Microbiology* eingereicht, und befindet sich in Begutachtung.

4.2. *Desulfovibrio oxyclini* sp. nov., a sulfate-reducing bacterium from the oxic layer of a microbial mat from Solar Lake, Sinai.
Daniel Krekeler, Pavel Sigalevich, Andreas Teske, Yehuda Cohen, Heribert Cypionka. Diese Publikation befindet sich in Vorbereitung und soll in der Zeitschrift *FEMS Microbiology Ecology* zur Veröffentlichung eingereicht werden.

4.3. Molecular and microbiological analysis of sulfate-reducing bacterial populations in the surface layer of a hypersaline cyanobacterial mat of Solar Lake, Sinai.
Andreas Teske, Niels B. Ramsing, Manabu Fukui, Yehuda Cohen. Diese Publikation befindet sich in Vorbereitung und soll zur Veröffentlichung in der Zeitschrift *Applied and Environmental Microbiology* eingereicht werden.

4.4. Distribution of sulfate-reducing bacteria in a stratified fjord (Mariager Fjord, Denmark) as evaluated by Most-Probable-Number counts and denaturing gradient gel electrophoresis of PCR-amplified ribosomal DNA fragments.
Andreas Teske, Cathrin Wawer, Gerard Muyzer, Niels B. Ramsing. Diese Arbeit wurde auf der Tagung *New Approaches in Microbial Ecology* (Helsingør, Dänemark, 21-25. August 1994) in einem Kurzvortrag vorgestellt, im September 1995 zur Veröffentlichung in der Zeitschrift *Applied and Environmental Microbiology* angenommen, und befindet sich im Druck.

4.5. *Desulforhopalus vacuolatus* gen. nov., sp. nov., a new psychrotrophic sulfate-reducing bacterium with gas vacuoles isolated from a temperate estuarium, Kysing Fjord, Denmark.
Mai F. Isaksen, Andreas Teske. Dieser Artikel wird zur Veröffentlichung in der Zeitschrift *Archives of Microbiology* vorbereitet.

------------------------------ **Sulfidoxidierende marine Bakterien** ------------------------------

4.6. Phylogeny of *Thioploca* and related filamentous sulfide-oxidizing bacteria. Andreas Teske, Niels B. Ramsing, Jan Küver, Henrik Fossing. Diese Arbeit wurde auf der Frühjahrstagung der Vereinigung für Allgemeine und Angewandte Mikrobiologie e.V. (Stuttgart, 26.-29.März 1995) in einem Kurzvortrag vorgestellt, und veröffentlicht im vierten Quartal 1995 der Zeitschrift *Systematic and Applied Microbiology,* Vol. 18(4):517-526.

4.7. Phylogenetic relationships of *Thiomicrospira* species and their identification in deep-sea hydrothermal vent samples by denaturing gradient gel electrophoresis of 16S rDNA fragments.
Gerard Muyzer, Andreas Teske, Carl O. Wirsen, Holger W. Jannasch. Diese Arbeit wurde am 27. April 1995 in einem Seminarvortrag im Kluyver Laboratory of Biotechnology an der Technischen Universität Delft vorgestellt. Veröffentlicht im September 1995 in der Zeitschrift *Archives of Microbiology,* Vol. 164:165-172.

4.8. Late Proterozoic rise in atmospheric oxygen from phylogenetic and stable isotopic studies.
Donald E. Canfield, Andreas Teske. Diese Arbeit ist im Januar 1996 bei der Zeitschrift *Nature* eingereicht worden und wird gegenwärtig begutachtet.

Die hier vorliegenden Artikel entsprechen dem Inhalt nach den eingereichten Arbeiten. Vor der Veröffentlichung in den oben genannten Zeitschriften werden Begutachtungen der eingereichten Manuskripte durchgeführt. Das kann dazu führen, daß in den Arbeiten, die noch nicht zur Veröffentlichung angenommen und im Druck befindlich sind, nachträgliche Änderungen der Artikel nötig werden.

Weitere Publikationen, für die Einzelergebnisse aus verschiedenen Projekten und Vorhaben der Doktorarbeit beigetragen worden sind, enthalten hauptsächlich Beiträge von anderer Seite, oder sind für dieses Thema eher von peripherer Bedeutung , wie z.B. der Artikel über *Halobaculum*.

Concentration and transport of nitrate by the mat-forming sulphur bacterium *Thioploca*.
Henrik Fossing, Victor A. Gallardo, Bo B. Jørgensen, Markus Hüttel, Lars P. Nielsen, Heide Schulz, Donald E. Canfield, Stefan Forster, Ronnie N. Glud, Jens K. Gundersen, Jan Küver, Niels B. Ramsing, Andreas Teske, Bo Thamdrup, Oswaldo Ulloa. 1995. Veröffentlicht in der Zeitschrift *Nature*, Vol. 374:713-715.

Denaturing gradient gel electrophoresis of PCR-amplified 16S rDNA - A new molecular approach to analyse the genetic diversity of mixed microbial communities.
Gerard Muyzer, Sabine Hottenträger, Andreas Teske, Cathrin Wawer. Im Januar 1995 zur Veröffentlichung eingereicht und angenommen in: A.D.L Akkermans, J.D. van Elsas, F.J. de Bruijn (eds.) Molecular Microbial Ecology Manual (2nd edition). Kluwer, Dordrecht, The Netherlands.

***Halobaculum gomorrhense*, gen. nov., sp. nov., a novel extremely halophilic archaeon from the Dead Sea.**
Aharon Oren, Peter Gurevich, Renia T. Gemell, Andreas Teske. Veröffentlicht 1995 in der Zeitschrift *International Journal of Systematic Bacteriology*, Vol.45:747-754.

Phylogenetic relationships and in situ detection of filamentous sulfate-reducing *Desulfonema* species
Manabu Fukui, Gerard Muyzer, Andreas Teske, Bernhard Assmus, Fritz Widdel. Diese Arbeit wird zur Veröffentlichung in der Zeitschrift *Systematic and Applied Microbiology* vorbeireitet.

1.1. DER MARINE SCHWEFELKREISLAUF : SULFATREDUKTION

Der marine Schwefelkreislauf, die Reduktion von Sulfat und Schwefel zu Sulfid sowie die Rückoxidation des Sulfids zu Sulfat, wird durch Bakterien katalysiert. Der reduktive Teil des Schwefelzyklus, die Sulfatreduktion, ist der wichtigste Biomineralisationsprozeß des Kohlenstoffs in anoxischen marinen Sedimenten. Obwohl die Schelfmeere nur 8.6% der Fläche des Weltmeeres ausmachen, konzentriert sich in ihren Sedimenten, die reich an organischen Substraten sind, über 90% der sulfatreduzierenden Aktivität der Meere. Hier liegt die Kohlenstoffoxidation durch bakterielle Sulfatreduktion in derselben Größenordnung wie diejenige durch aeroben bakteriellen Abbau (JØRGENSEN 1982b, 1983). Sulfatreduktionsraten von ca. 20-200 nmol SO_4^{2-} cm^{-3} d^{-1} sind für die nährstoffreichen Küstensedimente charakteristisch (JØRGENSEN 1978a,b, SKYRING 1987, JØRGENSEN & BAK 1991) und liegen um etwa zwei Größenordnungen höher als Sulfatreduktionsraten der äußeren Schelfs und Kontinentalabhänge, mit Sulfatreduktionsraten von ca. 0 - 2 nmol SO_4^{2-} cm^{-3} d^{-1} (LEIN 1984, CHRISTENSEN 1989, BATTERSBY ET AL. 1985). Zellzahlen sulfatreduzierender Bakterien folgen einer ähnlichen Verteilung: Während in küstennahen Sedimenten und im Nordseewatt 10^4 - 10^6 sulfatreduzierende Bakterien pro cm^3 gezählt worden sind (LAANBROEK & PFENNIG 1981, JØRGENSEN & BAK 1991), liegen die Zellzahlen in Sedimenten der äußeren Schelfs und der Kontinentalabhänge bei 10^2 - 10^4 Zellen cm^{-3} (PARKES ET AL. 1994, BATTERSBY ET AL. 1985, IVANOV ET AL. 1980), und sinken auf < 10 kultivierbare Zellen cm^{-3} in nährstoffarmen Tiefseesedimenten (IVANOV ET AL. 1980). Sulfatreduktionsraten nehmen im allgemeinen mit zunehmender Tiefe und Entfernung von der Küste ab, und spiegeln in ihrer weltweiten Verteilung insgesamt die küstennahen Zonen hoher Primärproduktion und hohen Nährstoffeintrags wieder (CANFIELD 1991, CANFIELD ET AL. 1993). Wesentliche Anteile der lokalen Primärproduktion an organischem Kohlenstoff werden durch Sulfatreduktion remineralisiert: 14-20% in Cyanobakterienmatten des Solar Lake, eines hypersalinen Strandsees am Roten Meer, Ägypten (JØRGENSEN & COHEN 1977), 11% der planktonischen Primärproduktion in Long Island Sound, USA (ALLER & YINGST 1980), 30% der planktonischen Primärproduktion in der Ostsee (LEIN ET AL. 1982), 53% in einem mit Seegras bewachsenen Sediment im Limfjord, Dänemark (JØRGENSEN & FENCHEL 1974, JØRGENSEN 1977b). Aktivität und Dichte sulfatreduzierender Bakterien werden durch lokale Faktoren wie Sedimentationsrate, Sedimentstruktur, Nährstoffzufuhr durch Flußmündungen, und anthropogene Verschmutzung stark beeinflußt (JØRGENSEN 1989b, CANFIELD 1991, IVANOV ET AL. 1993).

Das beinahe ubiquitäre Vorkommen und die Aktivität von sulfatreduzierenden Bakterien in verschiedensten marinen Habitaten erklärt sich durch die außerordentliche ökophysiologische Vielfalt dieser Bakteriengruppe, ihr weites Substrat- und Temperaturspektrum, und ihre Toleranz gegenüber Streßfaktoren, wie z.B. Sauerstoff.

Substratspektrum. Die ersten Messungen von Sulfatreduktionsraten im Vergleich zur Sauerstoffzehrung eines Sediments ergaben, daß die Sulfatreduktion mit einer vollständigen anaeroben Oxidation des Kohlenstoffs im Sediment verbunden sein muß (JØRGENSEN 1977b), was wiederum bedeutet, daß kurzkettige Fettsäuren und Alkohole, wie Propionat, Acetat und Ethanol, die Abbauprodukte anaerober fermentativer Bakterien, in anaeroben Sedimenten vollständig zu CO_2 oxidiert werden müssen. Tatsächlich sind diese Substrate, zusammen mit Wasserstoff, die bevorzugten Substrate sulfatreduzierender Bakterien in Meeres- und auch in Süßwassersedimenten (ABRAM & NEDWELL 1978, BALBA & NEDWELL 1982, BANAT & NEDWELL 1983, LOVELEY & KLUG 1983, SMITH & KLUG 1981, SANSONE & MARTENS 1982, SØRENSEN & JØRGENSEN 1981, WINFREY & WARD 1983). Sulfatreduzierende Bakterien, die Fettsäuren und Acetat teilweise oder vollständig zu CO_2 oxidieren, sind meistens aus marinen Standorten isoliert worden. Hierzu gehören sämtliche Spezies des ausschließlich Acetat oxidierenden Genus *Desulfobacter* (WIDDEL & PFENNIG 1981, WIDDEL 1987), sämtliche Spezies des Acetat und komplexere Fettsäuren abbauenden Genus *Desulfonema* (WIDDEL ET AL. 1983; FUKUI ET AL. 1995), Isolate der unvollständig Propionat oxidierenden Genera *Desulfobulbus* (WIDDEL 1980, WIDDEL & PFENNIG 1982, SAMAIN ET AL. 1984) und *Desulforhopalus* (ISAKSEN & TESKE 1995), sowie die meisten Spezies der verschiedenste aromatische und nichtaromatische Substrate einschließlich Acetat oxidierenden Genera *Desulfobacterium*, *Desulfococcus* und *Desulfosarcina* (SCHNELL ET AL. 1989, BRYSCH ET AL. 1987, SZEWZYK & PFENNIG 1987, BAK & WIDDEL 1986a, BAK & WIDDEL 1986b, IMHOFF-STÜCKLE & PFENNIG, 1983, WIDDEL 1988, 1980, PLATEN ET AL. 1990). Sogar extrem schwer angreifbare Substrate wie langkettige Alkane und Toluol, die in Öllagerstätten vorkommen, werden von sulfatreduzierenden Bakterien anaerob vollständig oxidiert (AECKERSBERG ET AL. 1991, RABUS ET AL. 1993, RÜTER ET AL. 1995).

Obwohl acetatoxidierende Sulfatreduzierer als Endglieder der vollständigen anaeroben Kohlenstoffmineralization in anaeroben marinen Sedimenten von großer ökologischer Bedeutung sind, wird ihre Anzahl in Zählungen, besonders im Vergleich zu den unvollständig oxidierenden Sulfatreduzierern, häufig weit

unterschätzt, da die eher langsam wachsenden Acetatoxidierer lange Inkubationszeiten in den Verdünnungsreihen erfordern, und außerdem, wie z.B. *Desulfobacter* und *Desulfosarcina*, schwierig zu dispersierende Klumpen und Aggregate bilden (BAK & PFENNIG 1991a,b; WIDDEL, 1988). 10^3 Zellen per ml sind wahrscheinlich zu niedrig angesetzt, 10^5 - 10^6 Zellen sind ein realistischerer Wert (LAANBROEK & PFENNIG 1981, JØRGENSEN & BAK 1991).

Temperaturspektrum. Neben ihrer Fähigkeit, unterschiedlichste Kohlenstoffsubstrate entweder in einem Schritt oder über unvollständig oxidierte Zwischenstufen anaerob vollständig zu CO_2 zu mineralisieren, sind die sulfatreduzierenden Bakterien durch ihre Anpassung an unterschiedliche Temperaturbereiche gekennzeichnet. Obwohl die Mehrzahl der bisher bekannten Sulfatreduzierer mesophil sind, wurden auch psychrophile, sowie gemäßigt bis extrem thermophile Sulfatreduzierer isoliert. Psychrophile Sulfatreduzierer haben einen Temperaturbereich von $< 0°$ bis $\leq 20°C$, und wachsen optimal bei $\leq 15°C$. Mesophile Stämme wachsen von ca. 10°C bis ca. 45°C, optimal zwischen 30° und 40°C. Thermophile sulfatreduzierende Bakterien wachsen im Temperaturbereich von ca. 40° bis 75°C, und haben ihr Wachstumsoptimum bei 55° - 70°C (ISAKSEN ET AL. 1994). Extrem thermophile oder hyperthermophile sulfatreduzierende Archaeen wachsen nicht unterhalb von 60°C, und erfordern zwischen 80° und 110°C (HUBER ET AL. 1990).

Als erster gemäßigt psychrophiler Sulfatreduzierer wird *Desulforhopalus vacuolatus* beschrieben, ein gasvakuolierter, Propionat zu Acetat und CO_2 oxidierender, mariner Sulfatreduzierer aus anaeroben Sedimenten von Kysing Fjord, Dänemark, mit Wachstumsoptimum bei 18-19°C, nicht mehr wachsend bei 24°C (ISAKSEN & TESKE 1995). Dieses Isolat stellt vermutlich nur die Spitze eines Eisbergs dar: In permanent -1.8° bis 0.5°C kalten antarktischen Küstensedimenten sind bei in-situ Temperaturen Sulfatreduktionsraten von 60 - 150 nmol SO_4^{2-} cm^{-3} d^{-1} gemessen worden, die mit denen in gemäßigten Zonen vergleichbar sind (NEDWELL ET AL. 1993). Vakuolierung, charakteristisch für exponentiell und auch linear wachsende Kulturen von *Desulforhopalus vacuolatus*, ist bei psychrophilen und psychrotrophen Bakterien häufig anzutreffen (STALEY ET AL. 1989). Vakuolen vergrößern das Verhältnis von Zelloberfläche zu Cytoplasma, und könnten damit möglicherweise Kältelimitationen des Stoffwechsels oder der Membrantransportprozesse entgegenwirken, neben ihrer klassischen Funktion als Auftriebskörper (WALSBY 1994).

Thermophile sulfatreduzierende Bakterien des grampositiven, sporenbildenden Genus *Desulfotomaculum* sind in marinen Sedimenten zwar nicht aktiv, aber dennoch in beträchtlichen Zellzahlen im Ruhezustand vorhanden. In Sedimenten der Bucht von Århus, Dänemark, wurden 10^4 Zellen per ml der thermophilen Spezies *Desulfotomaculum kuznetzovii* gezählt (ISAKSEN ET AL 1994; NAZINA ET AL. 1988). Andere thermophile sulfatreduzierende Spezies sind *Desulfotomaculum nigrificans* und *Desulfovibrio thermophilus* (CAMPBELL & POSTGATE 1965, ROZANOVA & KUDYAKOVA 1974), sowie das Genus *Thermodesulfobacterium* (ZEIKUS ET AL. 1983). Die optimalen Wachstumstemperaturen dieser thermophilen sulfatreduzierenden Bakterien liegen bei 60° - 70°C.
Die obere Temperaturgrenze für biologische Sulfatreduktion, 80° bis 105°C, wird von sulfatreduzierenden Archaeen des Genus *Archaeoglobus* in den schwefelreichen Geothermalquellen des Guaymas Basin im Golf von Kalifornien erreicht. Extrem thermophile sulfatreduzierende Archaeen des Genus *Archaeoglobus* (STETTER 1988, BURGGRAF ET AL. 1990) sind keine Bakterien, sondern gehören zum zweiten prokaryontischen Organismenreich Archaea, das sich als eigenständiges Organismenreich neben Bakterien und Eukaryonten entwickelt hat (WOESE 1987, WOESE ET AL. 1990). Sie sind mit den sulfatreduzierenden Bakterien nicht verwandt, und weisen einen biochemisch grundsätzlich anderen Sulfatreduktionsweg auf (MÖLLER-ZINKHAN ET AL. 1989). Diese Anaerobier können in kaltem, aeroben Meerwasser in einer Art Kältestarre überleben und werden verdriftet, sind aber nur in örtlich begrenzten, heißen anaeroben Habitaten wie geothermisch aufgeheizten Ölfeldern und vulkanischen Quellen aktiv (STETTER ET AL. 1993). Aus vulkanischem Schlamm und Eruptionsmaterial konnten ca. 10^4 Zellen pro Liter bestimmt werden, z.T unter Lebensgefahr für die Expeditionsteilnehmer (HUBER ET AL. 1990). Das extrem thermophile sulfatreduzierende Archaeon *Archaeoglobus profundus* , das aus den Sedimenten des Guaymas Basins isoliert wurde, ist mit großer Wahrscheinlichkeit der dominante extrem thermophile Sulfatreduzierer *in situ*, weil seine optimale Wachstumstemperatur von 80°-90°C genau mit dem Temperaturbereich maximaler Sulfatreduktion in denselben Sedimenten übereinstimmt (JØRGENSEN ET AL. 1990, BURGGRAF ET AL. 1990). Ein weiteres Maximum sulfatreduzierender Aktivität bei 105°C, bis ca. 110°C reichend, der höchsten Temperatur, bei der bisher biologische Sulfatreduktion gemessen wurde, konnte bisher mit keinem bekannten sulfatreduzierenden Organismus in Verbindung gebracht werden und verweist auf die Existenz weiterer, noch unbekannter hyperthermophiler Sulfatreduzierer (JØRGENSEN ET AL. 1992).

1.2. DER MARINE SCHWEFELKREISLAUF : SULFIDOXIDATION

Gegenüber der Sulfatreduktion ist der oxidative Teil des Schwefelzyklus, die Oxidation des Sulfids zum Schwefel und zum Sulfat, weniger umfassend verstanden, was auf seine außergewöhnliche Komplexität zurückzuführen ist. Der oxidative Teil des Schwefelzyklus verläuft nicht, wie die Sulfatreduktion, in einer einzigen, durch nur wenige Bakteriengruppen katalysierten Reaktion, sondern führt in unterschiedlichen Reaktionen vom Sulfid zu Elementarschwefel, Dithionit, Tetrathionat, Sulfit, Thiosulfat, und Sulfat, von denen zumindest Elementarschwefel und Thiosulfat nicht nur oxidiert, sondern auch zurückreduziert oder bakteriell disproportioniert werden können. Zur Quantifizierung der Sulfidoxidation sind ^{35}S-Tracer problematisch, da ^{35}S-markiertes Sulfid in einer Isotopenaustauschreaktion, mit Polysulfid als Mediator, binnen Minuten zwischen Sulfid- und Elementarschwefelanteilen im Untersuchungsmaterial ausgetauscht wird (FOSSING & JØRGENSEN 1990, FOSSING ET AL. 1992). Durch Sulfatreduktion produziertes Sulfid erreicht nur zu geringen Teilen die Sedimentoberfläche, in den meisten Fällen werden 50% bis über 95% des Sulfids innerhalb des Sediments reoxidiert (JØRGENSEN 1977b, 1987). Oft überlappen die sauerstoffhaltige Oberflächenzone und die sulfidhaltige Sulfatreduktionszone eines Sediments nicht, sondern lassen eine Lücke, die suboxische Zone, die keinen molekularen Sauerstoff, aber oxidierte Metallverbindungen, wie Fe^{3+} oder MnO_2, enthält und ein positives Redoxpotential aufweist (REVSBECH ET AL. 1980). Die Oxidation des Sulfids zum Elementarschwefel oder zum Pyrit verläuft in dieser suboxischen Zone, mit Nitrat, Fe^{3+} oder MnO_2 als Elektronenakzeptor (SØRENSEN & JØRGENSEN 1987). Eine ähnliche Zonierung wurde in der Chemokline des Schwarzen Meeres gefunden. Sulfid wurde an der Obergrenze der sulfidhaltigen Schicht anaerob oxidiert, einhergehend mit einem lokalen Maximum mikrobieller, lichtunabhängiger CO_2-Fixierung (JØRGENSEN ET AL. 1991). Die Mikrobiologie der anaeroben, nicht phototrophen mikrobiellen Sulfidoxidation ist wenig erforscht: Bisher sind zwei denitrifizierende anaerobe sulfidoxidierende Bakterien beschrieben worden, *Thiobacillus denitrificans* und *Thiomicrospira denitrificans* (TAYLOR ET AL. 1971a,b; TIMMER TEN HOOR 1975). Eisen-oder manganreduzierende anaerobe Sulfidoxidierer sind nicht bekannt (JØRGENSEN 1989a).
Die aerobe Sulfidoxidation wird von einigen ungewöhnlichen Bakteriengruppen ausgeführt, die mit der chemischen Oxidation des Sulfids kompetitieren müssen und daher an die Existenz in Sulfid-Sauerstoff-Gradienten angepaßt sind. Diese Anpassungen gehören zu den extravagantesten Phänomenen in der Mikrobiologie und seien hier in Kürze beschrieben.

Filamentöse sulfidoxidierende Bakterien des Genus *Beggiatoa* bilden auf Sediment-oder Schlammoberflächen Matten innerhalb steiler, gegenläufiger Gradienten von Sulfid und Sauerstoff. *Beggiatoa* ist ein mikroaerophiler Organismus: Die Filamente einer Beggiatoenmatte weichen hohen Sauerstoffkonzentrationen im überstehenden Wasser aus, indem sie sich auf die Sedimentoberfläche zurückziehen. Bei sinkender Sauerstoffkonzentration greifen die *Beggiatoa*-Einzelfilamente weiter in das sauerstoffarme Wasser aus, und die Matte dekomprimiert sich (NELSON ET AL. 1986, JØRGENSEN 1987).
Der filamentöse Sulfidoxidierer *Thioploca* unterscheidet sich von *Beggiatoa*, indem *Thioploca*-Trichome zu einem Bündel zusammengelagert sind, das von in einer Polysaccharidscheide umgeben ist. Die *Thioploca*-Bündel sind im Redoxgradienten des Sediments vertikal ausgerichtet. In der Bündelscheide pendeln *Thioploca*-Trichome zwischen den räumlich getrennten Pools von Nitrat, dem Atmungssubstrat, das an der Sedimentoberfläche im Meerwasser enthalten ist, und Sulfid, dem Elektronendonator, der tiefer im Sediment durch Sulfatreduktion produziert wird, auf und ab. Nitrat wird in großen cytoplasmatischen Vakuolen konzentriert und gespeichert, und zur Sulfidoxidation aktiv in das Sediment hineintransportiert, was die Zone der dissimilatorischen Denitrifikation 7-10 cm in das von *Thioploca* besiedelte Sediment ausdehnt (FOSSING ET AL. 1995). Dieser mikrobielle Transportmechanismus koppelt den marinen Schwefel- und Stickstoffzyklus in nährstoffreichen Auftriebsgebieten vor der Westküste Südamerikas, und möglicherweise auch anderer, vergleichbarer Küsten, wie vor Namibia, wo *Thioploca*-Vorkommen ebenfalls wahrscheinlich sind (GALLARDO 1977).
Freischwimmende, gradientenbildende *Thiovulum* Spezies sind, dem gegenwärtigen Kenntnisstand zufolge, aerobe, chemolithotrophe Sulfidoxidierer (WIRSEN & JANNASCH 1978). Als schnellster Schwimmer unter den Bakterien (GARCIA-PICHEL 1989) positioniert sich *Thiovulum* als freischwimmender Bakterienschwarm in Sauerstoff-Sulfid-Gradienten im Wasser und bildet eine schwimmende Phasengrenze, die den Gradienten stabilisiert (JØRGENSEN & REVSBECH 1983).
Das Genus *Thiobacillus* umfaßt eine Vielzahl ökophysiologisch und phylogenetisch sehr verschiedenartiger, im allgemeinen aerober, sulfid-, schwefel-, thiosulfat- und eisenoxidierender Bakterien. Das Genus sollte in physiologisch und evolutionär zusammenhängende Gruppen aufgeteilt und taxonomisch revidiert werden. Man unterscheidet neutrophile, obligat chemoautotrophe *Thiobacilli*, neutrophile, fakultativ chemoautotrophe Species, die zwischen chemolithotropher und heterotropher Ernährung umschalten können, und obligat chemolithotrophe acidophile *Thiobacilli*, die in extrem

sauren Mineralquellen und eisenhaltigen Minenabwässern gefunden werden können. *Thiobacilli* können aus sehr verschiedenartigen Habitaten isoliert werden; Boden, Süßwasser, marine Sedimente, terrestrische und marine vulkanische Areale wie Schwefelquellen, Schwefelablagerungen und sulfidische Erze (KUENEN ET AL. 1991).

Neutrophile, chemolithoautotrophe, microaerophile Schwefeloxidierer des Genus *Thiomicrospira* sind in marinen Habitaten weit verbreitet. *Thiomicrospira* Species sind aus niederländischem und britischem Wattermeerschlick, sowie aus Hydrothermalquellen des Guaymas Basin, Mexiko, isoliert worden (KUENEN ET AL. 1991; JANNASCH ET AL. 1985). *Thiomicrospira* ist in der Chemokline des Schwarzen Meeres, und sehr wahrscheinlich in ähnlichen teilweise anoxischen Meeresbecken, eines der dominierenden sulfidoxidierenden Bakterien (JANNASCH ET AL. 1991).

Obligat endosymbiontische sulfidoxidierende Bakterien sind mit verschiedenen marinen Invertebraten, wie Röhrenwürmer und Muscheln, assoziiert, und werden vom Wirtstier mit Sauerstoff und Sulfid versorgt. Extreme gegenseitige Anpassungen sind die Folge langer gemeinsamer Evolution von Symbiont und Wirt (DISTEL ET AL. 1994). Die Muschel *Solemya reidi*, ebenso wie der Röhrenwurm *Riftia pachyptila*, besitzt weder Mundöffnung noch Verdauungstrakt, sondern lebt "autotroph" von der CO_2-Assimilation durch ihre bakteriellen Symbionten. Jeder Wirt besitzt eine einzige, spezifische Spezies bakterieller Symbionten. Die Wirtstiere versorgen, z.T. über sulfidbindende Hämoglobine, die in den Kiemen oder in speziellen Geweben, dem Trophosom, lokalisierten Symbionten, mit Sulfid und Sauerstoff, und positionieren sich entsprechend, um in den fluktuierenden und lokal variablen Gradienten von Sulfid und Sauerstoff beide Substrate zur Verfügung zu haben. Diese Symbiosen sind bei marinen Invertebraten sulfidreicher mariner Habitate, z.B. Hydrothermal vents, aber auch in Wattenmeersedimenten anzutreffen (CAVANAUGH 1994). Ähnliches gilt für epibiontische, außen auf marinen Nematoden sitzende Bakterien, die von ihrem Wirtstier in Sauerstoff-Sulfid-Gradienten positioniert werden (OTT & NOVAK 1989, POLZ ET AL. 1992).

Bakterienpopulationen des oxidativen Schwefelzyklus können, ebenso wie die sulfatreduzierenden Bakterien, lokal hohe Zelldichten erreichen. Zählungen chemolithotropher, aerober thiosulfatoxidierender Bakterien im Meerwassser nahe an Hydrothermal Vents ergaben 10^5 - 10^6 Zellen pro ml, in Cyanobakterienmatten bis zu 10^9 Zellen pro ml (GALCHENKO ET AL. 1990, LEIN ET AL. 1993, VISSCHER ET AL. 1992). Unter suboxischen Bedingungen, ohne freien Sauerstoff im Sediment, ist ein alternativer Sulfidoxidationsweg wahrscheinlich: Durch Sulfatreduktion entstandenes Sulfid diffundiert im

Sediment in die suboxische Zone und wird dort abiotisch mit Fe^{3+} oder MnO_2 zu Elementarschwefel oxidiert, der bakteriell zu Sulfid und Sulfat disproportioniert wird (THAMDRUP ET AL. 1993., LOVLEY & PHILIPPS 1994). Die Disproportionierung führt über Fraktionierung des schweren Schwefel-isotops ^{34}S zu ^{34}S-verarmtem leichtem Sulfid und ^{34}S-angereichertem, schwererem Sulfat. Das durch Disproportionierung entstandene Sulfid wird im Sediment durch MnO_2 oder FeOOH anaerob zu Elementarschwefel reoxidiert und kann darauf wiederum disproportioniert werden (THAMDRUP ET AL. 1993). Die Rückoxidation und weitere Fraktionierung des ^{34}S-verarmten Sulfids erniedrigt den Anteil von ^{34}S des im Sediment verbleibenden S^{2-}, FeS, S° und FeS_2 gegenüber dem Ausgangsmaterial um 12-15 ‰. Diese Fraktionierung wurde in verschiedenen marinen Sedimenten und mit verschiedenen schwefel-disproportionierenden Reinkulturen gleichermaßen beobachtet (CANFIELD & THAMDRUP 1994; CANFIELD & THAMDRUP 1995). Schwefeldisproportionier-ende Bakterien sind in hoher Zelldichte, 10^4 - 10^6 Zellen pro ml, erstmals in Sedimenten der Salzmarschen und der Bucht von Århus nachgewiesen worden (THAMDRUP ET AL. 1993), und sind aus einer Vielzahl weiterer mariner Sedimente angereichert und isoliert worden (CANFIELD & THAMDRUP 1994).
Wie Schwefel ist auch Thiosulfat ein wichtiges, oft quantitativ dominantes Produkt der Sulfidoxidation, das sehr schnell oxidiert, reduziert, als auch disproportioniert werden kann. Thiosulfat wird unter Isotopenfraktionierung in ^{34}S-verarmtes Sulfid und ^{34}S-angereichertes Sulfat disproportioniert, und dieser "Thiosulfate Shunt" ist ein weiterer Reaktionsweg, um die isotopisch sehr leichten Sulfide mariner Sedimente herzustellen, zu deren Entstehung die Isotopendiskriminierung der bakteriellen Sulfatreduktion allein nicht ausreicht (JØRGENSEN 1990). Seit der Entdeckung der bakteriellen Thiosulfat-Disproportionierung (BAK & CYPIONKA 1987) wurde gezeigt, daß zahlreiche sulfatreduzierende Bakterien Thiosulfat disproportionieren können, und daß diese Bakterien in hohen Zelldichten (10^5 - 10^6 Zellen pro ml) in marinen und Süßwassersedimenten vorkommen (BAK & PFENNIG 1991a,b; JØRGENSEN & BAK 1991).

1.3 SULFATREDUZIERENDE BAKTERIEN IN MIKROBIELLEN MATTEN

Räumlich komprimierte, hochaktive Sulfatreduktion in mikrobiellen Matten. Sulfatreduktionsraten hängen von der Verfügbarkeit organischer Substrate durch Primärproduktion, Nährstoffeintrag, und Sedimentationsrate ab (CANFIELD 1989, 1991). Geringe Substratzufuhr, kombiniert mit aerobem Abbau während langer Sedimentationswege, z.B. im offenen Wasser der Ozeanbecken, begrenzen den organischen Eintrag in ein Sediment und seine Sauerstoffzehrung. Der verringerte Sauerstoffbedarf des Sediments erlaubt eine größere Eindringtiefe des Sauerstoffs, was wiederum die aerobe Biomineralisierung innerhalb des Sediments fördert. Sulfatreduzierende Bakterien in der anaeroben Zone des Sediments empfangen daher weniger, und bereits größtenteils oxidierte Substrate. Die sulfatreduzierende Aktivität im Sediment bleibt extrem niedrig, ca. 0.1 nmol SO_4^{2-} cm^{-3} d^{-1}, und verteilt sich gleichmäßig über einen Bereich in der Größenordnung eines Meters (IVANOV ET AL. 1976, IVANOV ET AL. 1980); in anderen Untersuchungen wurden sehr viel niedrigere Sulfatreduktionsraten von ca. 0.0001 nmol SO_4^{2-} cm^{-3} d^{-1} über 10 bis 100 Meter Sedimentdicke ermittelt (CANFIELD 1991). Sind organische Substrate durch hohe Primärproduktion, Nährstoffeintrag und Sedimentation reichlich vorhanden, so sinkt mit steigendem Sauerstoffbedarf des Sediments die Sauerstoffpenetration. Als Folge der verbesserten Substratversorgung ist einerseits die Aktivität sulfatreduzierender Bakterien absolut erhöht, andererseits ist der stärkste Anstieg der sulfatreduzierenden Aktivität, über mehrere Größenordnungen, nahe der nährstoffreichen Sedimentoberfläche zu verzeichnen. In tieferen, schlechter versorgten Sedimentschichten fallen die Sulfatreduktionsraten rasch wieder ab. Mit verbessertem Substratangebot konzentriert sich die absolut zunehmende Aktivität sulfatreduzierender Bakterien in einem stets schmaleren Bereich des Sediments, immer näher an der Oberfläche. Beispiele hierfür sind die Sedimente des Kattegat und des Limfjord, in denen die jeweiligen Maxima sulfatreduzierender Aktivität, in der Größenordnung von 20 - 200 nmol SO_4^{2-} cm^{-3} d^{-1} , in den obersten 10 - 20 cm oder 1 - 10 cm des Sediments konzentriert sind (JØRGENSEN 1982b). Marine Cyanobakterienmatten stellen den Extremfall dieser Entwicklung dar, sulfatreduzierende Bakterien und ihre Aktivität auf kleinstem Raum aufs höchste zu konzentrieren. Cyanobakterienmatten werden von photosynthetischen filamentösen Cyanobacterien, z.B. *Microcoleus*, *Oscillatoria* oder *Lyngbya*, gebildet, die als Pionierorganismen extrem unterschiedliche Habitate, Wüstenböden ebenso wie Sanddünen, Wattflächen und hypersaline Lagunen, kolonisieren, und mit ihren Filamenten eine zusammenhängende, mechanisch widerstandsfähige Matrix ausbilden, die eine

Vielzahl weiterer Mikroorganismen unterschiedlicher physiologischer Gruppen beherbergt und mit zunehmender Entwicklung ein komplexes mikrobielles Ökosystem, auf kleinstem Raum, mit höchster Bakteriendichte, darstellt (VAN GEMERDEN 1993, STAL & CAUMETTE 1994). In marinen Cyanobakterienmatten wurden sowohl die bisher höchsten Sulfatreduktionsraten, in der Größenordnung von 500 bis 15.000 nmol SO_4^{2-} cm^{-3} d^{-1}, als auch die höchsten bekannten Zelldichten sulfatreduzierender Bakterien, ca. 10^6-10^8 Zellen cm^{-3}, beinahe unmittelbar an der Oberfläche, in den obersten fünf bis zehn Millimetern der Cyanobakterienmatte, gefunden (JØRGENSEN & COHEN 1977, CANFIELD & DES MARAIS 1991, 1993, FRÜND & COHEN 1992, VISSCHER ET AL. 1992, CAUMETTE ET AL. 1994). In den benthischen Cyanobakterienmatten des Solar Lake, eines hypersalinen Sees im Sinai, finden 90% der über die gesamte Mattendicke von 80 cm integrierten Sulfatreduktion in den obersten 3 cm der Matten statt (JØRGENSEN & COHEN 1977).

Sauerstofftoleranz. Aktive sulfatreduzierende Bakterien, die in den oberflächennahen, substratreichen Schichten mariner Sedimente und Cyanobakterienmatten leben, geraten in Kontakt mit Sauerstoff, und bleiben unter oxischen Bedingungen aktiv. Sulfatreduktion in Gegenwart von Sauerstoff ist ein bereits mehrfach beobachtetes und diskutiertes, allerdings bisher unverstandenes Phänomen. Erstmals wurde sauerstofftolerante Sulfatreduktion in marinen Sedimenten des Limfjords nachgewiesen (JØRGENSEN 1977a). Anaerobe Mikronischen im oxischen Sediment, die zur Erklärung postuliert wurden (JØRGENSEN 1977a), sind bisher nicht gefunden wurden (JØRGENSEN & BAK 1991). In marinen Sedimenten ist die sulfatreduzierende Aktivität in der oxischen Oberflächenschicht erkennbar geringer als in den tieferen, anoxischen Schichten: JØRGENSEN & BAK (1991) haben in der oxischen Oberflächenschicht (ca. 1 cm) von marinen Sedimenten des Kattegat Sulfatreduktionsraten von 5 nmol cm^{-3} d^{-1} gemessen, die im anoxischen Bereich dieses Sediments auf 20 nmol cm^{-3} d^{-1} anstieg.
Sauerstofftolerante Sulfatreduktion ist am extremsten ausgeprägt in marinen Cyanobakterienmatten, deren oberste Schichten, in der Größenordnung weniger mm, im Tagesrythmus durch Photosynthese mit Sauerstoff angereichert sind (COHEN 1984, SKYRING 1984) Die höchsten sulfatreduzierenden Aktivitäten, die überhaupt bekannt sind, sind in der Oxykline und in der photosynthetisch aktiven, oxischen Oberflächenschicht mariner Cyanobakterienmatten gemessen worden. In hypersalinen Cyanobakterienmatten der meerwassergespeisten Verdunstungsteiche der Salinen von Guerrero Negro in Baja California, Mexiko, wurden in den tagesperiodisch sauerstoffübersättigten (200 bis 500 µM) Ober-

flächenschichten der Matte Sulfatreduktionsraten von maximal 7000-11.000 nmol cm^{-3} d^{-1} gemessen (CANFIELD & DES MARAIS 1991, 1993). Sulfatreduktionsrate und Zellzahl sulfatreduzierender Bakterien sind in den obersten Millimetern der Cyanobakterienmatten des Solar Lake maximal: 5400 nmol SO_4^{2-} cm^{-3} d^{-1} und 2.5 x 10^6 sulfatreduzierende Bakterien cm^{-3} (JØRGENSEN & COHEN 1977). FRÜND & COHEN (1992) untersuchten in einer detaillierten Studie die Oberflächenschichten einer hypersalinen Cyanobakterienmatte in einem künstlich angelegten, mit Meerwasser gespeisten Verdunstungsteich am Interuniversity Institute, Eilat, Israel. In den obersten zwei Millimetern der Matte fielen genau lokalisierte Maxima der Sauerstoffkonzentration, ca. 500 bis 1000 µM, mit Sulfatreduktionsraten von 200 - 400 nmol cm^{-3} d^{-1} zusammen. Spitzenwerte der Sulfatreduktion in der oxischen Oberflächenschicht dieser Matte erreichten 1-2 µmol cm^{-3} d^{-1}. Für all diese Beispiele gilt, daß die Sulfatreduktionsraten in der oxischen Oberflächenschicht tagsüber mindenstens gleich oder doppelt so hoch waren wie die entsprechenden Raten bei Nacht, wenn die photosynthetische Sauerstoffproduktion aussetzt und die Oberflächenschicht der Cyanobakterienmatte anoxisch wird (CANFIELD & DES MARAIS 1991, 1993; FRÜND & COHEN 1992; JØRGENSEN 1994). Ein direkter Zusammenhang zwischen photosynthetischer Aktivität der Cyanobakterienmatten und Sulfatreduktion ist möglich, andererseits ist ein Temperatureffekt nachgewiesen. Der nächtliche Rückgang der sulfatreduzierenden Aktivität folgt einer temperaturabhängigen Arrhenius-Funktion $V=A*e^{-(Aktivierungsenergie/R*T)}$ (JØRGENSEN 1994). Bei konstantgehaltener Temperatur schwächen sich die Tages-Nacht-Schwankungen der Sulfatreduktion ab oder verschwinden ganz (CANFIELD & DES MARAIS 1991).

Substrate. In der marinen Cyanobakterienmatte des Solar Lake stammen die Kohlenstoffsubstrate der Sulfatreduktion direkt aus der photosynthetischen Primärproduktion der Cyanobakterienmatte. Der Substrateintrag von außen ist durch die isolierte Lage des Sees in der Wüste des Sinai vernachlässigbar gering. 14-20% der Primärproduktion der Cyanobakterienmatten des Solar Lake wird über Sulfatreduktion zu CO_2 reoxidiert (JØRGENSEN & COHEN 1977). Photosynthetisch fixiertes CO_2 wird durch Sulfatreduktion und andere Atmungsprozesse sofort wieder freigesetzt, so daß die oberen cm der Matte ein offenes System darstellen. Erst in tieferen Schichten, ab ca. 10 - 20 cm, verhält sich die Matte wie ein geschlossenes System, in dem die Biomasse der Cyanobakterienmatten über Sulfatreduktion mineralisiert und als $CaCO_3$ akkumuliert wird (JØRGENSEN & COHEN 1977). Neben dem photosynthetischen Beitrag der Cyanobakterien ist wahrscheinlich auch die anoxygene Photosynthese durch

sulfidoxidierende phototrophe Bakterien, und die aerobe chemolithotrophe CO_2-fixierung durch nichtphotosynthetische sulfidoxidierende Bakterien, wichtig für das Gesamtbudget der Kohlenstoffassimilation der Matte (JØRGENSEN & COHEN 1977, CANFIELD & DES MARAIS 1993), zumal anoxygene phototrophe und chemolithoautotrophe sulfidoxidierende Bakterien in Maximaldichten in der oberflächennahen oxisch-anoxischen Grenzschicht von Cyanobakterienmatten vorkommen (VISSCHER ET AL. 1992). Schließlich tragen die Abbauprodukte heterotropher aerober und fermentativer anaerober Bakterien, sowie dunkelakkumulierte Fermentationsprodukte von Cyanobakterien (STAL ET AL. 1989, 1991, HEYER & KRUMBEIN 1991) zur maximalen Dichte und Aktivität sulfatreduzierender Bakterien in der oxisch-anoxischen Grenzschicht bei.

Als spezifisches Kohlenstoffsubstrat der sauerstofftoleranten Sulfatreduktion in Cyanobakterienmatten wird Glykolat diskutiert, das in einer Seitenreaktion der Photosynthese, der Photorespiration, entsteht (FRÜND & COHEN 1992). Das CO_2-fixierende Enzym Ribulose-1,5-bisphosphat-carboxylase, das sowohl photosynthetische wie auch chemolithotrophe schwefel-und wasserstoffoxidierende Bakterien besitzen, reagiert unter CO_2-Limitation und Sauerstoffüberschuß als Oxygenase und produziert anstatt des C_3-Moleküls Phosphoglycerat das C_2-Molekül Phosphoglykolat. ^{13}C-Isotopendaten sprechen dafür, daß dieser Prozeß in photosynthetisch sehr aktiven, CO_2-limitierten, sauerstoffübersättigten Cyanobakterienmatten stattfindet. CO_2-Fixierung durch Ribulose-1,5-bisphosphat-carboxylase selektiert gegen das schwere Kohlenstoffisotop ^{13}C, was zu einer charakteristischen $\partial^{13}C$ Isotopensignatur -29.2‰ bei grünen Pflanzen und -17‰ bis -31‰ bei marinem Plankton führt (ROESSKE & O'LEARY 1984; DEINES 1990). Der Kohlenstoff der Cyanobakterienmatten von Solar Lake und Guerrero Negro ist mit $\partial^{13}C$-Werten von -6‰ bis -13‰ isotopisch schwerer, was als durch CO_2-Limitation abgeschwächte Isotopendiskriminierung bei der CO_2-Fixierung interpretiert wird (AIZENSHTAT ET AL. 1984, DES MARAIS ET AL. 1989, COHEN ET AL. 1994).

Die stimulierende Wirkung von Glykolat auf die sulfatreduzierende Aktivität in der sauerstoffhaltigen Mattenoberfläche deutet auf glykolatverwertende sulfatreduzierende Bakterien, die allerdings bisher nicht isoliert werden konnten, als eine dominante Population in der obersten Mattenschicht. Acetat, das auch als Fermentationsprodukt von Cyanobakterien exkretiert wird, wirkt ebenfalls induzierend (FRÜND & COHEN 1992). Wasserstoff wird stets als Nebenprodukt des stickstofffixierenden Enzymkomplexes der Cyanobakterien, der Nitrogenase, synthetisiert und stellt ein weiteres mögliches Substrat für die Sulfatreduktion dar (HOUCHINS 1984, STEWARD 1980).

2. FRAGESTELLUNG

Als Beitrag zur mikrobiellen Ökologie und Evolution der Sulfatreduktion soll das Problem der sauerstofftoleranten Sulfatreduktion bearbeitet werden. Mit molekularbiologischen und mikrobiologischen Methoden sollen Populationen sauerstofftoleranter sulfatreduzierender Bakterien identifiziert werden. Es soll untersucht werden, ob in oxischen Habitaten neue Entwicklungslinien sauerstofftoleranter oder fakultativ aerober sulfatreduzierender Bakterien vorliegen, oder ob bereits bekannte, klassisch-anaerobe Sulfatreduzierer durch spezifische Anpassungen, modifizierte Stoffwechselleistungen oder Vergesellschaftung mit aeroben, sauerstoff-abfangenden Bakterien auch oxische Habitate kolonisieren können und zur sauerstofftoleranten Sulfatreduktion befähigt werden. Zwei sehr unterschiedliche Habitate werden vergleichend untersucht:

Die Cyanobakterienmatten des Solar Lake, eines hypersalinen Wüstensees am Roten Meer im Sinai. In den benthischen Cyanobakterienmatten des Solar Lake finden 90% der über die gesamte Mattendicke von 80 cm integrierten Sulfatreduktion in den obersten 3 cm der Matten statt, und 50% im obersten Zentimeter. Sulfatreduktionsrate und Zellzahl sulfatreduzierender Bakterien sind in den obersten Millimetern der Mattenschicht, an der Mattenoberfläche maximal: 5400 nmol SO_4^{2-} cm^{-3} d^{-1} und 2.5×10^6 sulfatreduzierende Bakterien cm^{-3} (JØRGENSEN & COHEN 1977). Sulfatreduktion in der Oberflächenschicht in Gegenwart von 500 - 1000 µmol O_2 ist nachgewiesen worden. Die sulfatreduzierenden Bakterienpopulationen des obersten Zentimeters der Solar Lake-Matten sollen mit mikrobiologischen und molekularbiologischen Methoden untersucht werden (Kapitel 4.1, 4.2, 4.3).

Die Wassersäule des Mariagerfjord (Dänemark), eines teilweise anoxischen Meeresbeckens (FENCHEL 1990, JENSEN & NIELSEN 1993), als Beispiel für sauerstofftolerante Sulfatreduktion in der Wassersäule. Sulfatreduktion und sulfatreduzierende Bakterien sind in der Chemokline teilweise anoxischer Meeresbecken, z.B. im Schwarzen Meer und im karibischen Cariaco Trench, nachgewiesen (SOROKIN 1964, HASTINGS & EMERSON 1988, ALBERT ET AL. 1995). Diese Art sauerstofftoleranter Sulfatreduktion unterscheidet sich von ihrem Gegenstück in Cyanobakterienmatten bereits durch andere Dimensionen, wie die breite Sulfatreduktionszone (bis zu hundert Meter), und die niedrigen, nicht tagesperiodisch schwankenden Sauerstoffkonzentrationen von wenigen µM (Kapitel 4.4, ergänzend 4.5).

Als Beitrag zur mikrobiellen Ökologie und Evolution des oxidativen Schwefelzyklus werden schwefeloxidierende Bakterien untersucht, die trotz ihrer großen Bedeutung als Katalysatoren des marinen oxidativen Schwefelzyklus unzureichend bekannt sind: *Thiomicrospira*, *Thioploca*, *Beggiatoa* und *Thiothrix*.

Thioploca, ein fakultativ anaerobe filamentöse Sulfidoxidierer, bildet entlang der Küste Chiles und Perus über einen Bereich von ca. 3000 km die ausgedehntesten bekannten Bakterienmatten der Erde (GALLARDO 1977). *Thioploca*-Trichome pendeln zwischen den räumlich getrennten Pools von Nitrat, dem Atmungssubstrat, das an der Sedimentoberfläche im Meerwasser enthalten ist, und Sulfid, dem Elektronendonator, der tiefer im Sediment durch Sulfatreduktion produziert wird, auf und ab. Nitrat wird in großen cytoplasmatischen Vakuolen konzentriert und gespeichert, und zur Sulfidoxidation aktiv in das Sediment hineintransportiert, was die Zone der dissimilatorischen Denitrifikation 7-10 cm in das von *Thioploca* besiedelte Sediment ausdehnt (FOSSING ET AL. 1995). Die Phylogenie von *Thioploca*, *Beggiatoa* und *Thiothrix* wird untersucht (Kapitel 4.6).

Thiomicrospira, ein chemolithoautotrophes, mikroaerophiles Schwefelbakterium, ist in der marinen Umwelt sehr weit verbreitet. *Thiomicrospira* ist aus Wattermeerschlick, aus Hydrothermal Vents sowie aus der Chemokline des Schwarzen Meeres isoliert worden, wo es wahrscheinlich eines der dominanten schwefeloxidierenden Bakterien darstellt (JANNASCH ET AL. 1985, JANNASCH ET AL. 1991, KUENEN ET AL. 1991). Die Phylogenie von Thiomicrospira wird untersucht. Um die Verbreitung und ökologische Bedeutung von *Thiomicrospira* für die marine Schwefeloxidation abzuschätzen, werden Umweltproben mittels Denaturierender Gradiendengelelektrophorese (DGGE) auf ribosomale Ribonukleinsäuren von *Thiomicrospira* untersucht (Kapitel 4.7).

Thioploca, Beggiatoa, Thiothrix,* und *Thiomicrospira sollen durch vergleichende Analyse hochkonservierter ribosomaler Ribonukleinsäurengene (16S rRNA-Gene) untersucht werden, um ihre stammesgeschichtliche Stellung zu definieren. Diese Bakterien zählen zu den Hauptkatalysatoren des modernen oxidativen Schwefelzyklus. Ihre Evolution ist mit dem Beginn der aeroben, nichtphotosynthetischen bakteriellen Sulfid-und Schwefeloxidation verbunden. Dieser Teil des bakteriell katalysierten oxidativen Schwefelzyklus hat sich sehr wahrscheinlich erst im Gefolge der bakteriellen Sulfatreduktion und der anoxygenen photosynthetischen Schwefeloxidatioon entwickelt (Kapitel 4.8).

3. METHODIK

3.1. MIKROBIOLOGISCHE VERFAHREN

Populationsdichten kultivierbarer sulfatreduzierender Bakterien werden durch MPN (Most Probable Number) Verdünnungsreihen ermittelt. Hierbei wird eine Wasser- oder Sedimentprobe schrittweise in geeigneten Kulturmedien ausverdünnt, bis man in den obersten positiven Verdünnungsstufen die zahlenmäßig dominanten Bakterien erhält, die in dem jeweils gewählten Medium kultivierbar sind. Aus den obersten Verdünnungstufen von MPN-Reihen lassen sich die zahlenmäßig dominanten kultivierbaren Bakterien einer Probe isolieren, durch Agarshakes aufreinigen und charakterisieren. Ohne solche Verdünnungsschritte lassen sich in hohen Dichten vertretene, ökologisch dominante Bakterien oft nicht isolieren, da sie in einfachen Anreicherungen von Stämmen überwachsen werden, die in der Standortprobe in weniger hohen Zellzahlen vorkommen, aber im Labormedium optimale Wachstumsbedingungen vorfinden, sogenannte "Laboratory weeds" (WARD ET AL. 1994). Aus diesem Grund stellen Isolate aus hohen Verdünnungen oft neue Spezies oder Genera dar (OREN ET AL. 1995).

Für Zählungen sulfatreduzierender Bakterien wird das Mehrzweckmedium von WIDDEL & BAK (1991) verwendet. Der Salzgehalt des Mediums wird dem jeweils zu untersuchenden Standort angepaßt (Meerwasser, Salzwasser des hypersalinen Solar Lake). Die Inkubationen wurden bei Raumtemperatur über drei bis vier Monate hinweg fortgesetzt und überwacht, um sehr langsam wachsende, acetat-oxidierende Sulfatreduzierer, z.B. die filamentösen *Desulfonema*, die bisher nicht mit MPN-Zählungen quantifiziert worden waren, mit zu berücksichtigen.

Um sulfatreduzierende und andere Bakterien aus Solar-Lake-Matten oder MPN-Kulturen in Reinkultur zu erhalten, wird die Ausverdünnung in Agarreihen angewandt. In hohen Verdünnungstufen erhaltene Kolonien der gesuchten Bakteriengruppe werden durch weiteres wiederholtes Ausverdünnen in Agarreihen in Reinkultur gebracht. Wenn die bakteriellen Komponenten einer Mischkultur oder einer natürlichen Probe über Sequenzdaten identifiziert werden können, können darauffolgend spezifische Kulturbedingungen gewählt werden, um diese Organismen gezielt zu isolieren (siehe Abschnitt 4.1).

3.2. MOLEKULARBIOLOGISCHE VERFAHREN

Einige Grundlagen der molekularbiologischen Verfahren in der Ökologie und Evolution von Mikroorganismen sollen hier kurz erläutert werden, da diese Konzepte in jede einzelne der hier zusammengefaßten Arbeiten einfließen. Biologische Makromoleküle, wie Proteine und Nukleinsäuren, evolvieren auf molekularer Ebene. Besitzt eine Organismengruppe ein charakteristisches Molekül, daß sich parallel mit seinen Trägerorganismen entwickelt hat, so ermöglicht der Sequenzvergleich des Moleküls die Ableitung der Stammesgeschichte seiner Trägerorganismen (ZUCKERKANDL & PAULING 1965). Die Mutationen in einem Molekül akkumulieren sich als das Produkt aus Mutationsrate und Zeit, korrigiert für Rückmutationen. Bei konstanter Mutationsrate erscheint das Molekül als "molecular clock". Um die Evolution einer so alten Organismengruppe wie der Bakterien zu rekonstruieren, wählt man hochkonservierte, langsam evolvierende Moleküle, um auch sehr alte und entfernte Verwandschaftsbeziehungen zu erkennen. Bevorzugt werden ribosomale Ribonukleinsäuren, essentielle Bestandteile des Ribosoms, des universell verbreiteten, evolutionär sehr alten Proteinbiosynthesekomplexes jeder Zelle (HILL ET AL. 1990). Für phylogenetische Untersuchungen sind besonders die ribosomalen Ribonukleinsäuren (rRNA) geeignet, von denen es bei Prokaryonten drei gibt, die ca. 120 Nukleotide lange 5S rRNA, die ca. 1500 Nukleotide lange 16S rRNA, und die 2500 Nukleotide lange 23S rRNA. Ribosomale RNAs bilden das Strukturgerüst für die räumliche Orientierung und Wechselwirkung der ribosomalen Proteine. Ihre Nukleinsäuresequenz und ihre Sekundärstruktur, die aus der Primärsequenz hervorgehende räumliche Konfiguration des Moleküls, sind in allen Organismen weitgehend konserviert (WOESE ET AL. 1980, NOLLER ET AL. 1981), und ermöglichen den Vergleich homologer Sequenzregionen, unter Ausschluß hochvariabler Bereiche. Aus praktischen Gründen wird die 16S rRNA bevorzugt, da sie einerseits deutlich mehr phylogenetische Information liefert als die sehr kurze 5S rRNA, andererseits einfacher zu sequenzieren ist als die längere 23S rRNA. Durch automatische Sequenzierungstechniken ist jedoch auch die 23S rRNA in die Reichweite der Routinesequenzierung gerückt.

Die Substitutionsrate der 16S rRNA wird auf ca. 1% in 50-60 Millionen Jahren geschätzt, eine der langsamsten Raten von bakteriellen und eukaryontischen Genen überhaupt (OCHMAN & WILSON 1987, STACKEBRANDT 1994). Die Mutationen an einzelnen Nukleotidpositionen sind voneinander in gewissem Maß unabhängig, da die 16S rRNA in ihrer Sekundärstruktur additiv in ca. 50 strukturelle Untereinheiten (Helixschleifen) unterteilt ist, die bis auf wenige

Tertiärstruktur-Interaktionen unabhängig voneinander, und im Zusammenhang mit ihren jeweiligen ribosomalen Proteinbindungsstellen evolvieren. Diese relative wechselseitige Unabhängigkeit der Nukleotidsubstitutionen in der 16S rRNA begünstigt einen gleichmäßigen Lauf dieser "molecular clock" in den meisten Bakterien, obwohl bestimmte Bakteriengruppen eklatante Ausnahmen darstellen (YANG & WOESE 1989). Etwa 60% der Basenpaarungen der 16S rRNA bilden die Sekundärstruktur (WOESE ET AL. 1983, GUTELL ET AL. 1994).

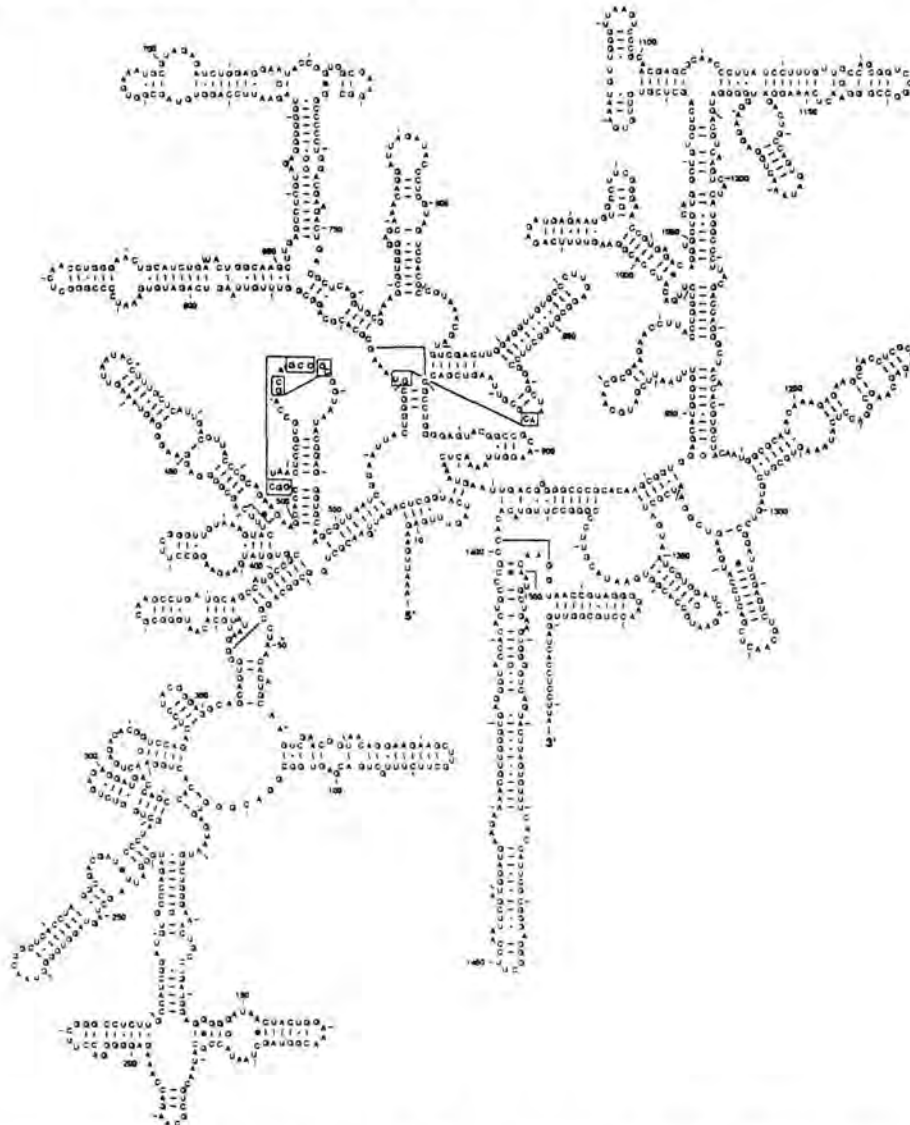

Abbildung 1A: Sekundärstrukturmodell der *E. coli* 16S rRNA. Watson-Crick (kanonische) Basenpaarungen (G-C, A-U) sind durch Striche miteinander verbunden. Nichtkanonische Basenpaarungen, die bei ribosomaler RNA häufig sind, werden durch Punkte (G-U), oder offene Kreise (A-G u.a.) dargestellt. Nukleotide, die auf Tertiärstrukturebene koevolvieren, sind durch Kästen und Linien verbunden. Die Numerierung der Nukleotidpositionen (No. 1 - 1542) kennzeichnet Primerpositionen und Sequenzabschnitte (GUTELL ET AL. 1994).

Die mosaik-ähnliche Struktur der 16S rRNA aus größtenteils hochkonservierten, doch auch variablen und hochvariablen Sequenzabschnitten (Abb. 1B), ermöglicht einerseits, Verwandtschaftsbeziehungen zwischen evolutionär weit voneinander entfernten Bakterien zu rekonstruieren, andererseits, auch nahe verwandte Organismen anhand hochvariabler Sequenzabschnitte zu unterscheiden.

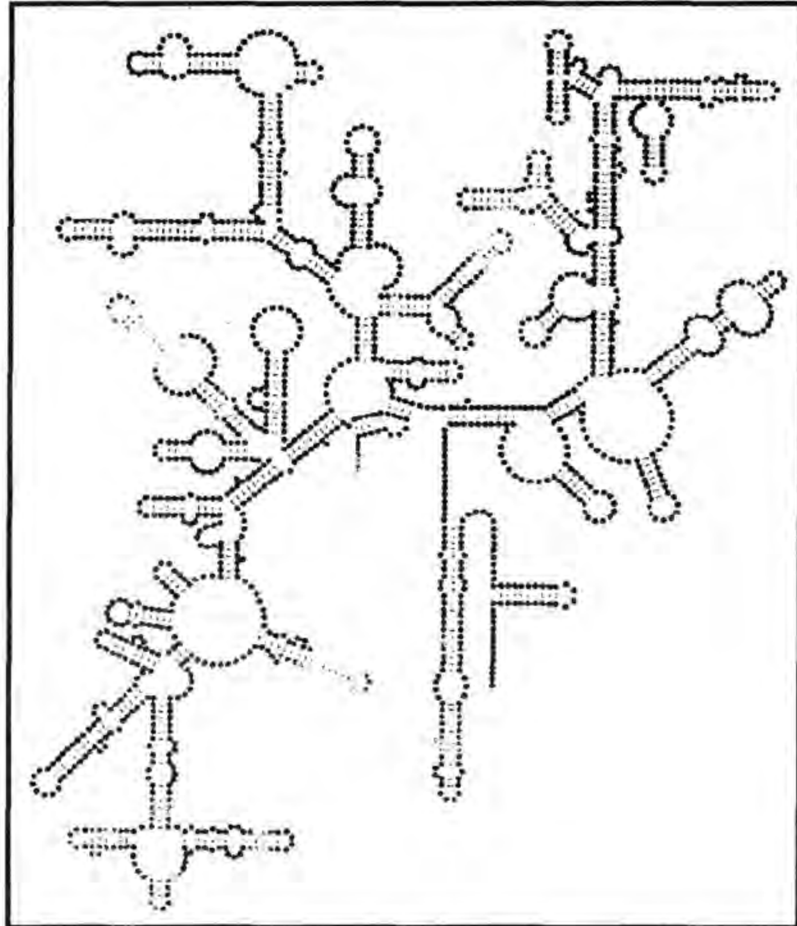

Abbildung 1B. Sekundärstruktur der 16S rRNA. Konservierte Sequenzregionen sind dunkler schattiert als variable Sequenzabschnitte. Die dunkelste Tönung entspricht etwa dem 90%-Konsensus bakterieller 16S rRNA-Sequenzen.

Die Sequenzbestimmung, Identifizierung und phylogenetische Einordnung eines unbekannten Bakteriums verläuft in folgenden Schritten: Das 16S rRNA Gen wird mittels der Polymerasekettenreaktion amplifiziert (SAIKI ET AL. 1988) und das Amplifikat sequenziert. Die Sequenz wird mit den in 16S rRNA-Datenbasen verfügbaren Referenzsequenzen verglichen. In den meisten Fällen kann sie einer bekannten Bakteriengruppe zugeordnet werden, kann sich aber auch als neuartige Sequenz einer bisher noch nicht bekannten Bakteriengruppe herausstellen. Als Referenzdatenbank wird die sehr umfassende und benutzerfreundliche Datenbank des Ribosomal Database Project an der University of Illinois at Urbana/Champaign verwendet, die zur Zeit (Version 5.0.

Mai 1995) ca. 2800 16S rRNA und 1400 23S rRNA-Sequenzen enthält (MAIDAK ET AL. 1994). Die evolutionären Distanzen der neuen Sequenz zu näher und entfernter verwandten Sequenzen werden quantifiziert, und die mit dieser Distanzmatrix am ehesten vereinbare evolutionäre Abfolge wird in einem phylogenetischen Baum dargestellt.

Das außerordentliche Potential ribosomaler RNAs für die mikrobielle Ökologie beruht auf dem Prinzip, ribosomale RNA aus Umweltproben zu extrahieren und mittels verschiedener Techniken zu identifizieren, und somit die Zusammensetzung der Bakteriengemeinschaft in situ zu analysieren, ohne die Bakterien selbst zu kultivieren (OLSEN ET AL. 1986). Ribosomale RNAs verschiedener Bakterienspezies können nach der Extraktion chromatografisch aufgetrennt und sequenziert werden, wenn nur wenige verschiedene Bakterienspezies in der Probe vorliegen (STAHL ET AL. 1985). Ribosomale RNA kann mit radioaktiv markierten Oligonukleotiden hybridisiert werden, die einem ribosomalen Sequenzmotiv bestimmter Bakteriengruppen komplementär sind, um die entsprechenden Anteile dieser RNAs, und damit der Bakterien, an der Gesamt-rRNA in der Probe zu quantifizieren (RISATTI ET AL. 1994). Sie kann ohne weitere Auftrennung mit reverser Transcriptase in ribosomale cDNA transcribiert und kloniert werden, um aus komplexen rRNA-Gemischen einzelne rRNA-Sequenzen zu erhalten (GIOVANNONI ET AL. 1990, WARD ET AL. 1990). Eine neue Entwicklung stellt die denaturierende Gradientengel-elektrophorese, DGGE, dar (MUYZER ET AL. 1993). PCR-Amplifikate heterogener Bakterienpopulationen, von gleicher Länge und Molekulargewicht, sind in der klassischen Agarosegelelektrophorese, die nach Molekulargewicht auftrennt, nicht voneinander zu unterscheiden, doch können sie aufgrund ihrer Sequenzdifferenzen in denaturierenden Gradienten elektrophoretisch separiert werden. PCR-Amplifikate sind durch Wasserstoffbrückenbindungen zusammengehaltene DNA-Doppelstränge, die nach Primärsequenz und Schmelzdomänenstruktur an unterschiedlichen Punkten des denaturierenden Gradienten in Einzelstränge denaturiert werden, deren Wanderungs-geschwindigkeit durch die Gelmatrix stark herabgesetzt ist. Jedes PCR-Produkt wandert bis zu seinem individuellen Schmelzbereich im denaturierenden Gradienten, so daß sich ein differenziertes Bandenmuster von separierten, PCR-amplifizierten ribosomalen RNAs ergibt (MUYZER & DE WAAL 1994). Das DGGE-Bandenmuster gibt eine qualitative Übersicht über die Komplexität eines mikrobiellen Ökosystems, und ermöglicht die systematische Identifizierung bestimmter Banden bzw. Bakterienpopulationen, durch das Screening des Bandenmusters mit spezifischen rRNA-Sonden, und durch Sequenzierung von DGGE-Banden (MUYZER ET AL. 1993).

3.3. DIE DENATURIERENDE GRADIENTENGELELEKTROPHORESE IN MIKROBIOLOGISCH-ÖKOLOGISCHEN FELDSTUDIEN

Die DGGE bietet sich für Feldstudien an, da mehrere Proben nebeneinander in einem denaturierenden Gel aufgetragen werden und die resultierenden DGGE-Bandenmuster direkt miteinander verglichen werden können. Die Bakterienpopulationen in verschiedenen Schichten einer mikrobiellen Matte oder einer stratifizierten Wassersäule können somit direkt miteinander verglichen werden, ebenso die zeitliche Dynamik von Bakterienpopulationen. Der Arbeitsgang läßt sich wie folgt zusammenfassen: Aus Umweltproben, z.B. marinem Sediment, Cyanobakterienmatte, Bakterien aus der Wassersäule, werden DNA und rRNA isoliert. Abschnitte eines bestimmten Gens (hier: des 16S rRNA-Gens) werden in der Polymerasekettenreaktion (PCR) vervielfältigt, wobei die Komplementarität der PCR-Primer zur Zielsequenz die Spezifität der Reaktion bestimmt. Die Amplifikate werden in denaturierender Gradienten-Gelelektrophorese (DGGE) aufgetrennt und durch Hybridisierungen mit rRNA-komplementären Oligonukleotidsonden identifiziert. Das DGGE-Bandenmuster wird mit rRNA-Sonden unterschiedlicher Spezifität hybridisiert: Eine hochspezifische Sonde, allein für eine nahe verwandte Gruppe von *Desulfovibrio*-Stämmen aus der oxischen Oberflächenschicht des Solar Lake, dient zum Nachweis von 16S rRNA-Amplifikaten allein dieser Bakteriengruppe in Solar Lake-Proben. Genusspezifische rRNA-Sonden sulfatreduzierender Bakterien (DEVEREUX ET AL. 1992), sowie eine unspezifischere Sonde, die die meisten sulfatreduzierenden Bakterien, einschließlich einiger nicht-sulfatreduzierender Gruppen, einschließt (AMANN ET AL. 1990), werden in den Solar Lake- und in den Mariagerfjord-Untersuchungen verwendet. Auffällige Banden, die z.B. in verschiedenen Proben wiederholt vorkommen und mit bestimmten rRNA-Sonden hybridisieren, werden ausgeschnitten, aus der Gelmatrix eluiert und reamplifiziert, sowie zur genauen Identifizierung sequenziert. Durch Vergleich dieser Sequenzen mit den in Datenbanken vorliegenden Referenzsequenzen der verschiedensten Organismen ist eine genaue Identifizierung möglich, sofern die DGGE-Bande nicht von einem völlig neuartigen Organismus stammt, und keiner bekannten Gruppe zuzuordnen ist.
Je nach Aufgabenstellung läßt sich die Untersuchung eines mikrobiellen Ökosystems auf unterschiedlichen Ebenen vornehmen: Der einfache visuelle Vergleich von DGGE-Bandenmustern gibt eine Vorstellung von der Komplexität eines mikrobiellen Ökosystems, der räumlichen oder temporären Abfolge verschiedener Bakterienpopulationen. Die Hybridisierung mit Oligonukleotid-Sonden unterschiedlicher Spezifität erlaubt eine weitergehende Zuordnung

bestimmter Banden zu denjenigen Bakteriengruppen, für die die Sonden spezifisch sind, und die je nach Sondenspezifität enger oder weiter gefaßt sein können. Schließlich ermöglicht die Sequenzierung von DGGE-Banden die genaueste mit molekularen Methoden in diesem Kontext erreichbare Identifizierung eines Bakteriums, und zugleich dessen phylogenetische Einordnung.

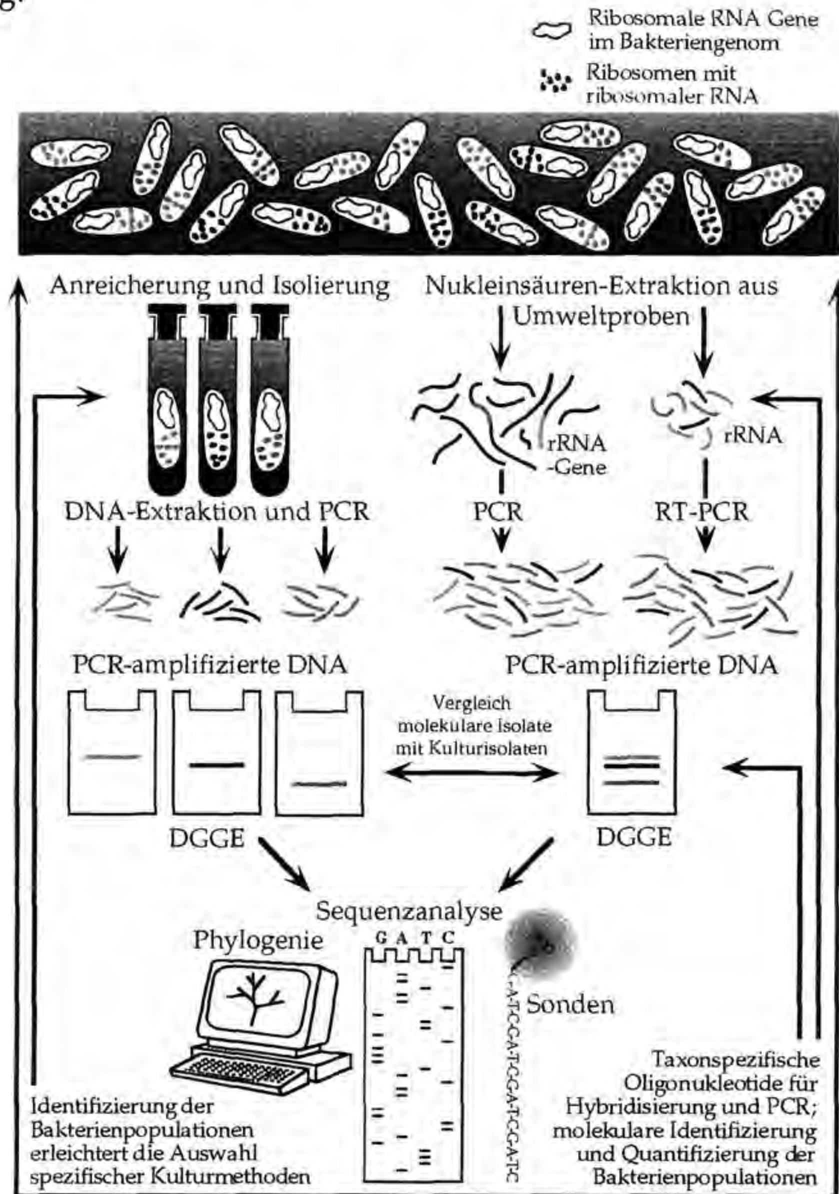

Abbildung 2. Integration mikrobiologischer und molekularbiologischer Arbeitstechniken in der mikrobiellen Ökologie. Die Analyse von ribosomaler rRNA einer Bakteriengesellschaft mittels PCR, DGGE und Sequenzierung identifiziert spezielle Bakterienpopulationen eines mikrobiellen Ökosystems, und erleichtert die Auswahl angemessener Kulturtechniken, um diese zu isolieren. Publikation 4.1 demonstriert diese Methodik an einem Fallbeispiel, einer Mischkultur aus der Solar Lake-Cyanobakterienmatte.

4.1 Molecular identification of bacteria from a co-culture by Denaturing Gradient Gel Electrophoresis of 16S ribosomal DNA fragments as a tool for isolation in pure cultures

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ABSTRACT

Molecular information about the bacterial composition of a co-culture, capable of sulfate reduction under anaerobic and microaerobic conditions, was used to identify and subsequently to isolate the components of the mixture in pure culture. PCR-amplification of 16S rDNA fragments from the co-culture, analyzed by denaturing gradient gel electrophoresis (DGGE), resulted in two distinct 16S rDNA bands, indicating two different bacterial components. The bands were sequenced and identified as derived from a *Desulfovibrio* strain and an *Arcobacter* strain, a genus related to *Campylobacter*. Since the phylogenetic position of bacteria is often consistent with their physiological properties and culture requirements, molecular identification of the two components of this co-culture allowed to design specific culture conditions to separate and isolate both strains in pure culture. This approach facilitates the combined molecular and physiological analysis of mixed cultures and microbial communities.

INTRODUCTION

Analysis of ribosomal RNA has been widely used to determine the bacterial species composition of microbial communities. The methodology encompasses such different approaches as direct analysis of ribosomal RNA by chromatography and membrane blotting (RISATTI ET AL. 1994, STAHL ET AL. 1985), PCR-amplification and subsequent cloning and sequencing of reverse-transcribed environmental ribosomal RNA (GIOVANNONI ET AL. 1990, WARD ET AL. 1990), or denaturing gradient gel electrophoresis (DGGE) of PCR-products obtained from extracted bacterial DNA and ribosomal RNA (MUYZER ET AL. 1993, 1995, TESKE ET AL. 1995b,c). In nearly all cases, rRNA analysis of microbial communities has been a one-way-approach to infer the composition of a microbial ecosystem from ribosomal RNA sequence data. Nethertheless, molecular information from rRNA sequences also provides a starting point for classical, culture-dependent microbiological investigations, a guideline to identify and subsequently to isolate specific microorganisms from a natural bacterial community or from a co-culture (KANE ET AL. 1993). Prerequisite of this approach is that the phylogenetic position of a bacterium, as inferred from sequence data, is consistent with its physiology and culture requirements. Phylogeny and physiology are congruent for many major bacterial groups, such as the gramnegative sulfate reducers, the methanogens, or the *Campylobacter-Helicobacter* group (BALCH ET AL. 1979, DEVEREUX ET AL. 1989, VANDAMME ET AL. 1992).

We investigated a bacterial co-culture of unknown composition capable of conserving sulfate reduction activity after repetetive exposure to oxygen up to 20% saturation by DGGE of PCR-amplified 16S rDNA fragments. DGGE can separate PCR products of identical length on the basis of primary sequence and base composition (MUYZER ET AL. 1993, MYERS ET AL. 1987). Two different DGGE bands were found, indicating two different bacteria as components of the culture, and sequenced. The bands were identified as being derived from a *Desulfovibrio* strain and from an *Arcobacter* strain, a genus related to *Campylobacter, Wolinella,* and *Thiovulum*. After molecular identification of these two components by partial 16S rDNA sequences, this information was used to design selective culture conditions to isolate both bacterial strains in pure culture for subsequent physiological studies of the individual isolates. This approach is of wide application for the taxonomical identification, followed by the design of suitable enrichment and isolation procedures, and subsequent physiological analysis of isolated bacteria from unknown bacterial mixtures, environmental communities or microbial consortia.

MATERIALS AND METHODS

Origin of the co-culture. High sulfate reduction rates of up to 2 µmoles SO_4^{2-} reduced cm^{-3} d^{-1} have been found in the diurnally oxic surface layer of a hypersaline cyanobacterial mat in Solar Lake, Sinai, Egypt (FRÜND & COHEN 1992). The Solar Lake mats were introduced to an experimental hypersaline pond established at the Interuniversity Institute of Eilat in 1987 and were developed under evaporated Red Sea seawater at constant salinity of 9%±0.5 % total dissolved salt (TDS) for five years prior to this research (KRAUSE-MIRUS & COHEN, personal communication). The 3 mm thick surface layer exhibited marked diurnal fluctuations of up to 1200 µM O_2 and no detectable sulfide at noon, and 100 µM H_2S and no oxygen at midnight. This surface layer was used for the inoculation of redox gradient agar shake enrichments, in an attempt to isolate sulfate-reducing bacteria specifically adapted to oxygen exposure in shifting oxygen gradients.

Two ml of reducing 2% (wt/vol) agar, containing 0.1 % (wt/vol) ascorbic acid and (wt/vol) 0.1% sodium thioglycolate dissolved in filtered 9% TDS pond water were placed at the bottom of a 18 ml test tube. It was overlaid by 9 ml of filtered pond water supplemented with 20 mM sodium acetate, 0.1% (wt/vol) yeast extract and 1.5% (wt/vol) agar. This suspension was mixed with 1 ml of the homogenized surface layer of the cyanobacterial mat. A duplicate series of 5 sequential 10-fold dilutions of the overlaid enrichment was then carried out. The enrichment shake

agar series was later overlaid by a 2% (wt/vol) agar suspension of an axenic culture of a halophilic cyanobacterium *Synechococcus* sp. (BOLL-ARGUELLO & COHEN, in preparation) and were incubated at 35°C under illumination of 200 µE m^{-2} sec^{-1} for several days.

Oxygen profiles established in the enrichment shake agar were measured using oxygen microelectrodes in one of the dilution series (REVSBECH & JØRGENSEN 1986). The oxic layers of the other series were sliced aseptically under anaerobic conditions in 2 mm slices and the slices were placed on the surface of 2% (wt/vol) agar plates of modified Postgate medium C containing 10 mM sodium thiosulfate as sulfur source (WIDDEL & BAK 1991). After a week incubation at 25°C, black colonies were developed. The sulfidogenic colonies were transferred several times over agar plates with synthetic modified Postgate medium C containing 20 mM sodium lactate as carbon source and 10 mM sodium thiosulfate or 10 mM Na sulfate as sulfur sources, and incubated under anaerobic conditions at room temperature. Black colonies, sulfate-reducing bacteria obtained from the oxic zone of the cyanobacterial mat, appeared on both media and were transferred several times, until uniform colonies of curved bacteria were developed.

Culture conditions and Media. Modified Postgate medium C used for the shake agar enrichments contained (per liter of filtered pond water): NH_4Cl, 1.0 g; K_2HPO_4, 0.5 g; sodium citrate dihydrate, 1.0 g; yeast extract, 1.0 g; mineral solution SL7, 1 ml; vitamin solution, 1 ml; reductants solution, 10 ml; $FeSO_4$ solution, 5 ml; agar, 20g. Synthetic modified Postgate medium C used for the isolation procedure, for agar shakes, agar plates and electron donor/acceptor tests, contained (per liter of double distilled water): NaCl, 50.0 g; KCl, 1.0 g; $MgSO_4 * 7 H_2O$, 2.5 g; K_2HPO_4, 0.5 g; NH_4Cl, 1.0 g; $CaCl_2 * 2 H_2O$, 0.08 g; sodium citrate dihydrate, 1.0 g; yeast extract, 1.0 g; mineral solution SL7, 1 ml; vitamin solution, 1 ml; reductants solution, 10 ml; resazurin, 0.001 g. Five ml/l 5% (wt/vol) $FeSO_4$ solution was added as sulfide indicator. For the continuous culture, yeast extract was omitted.

Mineral solution SL7 contained per liter: 25%HCl, 10 ml; 1.5 g $FeCl_2 * 4 H_2O$, 1.5 g; $ZnCl_2$, 70 mg; $MnCl_2 * 4 H_2O$, 100 mg; H_3BO_3, 62 mg; $CoCl_2 * 6 H_2O$, 120 mg; $CuCl_2 * 2 H_2O$, 17 mg; $NiCl_2 * 6 H_2O$, 24 mg; $Na_2MoO_4 \times 2 H_2O$, 36 mg. Solution was filter sterilized.

Vitamin solution contained (g/l double distilled water): 4-aminobenzoic acid, 0.5 g; D(+)-biotin, 0.1 g; nicotinic acid, 1.0 g; Ca-D-panthothenate, 0.5 g; pyridoxamine hydrochloride, 1.5 g; thiamine chloride, 0.1 g; vitamin B_{12}, 0.5 g. Solution was filter sterilized. Reductants solution contained (g/l double distilled water): Sodium thioglycollate, 10.0; ascorbic acid, 10.0. Trace minerals, vitamins and

reductant solutions were added to the medium after sterilization; carbon and sulfur sources were introduced as sterile 1 M stock solutions.
The continuous culture experiments were carried out in an apparatus manufactured at the Biological Center of the University of Groningen, Haren, The Netherlands, including controls for temperature, pH and dissolved oxygen. The 1200 ml growth vessel was inoculated with 120 ml of the mixed culture. Growth temperature was maintained at 35°C. pH was maintained at 7.0-7.5. Stirring rate was 1500 RPM. Gas mixture containing 90% N_2, 5% O_2, 5% CO_2 was bubbled through the growth vessel at the rate of 0.1 l/min. Dissolved oxygen measured by a 900 series New Brunswick (Edison, N.J., USA) oxygen electrode was fluctuating between zero and 2.5 % air saturation. After 24 hours of batch incubation, the flow of the medium was commenced at a dilution rate D=0.1 h^{-1}. Samples were taken daily for inoculation on sulfate-lactate agar plates which were thereafter incubated under anaerobic conditions.

Media for separation of both components of the co-culture. The Defined Multipurpose medium with nonchelated trace element mixture, as described by WIDDEL & BAK (1991), was used for the isolation of the two components of the bacterial mixture. Agar shakes with four different substrate combinations were used for the analysis of the mixed culture and for further strain purification: a) 10 mM lactate, 0.01% (wt/vol) yeast extract, b) 5 mM lactate, 5 mM fumarate, 2 mM actetate, 0.01% (wt/vol) yeast extract, c) 10 mM fumarate, 2 mM acetate, 0.01% yeast extract, d) 10 mM malate, 2 mM acetate, 0.01% (wt/vol) yeast extract.

Oxygen growth tests. The ability of both strains of the co-culture to grow under various oxygen concentrations on agar plates was tested under increasing oxygen partial pressure a) 0%, b) 5%, c) 20% (vol/vol) oxygen in the gas phase. The agar plates for the oxygen growth tests were prepared with 1% (wt/vol) Bacto agar and defined multipurpose medium (WIDDEL & BAK 1991), with the following modifications: For growth under 5% and 20% oxygen, sulfide was omitted from the medium, since the oxidation of sulfide forms toxic by-products, and was replaced by 0.5 mM $Na_2S_2O_3$ as a sulfur source. Gas mixtures were checked by GC (% values in vol/vol): a) 10% CO_2, 90% N_2; b) 5% O_2, 10 % CO_2, 85% N_2; c) 20% O_2, 5% CO_2, 75% N_2. To complement for the lower CO_2 content of gas phase c, the medium was modified to contain 10 ml/l 1M $NaHCO_3$ and 0.5 g/l KH_2PO_4 .

DNA isolation. Proteinase K/SDS digestion followed by phenol extraction and ethanol precipitation was used for DNA isolation (SAMBROOK ET AL. 1989). DNA was extracted from several 0.5 ml samples of the continuous co-culture

after 144 hours, and from bacterial pure cultures after strain separation and purification.

PCR amplification of rDNA fragments. The 16S rRNA genes from mixed bacterial DNA were amplified by the polymerase chain reaction (SAIKI ET AL. 1985). The primer combination GM5F-GC clamp and DS907R (Table 1) amplified a 550 base pair fragment of the 16S rDNA, suitable for subsequent DGGE analysis, sequencing and phylogenetic analysis (MUYZER ET AL. 1993, 1995b). The two bands obtained from the mixed culture were excised from the DGGE gel, reamplified, sequenced and identified by comparison with 16S rRNA sequences of the Ribosomal Database Project (MAIDAK ET AL. 1994). After separation and pure culture isolation of the two bacterial types of the mixed culture, genomic bacterial DNA from these pure cultures was PCR-amplified using the primer pair GM5F-GC clamp and DS907R. These PCR-amplificates were DGGE-analysed side by side with the original sample and also sequenced, to ensure that the DGGE bands of the pure cultures are actually identical to the corresponding DGGE bands of the mixed culture (Fig. 1). For phylogenetic analysis, the nearly complete 16S rDNA genes were amplified with primer combination GM3 and GM4 (Table 1), and sequenced using primers complementary to conserved sequence regions.

TABLE 1. Primer sequences and positions

primer[a]	position[b]	sequence
GM3F	8 - 24	5' - AGAGTTTGATCMTGGC - 3'
GM4r	1492 - 1507	5′ - TACCTTGTTACGACTT - 3′
GM5F	341 - 357	5′- CCTACGGGAGGCAGCAG - 3′
907R	907-926	5′ - CCGTCAATTCCTTTRAGTTT - 3′
GC-clamp[c]		5′ - CGCCCGCCGCGCCCCGCGCCCGTCCCGCCGCCCCCGCCCG - 3′ [c]

[a] F is the forward primer; R is the reverse primer.

[b] Positions in the16S rRNA of E.coli (BROSIUS ET AL. 1981)

[c] The GC-clamp is attached to the 5′- end of the GM5F primer

PCR amplifications were performed with a Techne PHC-3 Temperature Cycler (Techne, Cambridge, United Kingdom) as follows: 10-20 ng of target DNA were mixed with 25 pmol of each of the appropriate primers, 4 µl 2.5 mM deoxyribonucleoside triphosphate solution, and 5 µl of 10x PCR buffer (i.e. 100 mM Tris-HCl [pH 9], 15 mM $MgCl_2$, 500 mM KCl, 0.1% (wt/vol) gelatin, 1% (vol/vol) Triton X-100) and sterile water in a final volume of 50 µl, and overlaid in a 0.5 ml vial with 2 drops of mineral oil (Sigma Chemicals Co Ltd). To minimize non-specific annealing of the primers to non-target DNA, 0.5U

SuperTaq DNA polymerase (HT Biotechnology Ltd, Cambridge, UK) was added to the reaction mixture after the initial denaturing step (94°C, 5 min), at a temperature of 80°C. In addition, to increase the specificity of the amplification and to reduce the formation of spurious by-products a "touchdown" PCR was performed (DON ET AL. 1991). This means that the annealing temperature was set 10°C above the expected annealing temperature (65°C) and decreased by 1°C every second cycle until a touchdown of 55°C, at which temperature 9 additional cycles were carried out. Primer extension was carried out at 72°C for 3 min. Five µl aliquots of the amplification products were analyzed by electrophoresis in 1% (wt/vol) FMC Nusieve agarose (Biozym Diagnostik, Hameln, Germany) gels containing 0.5 µg/ml ethidium bromide.

DGGE analysis. PCR products obtained with primer combination GM5F-GC clamp and DS907R were analysed by Denaturing Gradient Gel Electrophoresis. PCR products were prepared as follows: 300 ul of PCR product was precipitated with 0.1 vol 5 M NaCl and 2.5 vol ethanol at - 80°C for 1 hour. After centrifugation the pellet was washed with 70% (vol/vol) ethanol, dried under vacuum, dissolved in 15 µl water, and applied on the DGGE gel.
DGGE was performed with a BIO-RAD Protean II system (MUYZER ET AL. 1993, 1995a), using 6% (wt/vol) polyacrylamide gels in 0.5x TAE (20 mM Tris-acetate pH 7.4, 10 mM acetate, 0.5 mM Na_2EDTA) with denaturing gradients ranging from 20-70 % denaturant. 100% denaturant corresponds to 7 M urea and 40% (vol/vol) formamide, deionized with AG501-X8 mixed bed resin (Bio-Rad Laboratories, Inc., Hercules, California). Gels were made with 6% (wt/vol) acrylamide stock solutions (5.694 g acrylamide and 0.306 g Bis-acrylylcistamin, 20:1, per 100 ml solution) of 20% and 70% denaturing, using a Bio-Rad Econo pump and Bio-Rad Model 385 gradient former. DGGE gels were allowed to polymerize for at least 12 hours. Electrophoresis was performed for four hours at a constant voltage of 200 Volt and a temperature of 60°C. After electrophoresis the gels were incubated for 15 min in Milli-Q water containing ethidium bromide (0.5 mg/liter), rinsed for 10 min in Milli-Q water, and photographed on a UV (302 nm) transillumination table with a Cybertech CS1 digital camera (Cybertech, Berlin, Germany). Small pieces of selected DGGE bands were punched from the DGGE gel before the blotting procedure, the PCR products were eluted, reamplified and sequenced (MUYZER ET AL. 1995a).

Sequencing of PCR products. Re-amplified PCR products obtained from the excised DGGE bands were gel-purified as follows: a small well was cut into the agarose gel in front of the selected PCR-product. Electrophoresis was continued

until the PCR product migrated into the buffer-filled well. The product was taken up with a pipette and transferred into an Eppendorf vial. After incubation with 0.1 volume of 5M NaCl and 2.5 volumes of absolute ethanol for one hour at -80°C the PCR product was pelleted by centrifugation and redissolved in 50 µl bidestilled water. Purified PCR products were sequenced directly: 10-100 ng purified PCR-product were mixed with 2 µl 5x concentrated sequencing buffer (200 mM Tris-HCl pH 7.5, 100 mM $MgCl_2$, 250 mM NaCl), 1 µl 5% Nonidet P-40 (Sigma) aqueous solution, 2 pmol primer, double distilled water in a final volume of 10 µl. The double stranded PCR-product was denatured by heating the 10 µl volume to 95°C for two 5 min periods, with a short centrifugation in between to recollect the 10 µl volume. The sequencing reaction was started by adding 1.0 µl 0.1 M DTT, 2.0 µl dNTP-solution (200 nM dGTP, dATP, dTTP), 0.5 µl (a^{33}P)-dCTP; (10 µCi/µl; 3000 Ci/mmol), 2 µl (1Unit) Sequenase 2.0 (USB). After short centrifugation to collect and mix the sample volume (15.5 µl), the sample was incubated for 5 minutes at 37°C.
The sample volume was then divided into four 3.5 µl portions and added to dideoxynucleotide termination solutions (2.5 µl, containing 80 µM of each dGTP, dATP, cTTP, dCTP, and 8 µM dideoxynucleotide). After 5 min incubation at 37°C the reaction was terminated by adding 4 µl of a solution of 96% (vol/vol) formamide and 20 mM EDTA. The samples were heated for 5 min at 95°C and 2.5 µl of the samples were loaded onto a 0.25 to 0.40 mm wedge sequencing gel (6% [vol/vol] acryl/bisacrylamide [30:1] and 7M urea). After electrophoresis at constant power of 60 W for 4 hours, the gel was fixed, dried and exposed with an X-ray film (Kodak XAR5).

Sequence alignment and phylogenetic tree inference. The 16S rRNA sequences were aligned to those of other bacteria obtained from the Ribosomal Database Project (RDP) (MAIDAK ET AL. 1994). The SIMILARITY_RANK tool of the RDP was used to search for close evolutionary relatives. Sequence alignments were prepared with the sequence alignment editor SEQAPP (GILBERT 1992). Distance matrixes were calculated with DNADIST as implemented in the software package PHYLIP (version 3.5) developed by Felsenstein (FELSENSTEIN 1989, 1993), using the Jukes-Cantor model, which assumes independent change at all sites with equal probability (JUKES & CANTOR 1969). Phylogenetic trees were constructed from evolutionary distances, calculated with the algorithm of Fitch and Margoliash (FITCH & MARGOLIASH 1967) as implemented in the program FITCH in the software package PHYLIP (version 3.5). The sequences obtained in this study are available from Genbank under accession numbers L42994 and L42995.

RESULTS AND DISCUSSION

Growth characteristics of the co-culture. Although grown under microoxic conditions and repeatedly transferred on aerobic agar plates, the co-culture maintained its capabiliy of sulfate reduction as soon as it was transfered to anaerobic conditions: Subcultures obtained from the microoxic chemostat were able to reduce sulfate and thiosulfate, when incubated under anaerobic conditions in test tubes with reduced Postgate C medium. Isolated colonies were formed in anaerobic agar shakes and on the surface of aerobic agar plates with sulfate-free Postgate medium C. Ten plate transfers of anaerobically obtained cells under aerobic conditions did not deprive the culture of the ability to reduce sulfate after transfer to anaerobic Postgate medium. After several aerobic transfers, however, sulfate reduction commenced only after several days of prolonged anaerobic incubation. A steady state mixed culture of 10 mg protein l^{-1} was maintained under an atmosphere of 5% O_2, 5% CO_2, 90% N_2 supplied to the chemostat at a rate of 0.1 l min^{-1} at dilution rate of $D = 0.1\ h^{-1}$. Under these conditions all oxygen was removed in the growth vessel and no sulfate reduction or thiosulfate reduction could be detected. Subcultures obtained from the chemostat and incubated under anaerobic conditions in srew capped test tubes filled with reduced medium were able to reduce both sulfate and thiosulfate. Sulfate reduction or thiosulfate reduction was demonstrated in the chemostat immediately after removal of oxygen from the atmosphere by the stochiometric production of sulfide from either sulfate or thiosulfate. The viability of the culture under aerobic conditions, both in chemostat and on plates exposed to oxygen, combined with the maintained ability for sulfate reduction after transfer to anaerobic conditions, led to further investigations to clarify the composition of this culture.

Molecular identification. Denaturing gradient gel electrophoresis of PCR-amplified 16S rDNA fragments was applied for molecular identification of the components of the co-culture (Fig. 1). After DNA extraction, PCR-amplification of 16S rDNA fragments and DGGE, two PCR products were separated in the DGGE pattern, suggesting that the culture consisted of two different bacterial types. The PCR products were excised from the gel, reamplified and sequenced. The similarity search option of the RDP (MAIDAK ET AL. 1994) was used to search for bacteria phylogenetically affiliated to the DGGE-separated PCR products derived from the mixed culture. The 16S rDNA fragment in the upper position of the DGGE gel was derived from an epsilon-subdivision proteobacterium, related to the spirilloid sulfur reducing genera *Campylobacter, Wolinella* and *Arcobacter*

(VANDAMME ET AL. 1992). The 16S rDNA fragment in the lower gel position was derived from a *Desulfovibrio* strain, related to *Desulfovibrio salexigens*. A detailed phylogenetic analysis was postponed at this stage, since only partial 16S rDNA sequences were available.

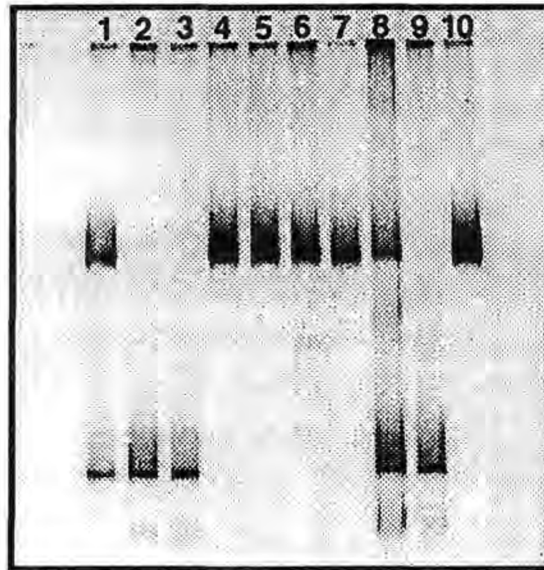

Figure 1. DGGE gel with 16S rDNA fragments derived from the mixed culture and from its two components in pure culture, the *Desulfovibrio* and the *Arcobacter* strain. Lane 1) Mixture of 16S rDNA fragments derived from both pure cultures. Lanes 2) and 3) Two independent isolates of the *Desulfovibrio* strain. Lanes 4), 5), 6), 7), 10) Five independent isolates of the *Arcobacter* strain, either picked from agar shakes with 5 mM lactate, 5 mM fumarate, 2 mM acetate before purification in 5 mM fumarate, 2 mM acetate agar shakes (lanes 4, 5), or isolated and purified directly in 5 mM fumarate, 2 mM acetate agar shakes (lanes 6, 7, 10). Lane 8) Original mixed culture. Lane 9) same as 2), for the ease of comparison with 8).

After isolation of the mixed culture components in pure culture, near-complete 16S rDNA sequences (approx. 1400 kb) of both pure strains were determined for phylogenetic analysis. The DGGE-separated 16S rDNA PCR amplificates were also recovered. The *Desulfovibrio* strain of this mixed culture was affiliated to *Desulfovibrio salexigens*. The epsilon-subdivision bacterium was by 16S rRNA sequence a member of the recently established genus *Arcobacter* (Fig. 2). *Arcobacter* consists of former *Campylobacter* species which were reclassified predominantly on the basis of 16S rRNA sequences (VANDAMME ET AL. 1991, 1992). The nitrogen-fixing species *Arcobacter nitrofigilis*, isolated from *Spartina* roots in a salt marsh, appears to be the closest relative of the strain obtained from the mixed culture (McCLUNG ET AL. 1983).

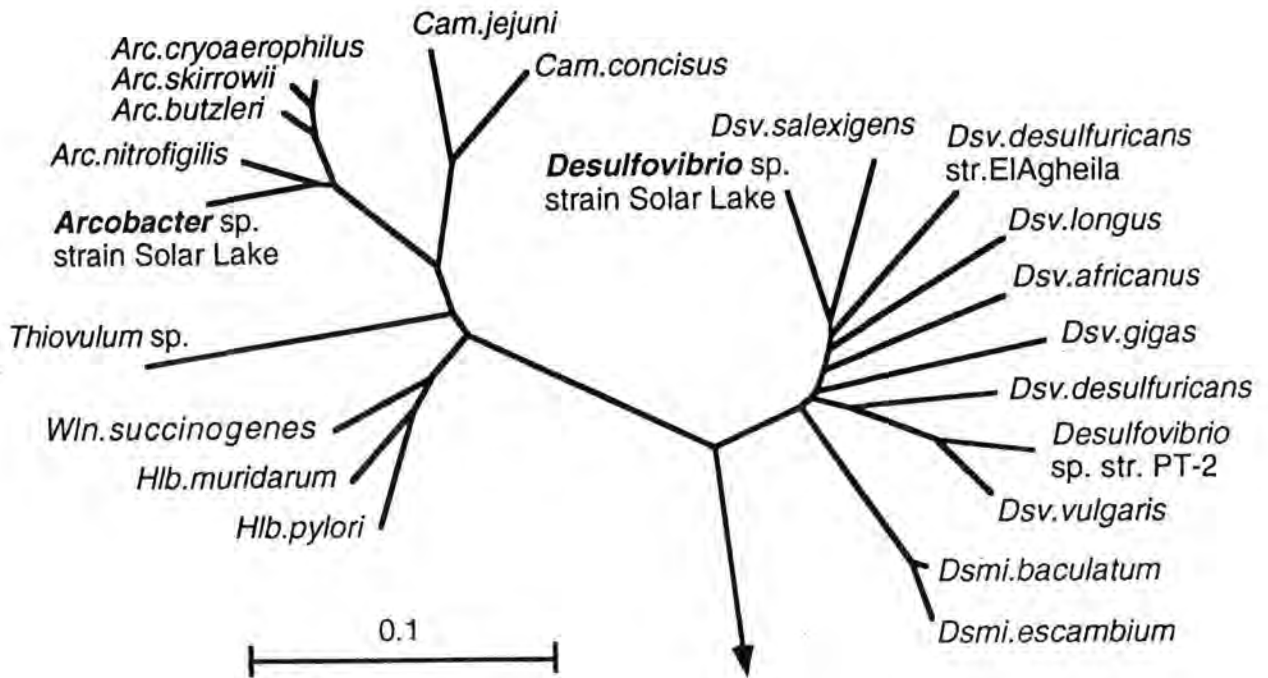

Figure 2. 16S rRNA distance tree of *Desulfovibrio, Arcobacter* and related genera of the delta and epsilon proteobacterial subdivisions, based on near-complete 16S rRNA sequences of approx. 1400 positions. The tree was rooted with *Desulfotomaculum ruminis* as outgroup. The scale bar corresponds to 0.1 mutations per nucleotide position. Sequences used in preparing this figure were derived from the Ribosomal Database Project (RDP) (MAIDAK ET AL. 1994). Abbreviations: *Arc., Arcobacter; Cam., Campylobacter; Dsmi., Desulfomicrobium; Dsv., Desulfovibrio; Hlb., Helicobacter; Wln., Wolinella.* The 16S rRNA sequences of the *Arcobacter* strain and of the *Desulfovibrio* strain are available from Genbank under accession numbers L42994 and L42995.

Isolation of bacterial components. The phylogenetic affiliation of the components of the co-culture determined the choice of selective culture conditions to separate both target organisms, the *Desulfovibrio* strain, and the epsilon-subdivision bacterium related to *Campylobacter, Wolinella* and *Arcobacter*. The *Desulfovibrio* strain of this bacterial mixture was isolated and purified in anaerobic agar shakes with defined sulfate-reducing bacteria multipurpose medium with 20 mM lactate as substrate, under a gas mixture of 10% CO_2 and 90% N_2 (WIDDEL & BAK 1991) The pure culture was grown in batch culture on the same medium. The cells showed the characteristic morphology of *Desulfovibrio* (Fig. 3a).

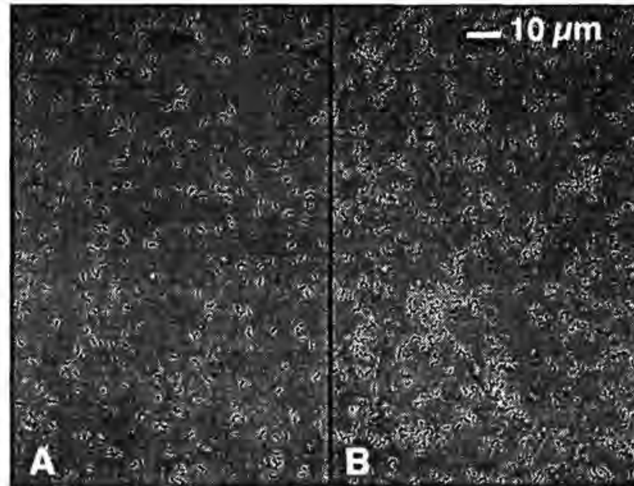

Figure 3. a) Phase contrast microphotograph of the *Desulfovibrio* strain. b) Phase contrast microphotograph of the *Arcobacter* strain. The scale bar corresponds to 10 µm.

Culture conditions for the epsilon-subdivision bacterium were adjusted with *Campylobacter* and *Wolinella*, the spirilloid sulfur reducers, as model organisms. Spirilloid sulfur reducers catalyze under anaerobic conditions the oxidation of H_2S to elemental sulfur, with fumarate being converted to succinate (WIDDEL 1988). *Wolinella succinogenes* has been shown to grow by this reaction (MACY ET AL. 1986). Therefore, sulfate-reducing bacteria multipurpose medium was supplemented with 10 mM fumarate as electron acceptor and 2 mM acetate as carbon source, since the spirilloid sulfur reducers do not oxidize succinate via an operating citric acid cycle, and cannot grow autotrophically (WIDDEL 1988). Agar shakes with this substrate combination favoured growth of the *Arcobacter* strain. Colonies were surrounded by a halo of sulfur grains, formed by oxidation of sulfide which was present in the medium at 1.5 mM. The cells were curved to spirilloid, characteristic for *Arcobacter*, *Campylobacter*, and *Wolinella* (Fig. 3b).

Colonies were picked and purified by further agar shakes, and cross-checked with lactate-medium for growth of *Desulfovibrio* contaminations. The *Arcobacter* strain was able to grow on agar plates anaerobically with a gas mixture of 10% CO_2, 90% N_2, as well as aerobically with 5% O_2, 10 % CO_2, 85% N_2; and with 20% O_2, 5% CO_2, 75% N_2. The *Desulfovibrio* strain grew only anaerobically with 10% CO_2, 90% N_2. Samples of the *Arcobacter* strain and of the *Desulfovibrio* strain were scratched off from the agar plates and checked by PCR and DGGE, showing again the respective 16S rDNA DGGE bands and thus confirming the identity of the samples.

The *Desulfovibrio* and the *Arcobacter* strain were checked in batch culture growth experiments for their range of electron donors and acceptors. The ability of the *Arcobacter* strain to reduce nitrate corresponds to this trait in the closely related species *Arcobacter nitrofigilis*, *Arcobacter butzleri* and *Arcobacter skirrowii* (VANDAMME ET AL. 1992). The physiological characteristics of the *Desulfovibrio* and *Arcobacter* isolates are summarized in Table 2.

Electron donors (20 mM)	*Arcobacter* sp.	*Desulfovibrio* sp.
lactate	+	+
formate	+	-
succinate	-	-
fumarate	-	-
malate	+	-
citrate	-	-
acetate	+	-
glycolate	-	-
glutamate	+	-
methanol	-	-
ethanol	-	+
hydrogen	n.d.	+
glucose	-	-
fructose	-	-

Electron acceptors (10 mM)	*Arcobacter* sp.	*Desulfovibrio* sp.
sulfate	-	+
sulfite	-	+
thiosulfate	-	+
nitrate	+	-
fumarate	+	-
2% oxygen*	+	-
5% oxygen*	+	-
20% oxygen*	+	-
air	-	-

*gas mixtures for testing oxygen tolerance contained 5-10% CO_2, since the *Arcobacter* strain required elevated CO_2 levels for growth under oxygen.

TABLE 2. Electron donors and acceptors. Electron acceptors used to determine the electron donor spectrum were 10 mM Na_2SO_4 for *Desulfovibrio* sp., 5% oxygen (with 5% CO_2 and 90% N_2) for *Arcobacter* sp. Electron donor for both isolates was 20 mM lactate. Fumarate served as electron acceptor also with 1.5 mM sulfide, which was oxidized to sulfur. Yeast extract was excluded from the medium. None of the isolates was capable of autotrophic growth on hydrogen and bicarbonate, and of fermentation with lactate, pyruvate and succinate.

In order to verify that these isolates were actually the two components of the investigated mixed culture, the two strains were checked by DGGE. PCR-amplified 16S rDNA fragments of the *Desulfovibrio* and of the *Arcobacter* strain were analysed side by side with the original sample by DGGE. The DGGE bands of the pure cultures match the two DGGE bands of the original co-culture (Fig. 1). The DGGE fragments of the two pure cultures were resequenced and found to be identical to their counterparts in the co-culture. This strategy is summarized in Fig.4: DGGE analysis of a bacterial sample (step 1) is followed by identification of the sequences of the bacterial components (step 2). This information facilitates to choose specific culture conditions for the bacterial groups to which the strains in the mixture are affiliated (step 3). After isolation of pure cultures, these strains are checked against the DGGE pattern of the original sample (step 4).

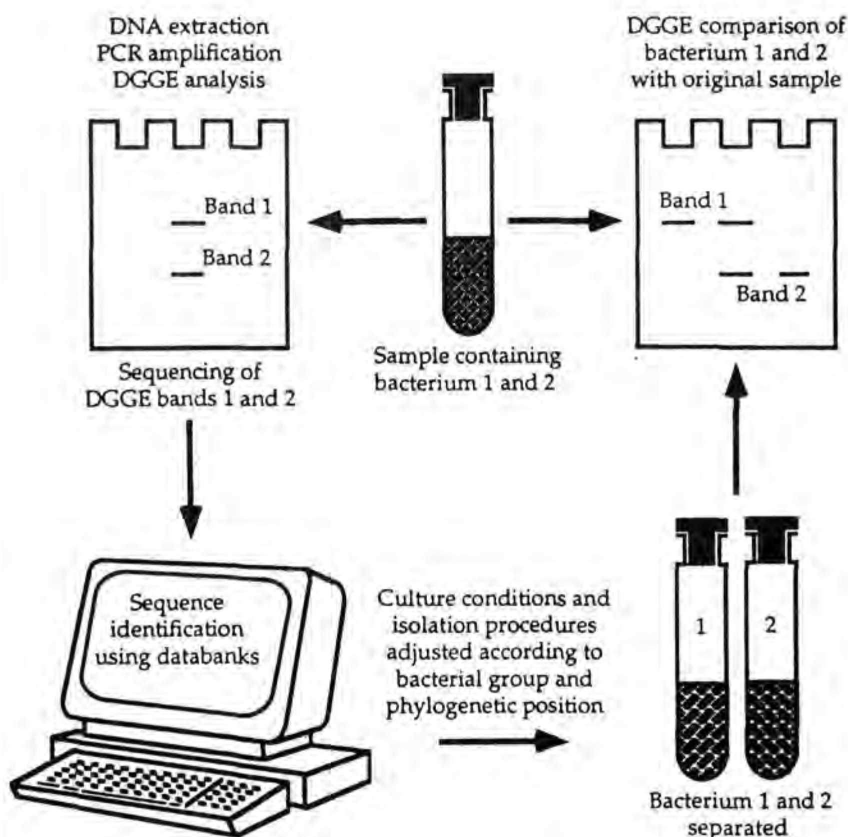

Fig. 4. Use of DGGE in the analysis of mixed cultures. After DNA isolation from the sample, PCR and denaturing gradient gel electrophoresis, the sequences of the DGGE bands are determined and compared to the sequence information available from databases, to identify the components of the microbial mixture, and subsequently to choose specific culture conditions to separate and isolate the bacterial components in pure culture. The DGGE bands of the pure cultures are compared to the DGGE pattern of the original sample.

Molecular analyses of co-cultures, unknown bacterial strains or environmental samples can be integrated with cultivation-based classical microbiology, and yield information of immediate relevance for ongoing microbiological work. Molecular identification of components of a co-culture allows to isolate and study them in pure culture, in turn providing valuable information and a better understanding of the properties of the mixed culture. The properties which had been observed for this co-culture, persistence under aerobic conditions combined with sulfate reduction after transfer to anaerobic conditions, are rationalized if the components of this culture are considered: An anaerobic, sulfate reducing *Desulfovibrio* strain, combined with an *Arcobacter* strain, capable of aerobic growth.

In many studies of microbial ecosystems, rather general media are used to cover a wide variety of bacteria. After DGGE separation of PCR-amplified marker genes and phylogenetic identification of these bacterial components, media can be optimized and adjusted to higher selectivity to isolate and separate particular members of a community or co-culture. This approach appears promising for the investigation of microbial communities and mixed cultures, and could also be useful in the analysis of bacterial consortia (TRÜPER & PFENNIG 1971) or syntrophic associations (BRYANT ET AL. 1967.).

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4.2 *Desulfovibrio oxyclini* sp. nov., a sulfate-reducing bacterium from the oxic layer of a microbial mat from Solar Lake, Sinai

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ABSTRACT

A sulfate-reducing bacterium, strain PIB, was isolated from a 10^7-fold dilution of the upper 3 mm of a hypersaline cyanobacterial mat (Solar Lake, Sinai) exposed to varying concentrations of oxygen and sulfide. The isolate appeared to be well adapted to this special environment, since it had very versatile eletron transport capacities. It grew with sulfate, sulfite or thiosulfate as electron acceptor using H_2, lactate, pyruvate, ethanol, propanol or butanol as electron donor. In the absence of these electron donors growth by disproportionation of sulfite or thiosulfate to sulfate and sulfide was obtained. If oxygen was added the bacterium switched to aerobic respiration with H_2, lactate, and even sulfide or sulfite as electron donor. The sulfur compounds were completely oxidized to sulfate. The highest respiration rates (260 nmol O_2 min^{-1} mg $protein^{-1}$) were obtained with H_2 at low oxygen concentrations. Additions of 1 % oxygen to the gas phase of a continuos culture resulted in flocculation. While the cells remained viable for a long time, a true steady state under oxic conditions was not obtained. It is concluded that strain PIB is rather oxygen-tolerent but does not carry out sulfate reduction in the presence of oxygen under the conditions tested. Analysis of the 16S rDNA sequence indicated that strain PIB belongs to the genus *Desulfovibrio*, but cannot be assigned to a known species. Therefore, a new species, *Desulfovibrio oxyclini*, is proposed.

INTRODUCTION

Microbial mats are characterized by varying oxygen and sulfide concentrations. During the light period oxygenic photosynthesis results in super-saturating oxygen concentrations, whereas in the dark oxygen is consumed and sulfide accumulates. These changes are a challenge for organisms living in such a habitat. Corresponding adaptations have been found in cyanobacteria which can switch from oxygenic to anoxygenic sulfide-dependent photosynthesis (COHEN ET AL. 1975a,b; DE WIT & VAN GEMERDEN 1978a, DE WIT ET AL 1988, STAL 1991) and purple sulfur bacteria which not only survive but even grow chemolithotrophically in the presence of molecular oxygen (DE WIT & VAN GEMERDEN 1978b, DE WIT & VAN GEMERDEN 1990, HERBERT & WELSH 1994). The occurrence of similar adaptations in sulfate-reducing bacteria has not been investigated. However, high numbers of sulfate reducing bacteria found in the oxic zones of microbial mats (VISSCHER ET AL. 1992, RISATTI ET AL. 1994) indicate that they can deal with high oxygen concentrations. Furthermore, physiological studies have shown that sulfate-reducing bacteria are able to use

oxygen for aerobic respiration resulting in ATP formation (DILLING & CYPIONKA 1990, DANNENBERG ET AL. 1992, MARSCHALL ET AL. 1993). However, all sulfate reducers so far studied revealed no or poor growth in aerated shaken cultures (MARSCHALL ET AL. 1993, SASS ET AL. 1995). Prolonged periods of oxygen exposition caused harmful effects (FUKUI & TAKII 1990, MARSCHALL ET AL. 1993). The strains were microaerophilic as indicated by the finding that the rates of aerobic respiration increased with decreasing concentrations of oxygen.

While in microbial mats or sediments high sulfate reduction rates have been measured in the oxic layers (CANFIELD & DESMARAIS 1991; FRÜND & COHEN 1992; VISSCHER ET AL. 1992; JØRGENSEN 1994), in pure culture studies no sulfate reduction was obtained in assays with oxygen concentration above 15 µM. Only low rates of sulfate reduction were observed in assays with low oxygen concentrations and electron donor in excess (MARSCHALL ET AL. 1993). Inhibition by oxygen could be explained by oxidation or inactivation of enzymes involved in the reduction of sulfate (POSTGATE 1979; DIJK ET AL. 1983) or by preference of oxygen as electron acceptor (MARSCHALL ET AL. 1993, KREKELER & CYPIONKA 1995). The occurrence of anaerobic microniches within the oxic layers appears unlikely since such microenvironments were not demonstrated by microelectrode studies. In a microscopic study of the distribution of sulfate-reducing bacteria using oligonucleotide probes for the 16S rRNA no aggregation of sulfate reducers within the oxic layer resulting in anaerobic microniches was observed (RAMSING ET AL. 1993).

In the present study we looked for sulfate-reducing bacteria being able to carry out oxygen-indifferent sulfate reduction. This would mean sulfate reduction independent of the occurrence of oxygen. Therefore we isolated an abundant sulfate reducer from the oxic zone of a microbial mat, established in the hypersaline experimental pond of the Interuniversity Institute of Eilat (inoculum mat material of the pond from Solar Lake, Sinai), where high sulfate reduction rates in the presence of oxygen have been measured (FRÜND & COHEN 1992, JØRGENSEN 1994). The physiology and the phylogenetic classification of this sulfate-reducing bacterium is presented.

MATERIALS AND METHODS

Source of organism. Strain PIB was isolated from the 3 mm thick surface layer of a cyanobacterial mat. This mat was transferred from the sediment surface of Solar lake (Sinai, Egypt) to an experimental hypersaline pond of the Interuniversity Institute of Eilat in 1987. Here, these mats developed under evaporated Red Sea seawater at a constant salinity of 9 ± 0.5 % TDS for five years prior to this research.

Cultivation and isolation. The medium used for isolation was based on Postgate's C medium as described by Widdel and Bak (1992) and contained per liter of filtered hypersaline pond water: NH_4Cl, 1.0 g; K_2HPO_4, 0.5 g; sodium citrate dihydrate, 1.0 g; yeast extract, 1.0 g; mineral solution SL9 (TSCHECH & PFENNIG 1984), 1 ml; vitamin solution (as described by PFENNIG 1978), 1 ml; reductants solution, 10 ml; 5% (wt/vol) $FeSO_4$ solution, 5 ml. The synthetic medium used for growth experiments contained all listed ingredients in double distilled water with the exception of $FeSO_4$ solution. This medium was completed by the following compounds (per l of distilled water): NaCl, 50.0 g; KCl, 1.0 g; $MgSO_4 * 7\ H_2O$, 2.5 g; $CaCl_2 * 2\ H_2O$, 0.08 g; Na_2SO_4, 1.44 g, and resazurin, 0.001 g.
The isolation was performed by sulfide gradient enrichments of the diluted inoculum followed by striking on agar plates. For sulfide gradient enrichments, 1 ml of agar solution (2% agar in filtered pond water) with 1 mM Na_2S was placed at the bottom of a 18 ml test tube. It was overlaid by 10 ml of homogenized and diluted (10^{-7}) surface layer of the cyanobacterial mat. The dilutions were prepared in medium supplemented with 5 mM ethanol as carbon and energy source and 2% agar. The test tubes were closed loosely so that exchange of air was possible and incubated at 35°C for up to 14 days.
The oxic layers of the gradient enrichments were cut aseptically under anoxic conditions in 2 mm slices. The slices were placed on agar plates containing 20 mM lactate as carbon and energy source. Sulfidogenic colonies were resuspended and striked on agar plates several times, which were incubated under anoxic conditions at room temperature.
For physiological tests strain PIB was cultivated in a chemostat as described (CYPIONKA & PFENNIG 1986). The medium contained H_2/CO_2 (80/20 vol/vol), acetate (2 mM) as additional carbon source, and sulfate at a limiting concentration of 20 mM. The marine medium was supplemented by yeast extract (1 g/l) and the pH was adjusted to 7.4. The cells were harvested by centrifugation and resuspended in a salt solution (NaCl 850 mM, KCl 13 mM and $MgCl_2$ 12.5 mM) under N_2.

Physiological tests. Utilization of energy sources, carbon sources and electron acceptors were tested using the concentrations of 20 mM for electron donors and 10 mM for electron acceptors. All these tests as well as the test for the determination of the optimum concentrations of NaCl were performed in completely filled crew cap tubes (16 ml). Growth was measured by turbidity determination.

Autotrophic growth and growth by disproportionation were checked with media containing no yeast extract which was not necessary for growth of strain PIB but caused an increase in cell yield. For checking autotrophic growth the medium contained carbonate (30 mM) as sole carbon source under a gas phase of H_2/CO_2 (80/20 vol/vol). Growth by disproportionation of sulfite or thiosulfate was tested in media containing acetate (2 mM) as carbon source. Besides turbidity, substrates consumed (sulfite and thiosulfate) and products formed (sulfate and sulfide) were measured. Sulfite was determined as described by PACHMAYR (1960) and sulfide as described by WIDDEL (1980). Sulfatre and thiosulfate were measured by ion chromatography. The reduction rates of oxygen and sulfur compounds were determined in a reaction chamber equipped with four different electrodes, oxygen, pH, sulfide, and redox electrode (CYPIONKA 1994). These measurements were performed at 30°C in N_2- or H_2-saturated salt solution (see above).

Isolation of nucleic acids , PCR and sequencing. Nucleic acids were isolated by Lysozyme/ProteinaseK/SDS digest, followed by phenol extraction and ethanol precipitation (SAMBROOK ET AL. 1989). The 16S rRNA gene was amplified py PCR, using primers GM3 (5'-AGAGTTTGATC(A/C)TGGCTCAG-3', corresponding to E. coli positions 8-23) and GM4 (5'-TACCTTGTTACGACTTG-3', corresponding to E. coli positions 1492-1507) (MUYZER ET AL. 1995b). PCR conditions, purification and sequencing of the PCR product were performed as described (OREN ET AL. 1995). The sequence is available from Genbank under accession number U33316.

Phylogenetic Tree Inference. 16S rRNA sequences related to PIB were searched and identified in the RDP database (MAIDAK ET AL. 1994) using the databases's SIMILARITY_RANK tool. The 16S rRNA sequences of the following *Desulfovibrio* and *Desulfomicrobium* strains and species were included in the phylogeny: *Dsv. salexigens* (DSM 2638^T), *Dsv. desulfuricans* strain E1 Agheila Z (DSM 1926), *Dsv. longus* strain SEBR 2582 (DSM 6739^T), *Dsv. africanus* strain Benghazi (DSM 2603^T), *Dsv. gigas* (DSM 1382^T), *Dsv. piger* (DSM 749^T), *Dsv. desulfuricans* (DSM 6949), *Dsv. vulgaris* subsp. *vulgaris* strain Hildenborough (DSM 644^T), *Dsv.* species strain PT-2, *Desulfomicrobium baculatum* strain X (DSM

1743), *Desulfomicrobium escambium*. The phylogenetic tree was inferred using the distance matrix programs DISTANCE and FITCH as implemented in the PHYLIP program package, version 3.5c (FELSENSTEIN 1993). In the program DNADIST, a matrix of evolutionary distances was computed from the sequence alignment, assuming independent change at all sites with equal probability. (JUKES & CANTOR 1969). The distance matrix was transformed into the phylogenetic tree with the least squares algorithm (FITCH & MARGOLIASH 1967) implemented in the program FITCH. Bootstrap resampling of the sequence data was performed with the program SEQBOOT, as implemented in PHYLIP. Bootstrapping tests the stability of a branching pattern by introducing random variations into the sequence data set and calculating the tree multiple times, to identify stable and unstable features of the tree topology. Here, 100 bootstrap reruns were performed.

RESULTS

Enrichment and isolation. For enrichment, sulfide gradient agar medium was inoculated with a 10-7 - fold dilution of the 3 mm thick surface layer of a cyanobacterial mat (Solar Lake, Sinai) which was exposed to diurnal changes of oxygen and sulfide (up to 1200 µM oxygen and no detectable sulfide during noon, and up to 100 µM sulfide at midnight). After an incubation period of up to two weeks at 35°C, colonies of morphologically diverse bacteria developed in the oxic layer of these shakes. From one these colonies strain PIB was isolated.

Physiological characteristics. Cells of strain PIB were motile and had a vibrio-like shape. The strain carried out incomplete oxidation of some organic acids and alcohols. Acetate was required for lithotrophic growth with H_2 and formate (Table 1). Strain PIB had a salt requirement of 2.5 - 22.5% NaCl with a growth optimum at 5 - 10 % NaCl. Growth was observed not only by reduction of sulfur compounds and fumarate but also by fermentation of organic substrates (pyruvate) and disproportionation of sulfite and thiosulfate.

Reduction of various electron acceptors. Washed cells of strain PIB catalyzed the reduction of five different electron acceptors with H_2 as electron donor (Tab. 1,2). The highest rates (1040 nmol electrons transferred min^{-1} mg $protein^{-1}$) were obtained with oxygen followed by the three different sulfur compounds. While sulfite and sulfate were reduced only at concentrations above 30 µM, thiosulfate and oxygen were also reduced at low (3 µM) concentrations. At 30 mM sulfate the rates of sulfate reduction were 20 times higher than at 0.3 mM.

Table 1. Characteristics of halophilic *Desulfovibrio* species.

	Desulfovibrio strain PIB	*Desulfovibrio halophilus*[a]	*Desulfovibrio salexigens*[b]
Shape	rods to curved rods	vibrio	vibrio
Size (µm width x length)	0.5 x 2-3	0.6 x 2.5 - 5	0.5-1 x 3.5
Motility	+	+	+
Salinity range (%)	2.5 - 22.5	3 - 18	2 - 4
Salinity optimum (%)	5 - 10	6 - 7	2 - 4
Vitamins required	-	-	-
Electron donors (with sulfate)			
$H_2 + CO_2$	-	-	-
H_2 + acetate	+	+	+
Formate + acetate	+	+	+
Lactate	+	+	+
Pyruvate	+	+	+
Citrate	-	n.d.	n.d.
Succinate	-	n.d.	n.d.
Fumarate	-	-	-
Malate	-	-	+
Acetate	-	-	-
Propionate	-	-	-
Butyrate	-	n.d.	-
Methanol	-	n.d.	n.d.
Ethanol	+	+	+
Propanol	+	+	-
Butanol	+	-	-
Glucose	-	n.d.	n.d.
Fructose	-	n.d.	n.d.
Glycolate	-	-	n.d.
Electron acceptors (with lactate as energy and carbon source)			
Sulfate	+	+	+
Sulfite	+	+	n.d.
Thiosulfate	+	+	n.d.
Oxygen	+[c]	n.d.	+[c]
Nitrate	-	-	n.d.
Fumarate	+	-	n.d.
Fermentation			
Thiosulfate	+	n.d.	n.d.
Sulfite	+	n.d.	n.d.
Formate	-	-	n.d.
Lactate	-	-	n.d.
Pyruvate	+	-	-
Malate	-	-	-
Methanol	-	n.d.	n.d.
Ethanol	-	-	n.d.
Propanol	-	-	n.d.
Butanol	-	n.d.	n.d.
i-Butanol	-	n.d.	n.d.

a) after CAUMETTE ET AL. (1991)

b) after POSTGATE (1979)

c) aerobic respiration was not coupled to growth, DANNENBERG ET AL. (1992)

Oxygen (30 µM) added to cells reducing sulfate or thiosulfate (370 µM) was consumed immediately while the sulfide concentration remained almost constant (Fig. 2). The high rates of oxygen consumtion (260 nmol O_2 min^{-1} mg $protein^{-1}$) indicated that under these conditions H_2 was the electron donor but not sulfide. The same results were obtained when the concentration of the sulfur compounds were increased to 3 mM while the oxygen addition remained the same.

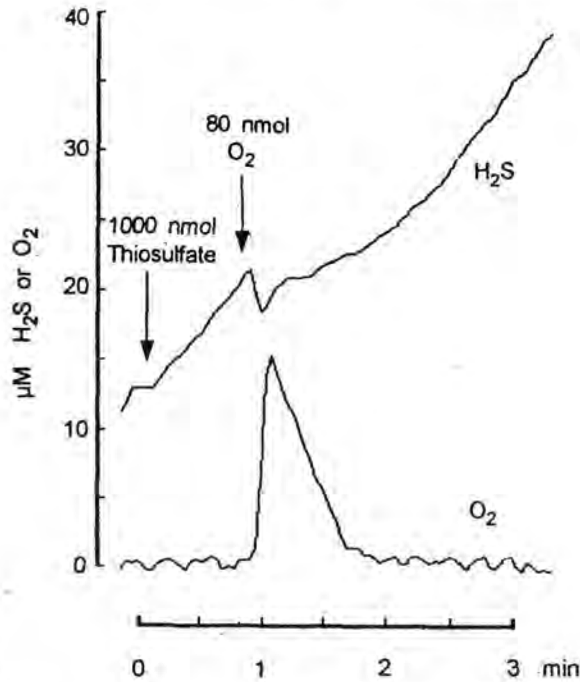

Fig. 1. Influence of oxygen on thiosulfate reduction by strain PIB. Washed cells, (2.7 ml, 0.13 mg protein ml^{-1}) were incubated in hydrogen-saturated salt solution (NaCl 850 mM, KCl 13 mM, and $MgCl_2$ 12.5 mM). Oxygen added to thiosulfate-reducing cells were immediately consumed while the sulfide concentration remained almost the same.

Table 2. Reduction of different electron acceptors in H_2-saturated solution by strain PIB.

	Rates of reactions (nmol electrons transferred min^{-1} mg $protein^{-1}$) Concentration of the electron acceptor			
Electron acceptors	30 mM	300µM	30 µM	3 µM
Sulfate	445	22	< 1	< 1
Sulfite	n.d.	255	< 1	< 1
Thiosulfate	n.d.	296	n.d.	124
Oxygen	n.d.	160	1040	≥ 1040

Oxidation of various substrates with oxygen. Various compounds were metabolized with oxygen as electron acceptor (Tab. 3). The highest rates were obtained with hydrogen as electron donor followed by the sulfur compounds sulfide and sulfite and the carbon compound lactate. No consumption of oxygen was found with acetate, thiosulfate and elemental sulfur (dissolved in methanol). Sulfide and sulfite were oxidized to sulfate as indicated by the stoichiometric ratio of sulfide and sulfite to oxygen. These results were confirmed by determination of products formed. 97% of the sulfide oxidized was found as sulfate while no thiosulfate was measured.

Table 3. Oxidation of various substrates with oxygen as electron acceptor by strain PIB[a)]

Electron donors	Maximum rates of oxygen consumption (nmol electrons transferred min^{-1} mg $protein^{-1}$)
Hydrogen	1040
Lactate	7
Acetate	0
Sulfide	152
Sulfite	56
Thiosulfate	0
Sulfur	0

[a)] Substrates were added to cell suspensions containung 10 μM oxygen.

Aerobic growth of strain PIB was checked in continuous culture experiments. First, strain PIB was grown anaerobically in the chemostat on lactate and sulfate with a dilution rate of 0.05 h^{-1} . Under these conditions sulfate was completely reduced to sulfide; 2 moles of lactate were oxidized to acetate for 1 mole of sulfate reduced. The yield was 2.48 mg protein per mol sulfate consumed. When oxygen (1%) was added to the gas phase, rapid flocculation began and true steady state was never established, although sulfate was consumed and high cell concentrations were maintained for hundreds of hours.

Phylogenetic classification. By 16S rRNA sequence, strain PIB falls into the genus *Desulfovibrio* within the phylogenetically defined family *Desulfovibrionaceae* (DEVEREUX ET AL. 1990), and belongs to the phylogenetic lineage of two salt-requiring species *Desulfovibrio salexigens* and *Desulfovibrio desulfuricans* strain El Agheila Z, which also includes the non-salt-requiring species *Desulfovibrio longus* (Fig. 1). The clustering of these *Desulfovibrio* species and strain PIB is confirmed by 82 % bootstrap. The salt-tolerant species *Desulfovibrio africanus* forms a slightly deeper branching lineage, affiliated to the *Dsv. oxyclini-salexigens-desulfuricans-longus* cluster. The 16S rRNA sequences of these *Desulfovibrio* species and of strain PIB are separated from each other by high evolutionary distances in the range of 0.105 - 0.135 mutations per site, which is characteristic for interspecies distances within the genus *Desulfovibrio* (DEVEREUX ET AL. 1990). These genetic differences support classification of strain PIB as a new species, *Desulfovibrio oxyclini*.

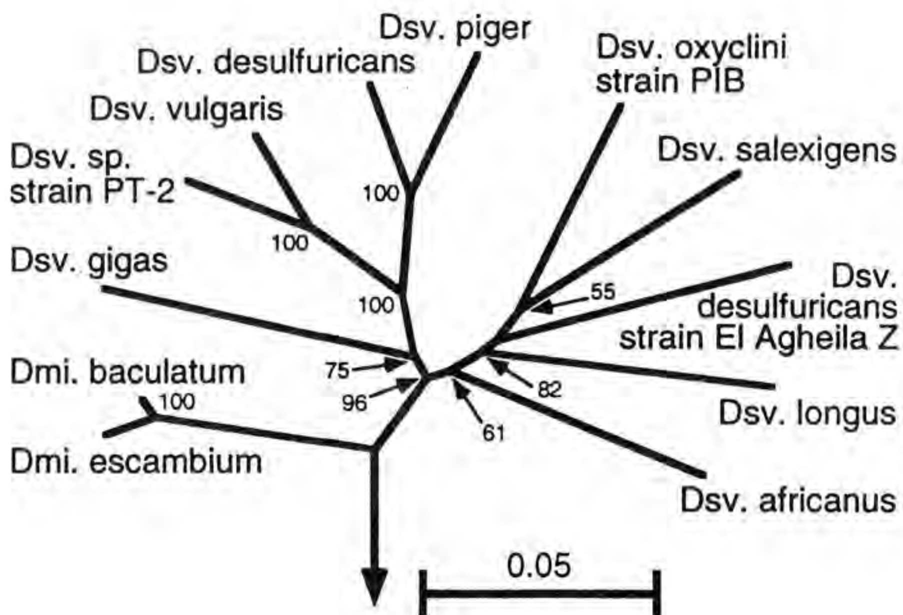

Fig. 2. 16S rRNA distance tree of *Desulfovibrio*, based on 16S rRNA positions 1 - 1380. The 5'-end of the 16S rRNA sequences were incompletely determined for most *Desulfovibrio* species, and therefore omitted from the analysis. The tree was rooted with *Desulfobacter vulgaris* as outgroup. Bootstrap values supporting the branching pattern in more than 50% of the resamplings are shown at the nodes. The scale bar corresponds to 0.05 mutations per nucleotide position. Sequences used in preparing this figure were derivedfrom the Ribosomal Database Project (RDP) on the anonymous ftp server at the University of Illinois in Urbana, Illinois updated on June 19th 1994 (MAIDAK ET AL. 1994).

DISCUSSION

In the present investigation we have studied for the first time the physiology and phylogenetic position of a sulfate-reducing bacterium that thrives in high numbers in the upper layer of a cyanobacterial mat. It turned out that strain PIB is well adapted to this extreme environment, since it can change its metabolism according to the varying conditions. The high abundance of this bacterium could be confirmed recently by reisolation and 16S rDNA analysis using denaturing gradient gel electrophoresis (TESKE ET AL. 1995d, MUYZER ET AL. 1993).

Compared to the marine sulfate reducers studied so far (DANNENBERG ET AL. 1992), strain PIB is remarkable in several aspects. It has the highest rate of aerobic respiration found so far among marine sulfate-reducing bacteria. The maximum rates (260 nmol O_2 min^{-1} mg $protein^{-1}$) are comparable to those of classical aerobic bacteria. Additionally, the numbers of electron donors that can be oxidized with O_2 is higher than in other marine sulfate reducers. However, strain PIB is clearly microaerophilic. The highest respiration rates were obtained at very low oxygen concentrations. While the bacteria possessed catalase activity and survived oxygen exposition over prolonged periods, we did not obtain continuous aerobic growth.

Strain PIB has also notable features of its sulfur metabolism. So far only sulfate reducers from freshwater origin were found to oxidize sulfur compounds completely (DANNENBERG ET AL. 1992, FUSELER & CYPIONKA 1995). Furthermore, strain PIB is the first marine sulfate reducer found to grow by disproportionation of thiosulfate or sulfite (BAK & CYPIONKA 1987, KRÄMER & CYPIONKA 1989). That thiosulfate might be an important natural substrate for this bacterium was indicated by the fact that even traces were metabolized, while sulfate was reduced only at concentrations above 30 µM.

With respect to the situation in the microbial mat we conclude that strain PIB is well adapted to survive oxic conditions. It can respire with oxygen at high rates and with various substrates. However, since oxygen blocked the reduction of sulfur compounds, we cannot explain by its activity sulfate reduction in the presence of oxygen as found in the mat (FRÜND & COHEN 1992, JØRGENSEN 1994). The same holds true for thiosulfate disproportionation which accounted for up to 50 % of the thiosulfate transformations in this zone (JØRGENSEN 1994).

Additional mechanisms and/or organisms must be involved in these processes. Strain PIB is clearly a member of the genus *Desulfovibrio*. Its closest relative is *Desulfovibrio salexigens* (Fig. 1, Tab. 1). However, the special physiological properties and the distinct 16S rRNA sequence of strain PIB do not allow to assign

this strain to a known *Desulfovibrio* species. Therefore, a new species, *Desulfovibrio oxyclini*, is proposed.

Description of *Desulfovibrio oxyclini* sp. nov.

O.xy.cli.ni. Gr. adj. oxys, acidic, Gr. v. clinein, decline; oxyclini referring to the oxycline as habitat. Straight or curved rods, 0.5 µm wide and 2 - 3 µm long. Gram-negative. Motile. Salinity range: 2.5 - 22.5 % NaCl; optimum at 5 - 10 % NaCl. Catalase is present.

Microaerophilic, reduces oxygen, sulfate, sulfite, thiosulfate and fumarate. Substrates used for aerobic respiration: hydrogen, lactate, sulfide and sulfite. Sulfide and sulfite are oxidized to sulfate. Substrates oxidized by sulfate reduction: Hydrogen, formate, lactate, pyruvate, ethanol, propanol and butanol. Ferments pyruvate, grows also by disproportionation of sulfite or thiosulfate. Desulfoviridin is present. Vitamins and growth factors are not required but may result in increased growth yields. Habitats: oxic zone and chemocline of hypersaline cyanobacterial mats. Type strain: PIB, deposited in Deutsche Sammlung von Mikroorganismen und Zellkulturen, Braunschweig, Germany.

4.3. Molecular and microbiological analysis of sulfate-reducing bacterial populations in the surface layer of a hypersaline cyanobacterial mat of Solar Lake, Sinai

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ABSTRACT

The sulfate-reducing bacterial populations within the surface layer of the hypersaline cyanobacterial mat of Solar Lake (Sinai) were investigated with molecular and culture-dependent approaches in parallel. Sulfate-reducing bacteria remain active during diurnal periods of oxygen supersaturation within the upper 0 - 2 mm of the mat. Within this layer, MPN counts indicated 10^5 - 10^6 lactate-utilizing sulfate-reducing bacteria, in part identified as *Desulfovibrio* species. The acetate-oxidizing, filamentous sulfate reducing bacterium *Desulfonema* was also found in high, although variable numbers, 10^4 - 10^6 cells per ml, within the surface layer, and in higher numbers, 10^6 - 10^7cells per ml, between 2 - 4 mm, the daytime position of the chemocline. Denaturing Gradient Gel Electrophoresis (DGGE) of PCR-amplified 16S rDNA fragments, subsequent hybridization with a 16S rRNA probe developed for *Desulfonema*, and sequencing, a dominant DGGE band was found in the upper 5 - 8 mm of the mat. This molecular isolate was related to the phylogenetic cluster of *Desulfonema, Desulfococcus, Desulfosarcina,* and *Desulfobotulus*. Thus, culture studies and molecular results indicate that not only *Desulfovibrio*, for which oxygen-tolerance has been dicussed lately, but also completely oxidizing sulfate-reducing bacteria, among them the filamentous genus *Desulfonema*, are dominant members of the sulfate-reducing population in the oxic surface layers of the Solar Lake cyanobacterial mat.

INTRODUCTION

Sulfate-reducing bacteria are almost universally distributed in marine sediments and microbial mats. Depth of the sulfate-reducing zone, population density and activity of sulfate-reducing bacteria are inversely related: In nutrient-low marine sediments, i.e. in the deep sea and the oceanic basins, the zone of sulfate reduction extends over a scale of many meters, but involves low numbers of sulfate-reducing bacteria and low activities (IVANOV ET AL. 1976, IVANOV ET AL. 1980, CANFIELD 1991). With improved supply of organic nutrients, increased primary production, or higher sedimentation rate, sulfate reducing bacteria find better conditions, and occur in higher numbers and activity. Increasing bacterial activity and numbers are compressed into a narrowing spatial scale, since nutrient supply improves towards the upper sediment layers, and energy-rich substrates are rapidly consumed before diffusing into deeper layers. Examples are provided by sulfate-reducing bacterial populations of organic-rich coastal sediments of Kattegat and Limfjord (Denmark), with highest values of sulfate-reducing activity (20 - 200

nmol SO_4^{2-} cm^{-3} d^{-1}) and bacterial numbers (10^5 - 10^6 cells per ml) within the upper 10 - 20 cm or 1 - 10 cm of the sediment (JØRGENSEN 1982b, JØRGENSEN & BAK 1991). The most extreme example for spatially compressed and highly stratified communities of sulfate reducing bacteria is found in hypersaline cyanobacterial mats. Allmost all recent investigations of sulfate reducing bacterial populations in microbial mats have confirmed highest cell numbers in the range of 10^6-10^8 cm^{-3}, and high sulfate-reducing activity, in the range of 500 - 15.000 nmol SO_4^{2-} cm^{-3} d^{-1}, within the uppermost 10 millimeters of cyanobacterial mats (JØRGENSEN & COHEN 1977, CANFIELD & DES MARAIS 1991, 1993, FRÜND & COHEN 1992, VISSCHER ET AL. 1992, CAUMETTE ET AL. 1994). The matrix of filamentous cyanobacteria, such as *Microcoleus*, *Oscillatoria* or *Lyngbya*, harbors within a millimeter scale bacterial ecosystems of highest complexity, interacting and shaped by steep opposed chemical gradients (VAN GEMERDEN 1993). Sulfate-reducing bacteria of different trophic levels inhabit specific mat layers; i.e. incompletely oxidizing *Desulfovibrio* are dominant 7 to 15 mm below the surface of Baja California cyanobacterial mats, while acetate-oxidizing sulfate reducers of the genera *Desulfobacter* and *Desulfobacterium* dominate the sulfate-reducing bacterial community below 3 cm (RISATTI ET AL. 1994).

A microbiological mystery has been the nature of sulfate-reducing bacterial populations in oxic surface layers of photosynthetic cyanobacterial mats, which are highly active under in-situ oxic conditions: 200 bis 500 µM oxygen in the surface layer of Baja California mats coincides with sulfate reduction rates of 7000 - 11.000 nmol cm^{-3} d^{-1} (CANFIELD & DES MARAIS 1991, 1993). In the benthic cyanobacterial mats of Solar Lake, Sinai, maximum cell numbers of 2.5 x 10^6 sulfate reducing bacteria cm^{-3}, and highest sulfate-reducing activities of 5400 nmol SO_4^{2-} cm^{-3} d^{-1}, are found in the top layer (JØRGENSEN & COHEN 1977). Localized oxygen maxima, between 500 and 1000 µM, coincided with considerable sulfate-reducing activity, 200 - 400 nmol cm^{-3} d^{-1} (FRÜND & COHEN 1992).

Population profiles obtained with 16S rRNA oligonucleotide probes indicated the phylogenetic lineages of *Desulfovibrio* and the *Desulfococcus-Desulfosarcina-Desulfobotulus* cluster as predominant sulfate reducing populations in the uppermost millimeter of Baja California cyanobacterial mats (RISATTI ET AL. 1994). *Desulfovibrio* is to some degree tolerant to oxic conditions: Several *Desulfovibrio* species can respire aerobically (DILLING & CYPIONKA 1990), or even use oxygen as their preferred electron acceptor (KREKELER & CYPIONKA 1995), although growth by oxic respiration, defined as more than doubling of protein, has not yet been observed in *Desulfovibrio* (MARSHALL ET AL. 1993). Specific sulfate reducers of the *Desulfococcus-Desulfosarcina-Desulfobotulus* lineage have not been discussed so far.

In this study, we investigated sulfate reducing bacterial populations in the surface layer of the Solar Lake cyanobacterial mat, using microbiological and molecular methods in parallel. Besides *Desulfovibrio*, filamentous sulfate reducers of the genus *Desulfonema* were among the dominant isolates. A specific phylotype of the *Desulfococcus-Desulfosarcina-Desulfobotulus* cluster, which also includes *Desulfonema* in its phylogenetic range (FUKUI ET AL. 1995), was found as consistently recurring molecular isolate in surface mat samples.

MATERIALS AND METHODS

Sampling of Bacteria. Numbers of sulfate reducing bacteria in the Solar Lake cyanobacterial mat were determined by Most-Probable-Number counts in November 1994, using samples of undisturbed shallow mat (approx. 0.5 m depth) from the eastern bank of Solar Lake. Mat blocks were cut out and transferred to the Experimental pond of the Heinz Steinitz Marine Biological Laboratory, Eilat, Israel, for taking subsamples and further processing. Solar Lake mat cores were sliced into the following layers: surface layer 0 - 2 mm, corresponding to the oxygenated zone at daytime, 2 - 4 mm, the chemocline layer at daytime, and the permanently anoxic layers 4 - 7 mm, 7 - 10 mm, 10 - 13 mm. The rubber-like mat slices were homogenized manually in 9 volumes Solar Lake water for approx. 20 minutes, using a 10-ml potter homogenizer with a teflon-coated piston. The resulting tenfold diluted mat homogenate was used as inoculum for Most-Probable-Number (MPN) dilution series. The first step of each MPN series was inoculated with 0.1 ml mat volume, followed by eight tenfold dilution steps.
MPN counts of Solar Lake mat were made at noon and at midnight, to represent the changing diurnal regimes of the mat surface layer, oxygen supersaturation at daytime and anoxia at night.

Migration experiment. Sulfate reducing bacteria might possibly migrate into the surface layer of the mat at night and retreat again as soon as oxygen penetrates into the surface layer. To test this migration pattern, a mat core was sliced into layers 0 - 2 mm, 2 - 4 mm, 4 - 7 mm, 7 - 10 mm, 10 - 13 mm at noon. Aluminiumoxide filters were placed between the layers, to prevent bacterial migrations between the layers, especially into the surface layer from below. Mat layers, including filters, were carefully put together and pushed back into the plastic core, and incubated in situ until midnight. MPN counts of the layers were made at midnight.

Media for MPN counts of sulfate reducing bacteria. Numbers of cultivable, viable sulfate-reducing bacteria were estimated with most-probable-number (MPN) dilutions in liquid medium (AMERICAN PUBLIC HEALTH ASSOCIATION 1969). Media were made with artificial seawater, which contained per liter of distilled water: 40 g NaCl, 5.67 g $MgCl_2 * 6 H_2O$, 6.8 g $MgSO_4 * 7 H_2O$, 1.47 g $CaCl_2 * 2 H_2O$, 0.19 g $NaHCO_3$, 0.66 g KCl, 0.09 g KBr. Compared to normal artificial seawater, which contains 26.37 g NaCl per liter, the NaCl concentration was increased to 40 g/l, as an approximate to the winter chlorosity and salinity of Solar Lake surface water, approx. 4 and 7 % (COHEN ET AL. 1977). Multipurpose medium for sulfate-reducing bacteria contained per liter artificial sea water 1 ml non-chelated trace element mixture No. 1, 1 ml selenite-tungstate solution, 30 ml $NaHCO_3$ solution, 1 ml vitamin mixture, 1 ml Thiamine solution, 1 ml Vitamin B_{12} solution, and 7.5 ml Na_2S solution (WIDDEL & BAK 1991). The salt concentrations of the modified sea water medium used in this study are higher than those of many standard brackish or marine sulfate reducer media, and enabled growth of several rarely isolated sulfate reducers, such as *Desulfonema*, which require elevated concentrations of Ca^{2+} and Mg^{2+} (WIDDEL & BAK 1991).
MPN counts were performed on lactate (20 mM), acetate (20 mM), and formate (10 mM) plus 2 mM acetate to account for non-autotrophic bacteria. The media were prepared anaerobically in a pressure-proof modified Erlenmeyer flask, and dispensed into culture vials (Kimex). After dispensing the medium, each MPN vial was immediately gassed with a gassing syringe according to the Hungate technique, and sealed with a butyl stopper. Lactate and acetate MPN vials were gassed with a mixture of 90% (vol/vol) N_2 and 10% (vol/vol) CO_2. Sulfate reducers were counted by triplicate MPN dilution series from the mat layers 0 - 2 mm, 2 - 4 mm, 4 - 7 mm, 7 - 10 mm and 10 - 13 mm. The 0 - 2 mm layer was oxygenated at daytime. The 2 - 4 mm layer represents the chemocline at daytime, whereas the deeper layers remain fully anoxic at all times. MPN dilution series were inoculated with 1 ml of water or sediment sample, respectively, and subsequently diluted in eight 1:10 dilution steps.

Sampling for Nucleic Acid Extraction: Solar Lake mat cores were taken in June 1994 from samples of undisturbed shallow mat (approx. 0.5 m depth) from the eastern bank of Solar Lake. Mat blocks were cut out and transferred to the experimental pond of the Heinz Steinitz Marine Biological Laboratory, Eilat, Israel, for taking subsamples and further processing. Three sets of subsamples were taken at different times of the day: 4^{00} am, representing the night status of the mat (anoxic surface layer), 12^{00} am and 5^{00} pm, representing the day status of the mat (oxic surface layer).

Nucleic Acid Extraction. Nucleic acids were extracted from Solar Lake mat by hot phenol extraction: The surface layer (1 cm) of a Solar Lake mat core (1 cm diameter) was sliced into 1 mm layers. 0.2 cm^3 of each slice were homogenized and mixed with the same volume ice-cold AE buffer (20 mM Na-Acetate, 1 mM EDTA, pH 5.5), and kept on ice. To each sample, 500 µl phenol-chloroform-isoamylalcohol (pH 5) and 5 µl 25% SDS were added. Phenolic and aequeous phases are mixed by approx. 1 minute vortexing. After 5 min of incubation at 60°C in a waterbath the samples were cooled on ice and then centrifuged for 5 min at 4000 *g*. The aequous phases were transferred to new vials containing 25 µl of 2 M Na-acetate, pH 5.2. Contaminating proteins and lipids were removed by subsequent twofold extraction of the aequous phase with 500 µl phenol-chloroform-isoamylalcohol. Nucleic acids were precipitated with 2.5 vol 96% (vol/vol) ethanol overnight at -70°C, followed by 10 min centrifugation at 4000*g*. The resulting white pellets were overlaid with 100 µl ethanol and stored at -70°C during the stay in Eilat. During the flight to Bremen, the samples were kept on ice-salt-mixture, at temperatures of -20 to 0°C. All chemicals and buffers used for the isolation of nucleic acids from Solar Lake mat, except the Phenol-Chloroform-isoamylalcohol mixture, were treated with diethyl pyrocarbonate (DEPC), to remove DNAse and RNAse activity (SAMBROOK ET AL. 1989).

PCR amplification of rDNA fragments. The 16S rRNA genes from mixed bacterial DNA or cDNA were amplified by the polymerase chain reaction (PCR). Two different primer combinations were used: The primer combination GM5-GC clamp and DS907R amplifies a 550 basepair fragment of the 16S rRNA gene. The primer combination 385-GC clamp and DS907R amplifies a 520 base pair fragments of the 16S rRNA (Table 1). Both fragments are suitable for subsequent DGGE analysis, membrane hybridization and sequencing, since they yield sufficient sequence information for phylogenetic analysis (MUYZER ET AL. 1995b). PCR amplifications were performed with a Techne PHC-3 Temperature Cycler (Techne, Cambridge, United Kingdom) as follows: Approx. 50 ng of target DNA were mixed with 25 pmol of each of the appropriate primers, 5 µl of 10x PCR buffer (i.e. 100 mM Tris-HCl [pH 9], 15 mM $MgCl_2$, 500 mM KCl, 0.1% [wt/vol] gelatin, 1% [vl/vol] Triton X-100), 4 µl 2.5 mM deoxyribonucleoside triphosphate solution, and sterile water in a final volume of 50 µl, and overlaid in a 0.5 ml vial with 2 drops of mineral oil (Sigma Chemicals Co Ltd). To minimize non-specific annealing of the primers to non-target DNA, 0.5 U SuperTaq DNA polymerase (HT Biotechnology Ltd.) was added to the reaction mixture after the initial denaturing step (94°C, 5 min), at a temperature of 80°C. In addition, to increase the specificity of the amplification and to reduce the

formation of unspecific by-products a "touchdown" PCR (DON ET AL. 1991) was performed where the annealing temperature was set to 65°C , which is 10°C above the expected annealing temperature, and decreased by 1°C every second cycle until a touchdown of 55°C, at which temperature 9 additional cycles were carried out. Denaturation was carried out at 95°C for 1 min, the annealing time was 2 minutes, primer extension was carried out at 72°C for 3 min. The ramp times were set to the fastest mode of the PHC3 Thermocycler. Aliquots of 5 µl of the amplification products were analyzed by electrophoresis in 1% (wt/vol) Nusieve agarose (FMC) gels containing 0.5 µg/ml ethidium bromide.

DGGE analysis, blotting and hybridization analysis of DGGE gels. PCR products obtained with primer combination GM5F-GC clamp and DS907R, and 385-GC clamp and DS907R, were analysed by Denaturing Gradient Gel Electrophoresis. DGGE was performed with a BIO-RAD Protean II system, using a denaturant gradient of 30-70 %, with 100% corresponding to 7 M urea and 40% [vol/vol] formamide (MUYZER ET AL. 1993, MUYZER ET AL. 1995a). Electrophoresis was modified to eight hours at a constant voltage of 100 Volt and a temperature of 60°C. Electroblotting of The DGGE pattern was electroblotted to a nylon membrane (Hybon-N+: Amersham, Amersham, UK), using a Trans-Blot SD semi Dry Transfer Cell (BIO-RAD Laboratories, Inc.), followed by UV-crosslinking of the DNA to the membrane (MUYZER ET AL. 1993, MUYZER ET AL. 1995a).
The DGGE-separated, membrane-crosslinked PCR products were analysed by hybridization with rRNA-targeted probes (Table 1.). The 16S rRNA region amplified by primers GM5F and DS907R, or 385GC and DS907R, respectively, includes several target sites for general, genus- and species-specific oligonucleotide probes for sulfate-reducing bacteria (DEVEREUX ET AL. 1992, FUKUI ET AL. 1995, KREKELER ET AL. 1995). Probes were labeled with digoxigenin, using the digoxigenin oligonucleotide labeling kit and protocols from manufacturer Boehringer Mannheim, Germany, or were obtained with 5'-digoxigenin-label from Biometra, Göttingen. Digoxigenin-labeled probes were detected by an antibody coupled with alkaline phosphatase, which gives a chemiluminescent reaction with CSPD (Serva Tropix). Hybridization was performed as described by MUYZER ET AL. (1995a). For probe 657, hybridization and washing temperature 47°C was used (FUKUI ET AL. 1995). Probe 636 was hybridized at 40°C and washed at 45°C.
To sequence specific bands which had in previous experiments hybridized with probes, PCR and DGGE were repeated, and pieces of the selected DGGE bands were punched out from the DGGE gel. The PCR products of the cutouts were eluted, re-amplified and sequenced directly as described (MUYZER ET AL. 1995a).

Table 1: Primers and Oligonucleotides used in this study

PRIMER	POSITION	SEQUENCE	
GM5F-GC-clamp*	341 - 357	5'- GC-clamp-CCTACGGGAGGCAGCAG -3'	general eubacterial primer for 16S rRNA gene DGGE-fragment amplification
385-GC-clamp*	385-402	5'- CGGCGT(C/T)GCTGCGTCAGG-3'	selective, but not specific: mainly delta-subdivision proteobacteria, incl. sulfate reducers
DS907-reverse	924 - 907	5'- GTCAATTCCTTTGAGTTT -3'	general eubacterial primer for 16S rRNA gene DGGE-fragment amplification
Probe 657	657 - 676	5'- TTCCG(C/T)TTCCCTCTCCCATA -3'	rRNA oligonucleotide probe for the genus *Desulfonema* (FUKUI ET AL. 1995)
Probe 636	636 - 653	5'- CTCAAGCACAGCAGTATCA-3'	*Desulfovibrio oxyclini* and closely related *Desulfovibrio* strains S9 and T10 (KREKELER ET AL. 1995)

*GC-clamp: 5'- CGCCCGCCGCGCGCGGCGGGCGGGGCGGGGGCACGGGGGG - 3'

Sequence alignment and phylogenetic tree inference. The 16S rRNA sequences were aligned to those of other bacteria obtained from the Ribosomal Database Project (MAIDAK ET AL. 1994). The SIMILARITY_RANK tool of the RDP was used to search for close evolutionary relatives. Sequence alignments were prepared with the sequence alignment editor SEQAPP (GILBERT 1992). Distance matrixes were calculated with DNADIST as implemented in the software package PHYLIP (version 3.5) developed by FELSENSTEIN (1989), using the Jukes-Cantor model, which assumes independent change at all sites with equal probability (JUKES & CANTOR 1969). Phylogenetic trees were constructed from evolutionary distances, calculated with the algorithm of Fitch and Margoliash as implemented in the program FITCH in the software package PHYLIP (version 3.5). The sequences obtained in this study are available from Genbank under accession numbers.

RESULTS

Most Probable Number counts. All MPN counts were based on three sliced cores, one for the night counts, one for the noon counts, and one for the migration experiment, which were taken from a single piece (approx. 10 x 10 cm) of Solar Lake Mat, selected for an even surface with a no waves and faults, and showing the characteristic, undisturbed lamination of Solar Lake mats.

Independently of the sampling time, the highest density of lactate-utilizing sulfate-reducing bacteria, in the order of 2 - 4 x 10^6 cells per ml, occurred in the 0 - 2 mm oxic surface layer and in the 2 - 4 mm chemocline layer (Figs. 1 A,B). At 4 -7 mm, 1 x 10^6 SRBs ml^{-1} were found day and night. At 7 - 10 mm, 2 x 10^6 SRBs ml^{-1} were found in the day counts, but only 0.4 x 10^6 SRBs ml^{-1} at night. An unusually low number is also found in the acetate count of this sample. The 10 - 13 mm sample shows approx. 1 x 10^6 lactate-utilizing SRBs ml^{-1} in both samples, day and night (Figs. 1 A,B).

Acetate-oxidizing sulfate reducing bacteria occurred in densities of 2 - 4 x 10^5 cells ml^{-1} the 0 - 2 mm surface layer, in the noon as well as in the night sample (Figs. 1 C,D). This number is a conservative estimate: Due to single gaps in the positive dilutions, the resulting Most Probable Number might underestimate actual cell densities by factor ten. For example, a single negative 10^5 dilution reduces the most probable number of the noon count, with positive dilutions at 10^6, 10^7, 10^7, from 9.3 x 10^6 to 4.4 x 10^5 cells ml^{-1}. Similar gaps reduce the most probable number of the night counts from possibly 2.1 x 10^6 to 2.0 x 10^5 cells ml^{-1}. Clumping and colony formation, which are common in acetate-utilizing sulfate-reducing bacteria, could interfere with counts of these populations by dilution series. The 2 - 4 mm layer of the noon counts might still be affected by this, with a conservative most probable number of 4 x 10^5 cells ml^{-1}. In striking contrast, the night counts for the 2 - 4 ml layer are, with gaps absent, very high, 9.3 x 10^7 cells ml^{-1}. Most Probable Numbers in this order of magnitude are reached if positive dilutions are not interrupted by single gaps. In deeper layers, the numbers of acetate-oxidizing sulfate reducers are unambiguous, since gaps are absent. Day and night counts showed different patterns: The day counts increase gradually with depth, towards 10^6 cells per ml within the 4 - 7 mm layer, and maintained in deeper layers (Fig. 1C). The night counts remain between 10^5 and 10^6 cells ml^{-1} , with a minimum of 0.75 x 10^5 cells ml^{-1} in the 7 - 10 mm layer, in parallel to the unusually low lactate counts for this particular sample (Fig. 1D).

Figs. 1 D,C show the conservative Most Probable Number estimates on acetate. The counts of acetate-oxidizing sulfate-reducing bacteria in the surface layer (0 - 2 mm) are potentially one order of magnitude higher.

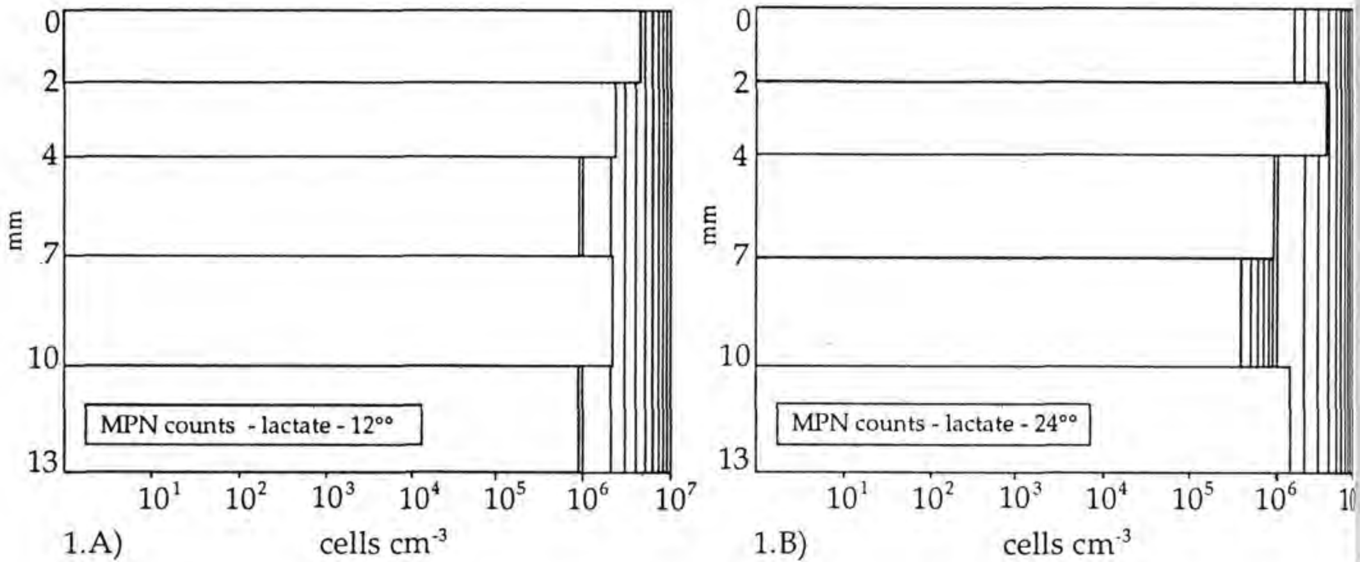

Fig. 1 A. Lactate MPN estimates of sulfate-reducing bacteria from Solar Lake cyanobacterial mat, at noon. **B.** At midnight. Lactate-oxidizing SRBs cm^{-3} of the mat layers 0 - 2 mm, 2 - 4 mm, 4 - 7 mm, 7 - 10 mm, 10 - 13 mm, are plotted on a logarithmical scale.

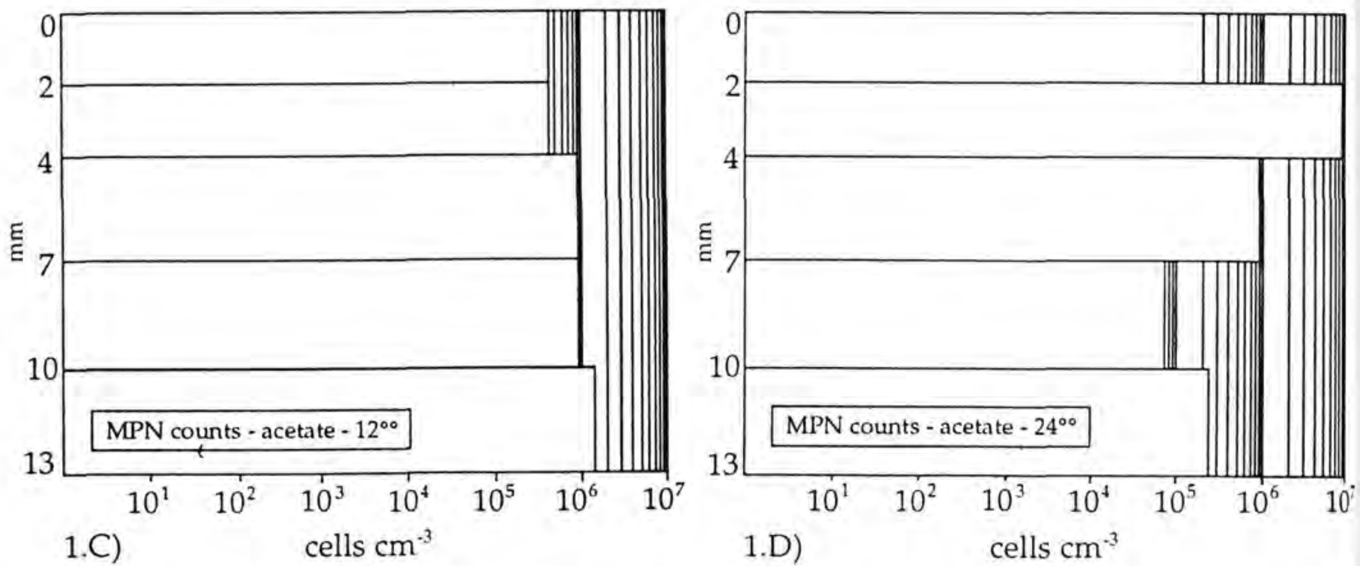

Fig. 1 C. Acetate MPN estimates of sulfate-reducing bacteria from Solar Lake cyanobacterial mat, at noon. **D.** At midnight. Acetate-oxidizing SRBs cm^{-3} of the mat layers 0 - 2 mm, 2 - 4 mm, 4 - 7 mm, 7 - 10 mm, 10 - 13 mm, are plotted on a logarithmical scale.

Migration experiment. Mat layers were sliced at noon and separated by filters, to preserve the bacterial distribution at noon. The core was reassemled and incubated in situ until midnight, when the slices were used for MPN on lactate. These counts should reflect the noon distribution of bacteria, since possible bacterial migrations were interrupted by the filters. Surprisingly, bacterial numbers were extremely high in the chemocline layer (4.3×10^7 SRBs cm^{-3}), and two orders of magnitude lower in the surface layer (4.4×10^5 SRBs cm^{-3}), and one order of magnitude lower compared to the lactate counts of the surface layer at noon and midnight. Counts in the deeper layers remained around $1 - 1.5 \times 10^6$ SRBs cm^{-3}, the same range as the other lactate counts. Either the slicing and the incubation of the reassembled core over 12 hours has affected the numbers of lactate-utilizing bacteria in the two upper layers, or the 2 - 4 mm mat slice contained a very dense cluster, a spatial inhomogeneity in the distribution of sulfate reducers. At least two (10^7) of the three highest dilutions of the chemocline layer contained *Desulfonema,* a completely oxidizing filamentous sulfate reducer, which due to its morphology is probably not homogeneously distributed in the mat.

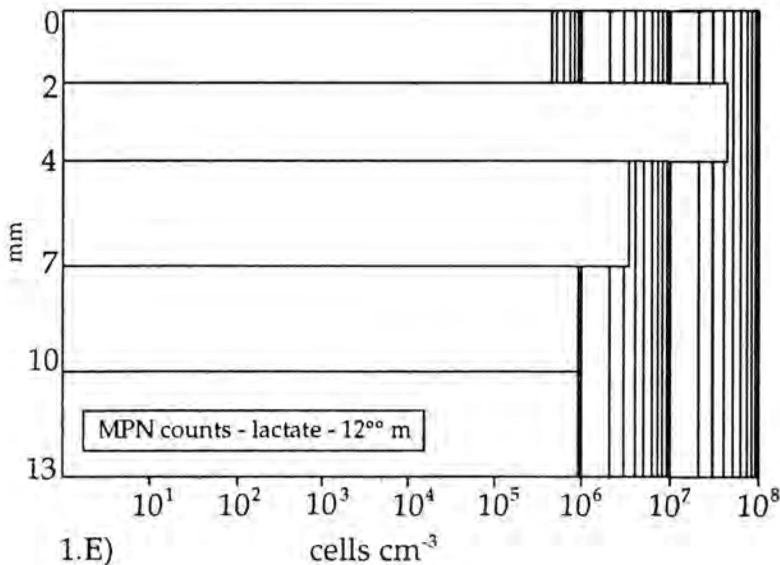

Fig. 1 E. Lactate MPN estimates of sulfate-reducing bacteria from Solar Lake cyanobacterial mat of the migration experiment. Lactate-oxidizing SRBs cm^{-3} of the mat layers 0 - 2 mm, 2 - 4 mm, 4 - 7 mm, 7 - 10 mm, 10 - 13 mm, are plotted on a logarithmical scale.

Counts of *Desulfonema*. Filamentous sulfate reducers of the genus *Desulfonema* (WIDDEL ET AL. 1983) developed in high MPN dilutions, on acetate, and also on lactate. Here, *Desulfonema* grew probably on acetate, propionate or other compounds excreted by lactate-utilizing sulfate reducing bacteria. The MPN dilutions in which *Desulfonema* developed are listed in Table 2. Due to gaps in the ocurrences of *Desulfonema* in the lactate MPN, most probable numbers for *Desulfonema* are not inferred. Nethertheless, *Desulfonema* is found in high cell densities, reaching 10^6 cells per ml in the 0 - 2 mm surface layer. *Desulfonema* occurs in maximal density in the 2 - 4 mm chemocline layer; two acetate and two lactate dilution series yield *Desulfonema* in the seventh dilution, indicating cell densities around 10^7 cells per ml. Towards deeper layers, the numbers decrease to approx. 10^5 and 10^4 cells per ml. Since *Desulfonema* grows in filaments, and not in single, independently dispersible cells, these numbers are probably an underestimate of its real abundance in the Solar Lake cyanobacterial mat. Direct microscopical observations (COHEN, unpublished) suggest high densities of this filamentous sulfate reducers in the upper mat layers.

	Lactate			Acetate	
	12^{oo}	12^{oo}m[1]	24^{oo}	12^{oo}	24^{oo}
0 - 2 mm	10^5	-	-	10^3	10^3
	10^5	10^4	-	10^5	10^4
	10^6	10^6	10^3	10^6	10^6
2 - 4 mm	10^5	10^6	10^3	10^4	10^5
	10^5	10^7	10^5	10^6	10^6
	10^6	10^7	10^6	10^7	10^7
4 - 7 mm	10^3	-	10^3	10^4	10^4
	10^4	-	10^4	10^5	10^5
	10^4	10^6	10^5	10^5	10^5
7 -10 mm	10^4	-	-	10^4	10^4
	10^5	10^4	-	10^5	10^4
	10^5	10^4	10^4	10^5	10^4
10-13mm	-	-	-	10^4	10^3
	-	10^3	10^3	10^4	10^4
	-	10^4	10^4	10^4	10^5

[1] Migration experiment.

Table 2. *Desulfonema* in Most-Probable-Number counts on lactate and acetate.

Denaturing Gradient Gel Electrophoresis and membrane hybridization. PCR-amplified 16S rDNA fragments of bacterial DNA, extracted from each millimeter layer of the upper ten millimeters of the Solar Lake cyanobacterial mat, were analyzed by Denaturing Gradient Gel Electrophoresis (DGGE). Each lane of the resulting DGGE patterns (Fig. 1) represents a millimeter layer of the uppermost cm of the Solar Lake cyanobacterial mat. The DGGE patterns were blotted to a nylon membrane and hybridized with 16S rRNA probe 657, and probe 636. Probe 657 was designed as a specific 16S rRNA probe for fluorescent *in-situ* hybridization of the genus *Desulfonema*, encompassing the species *D. magnum*, *D. limicola*, and *D. ishimotoei* (WIDDEL ET AL. 1983, FUKUI ET AL. 1995). Here, probe 657 was applied for membrane hybridization under sub-specific conditions, to search the Solar Lake DGGE band pattern for *Desulfonema*, and also for *Desulfonema*-related sulfate reducers which might show minor sequence variations, compared to the three known *Desulfonema* species and the target site of the probe (Table 3). Probe 636 was designed as a specific probe for the oxygen-adapted *Desulfovibrio* species *Desulfovibrio oxyclini*, originally isolated from high dilutions of the 0 - 2 mm surface layer of hypersaline cyanobacterial mat in the experimental pond of the Heinz Steinitz Marine Biological Laboratory, Eilat, Israel, and also found in high dilutions of the Solar Lake mat surface layer (KREKELER ET AL. 1995). Under the conditions used here, probe 636 is specific for *Desulfovibrio oxyclini*, and does not hybridize with its closest relative,*Desulfovibrio salexigens*, and other sulfate reducing bacteria.

In all DGGE patterns, a specific band of the DGGE pattern hybridized with probe 657 (Fig. 2A, B, C). Within the DGGE pattern derived from DNA extracted at 5^{oo} am, with the mat surface layer almost completely anoxic, this band is most prominent in the upper 5 mm of the mat, and only faintly visible in the deeper layers, 6, 7 and 8 mm. DNA extractions of the upper 5 mm had been performed in duplicates: Two cores were taken from the same mat piece, sliced and analysed. Both sample sets show the band reproducibly in the upper 5 mm of the mat (Fig. 2A). Within the DGGE pattern of DNA extracted at 12^{oo} am, the band remains most intense in the upper 3 mm surface layers of the mat, and is detectable until 6 mm depth. In this sample set, the upper 2 - 3 mm of the mat are oxic, in part supersaturated (Fig. 2B). The DGGE pattern obtained from DNA extracted at 5^{oo} pm, after day-long exposure of the surface layers to oxic conditions, shows a slightly different situation. The most intense bands were found below 2 or 3 mm, and above 10 mm. This experiment was performed in duplicate: Two cores were taken and sliced for DNA extraction (Fig. 2C).

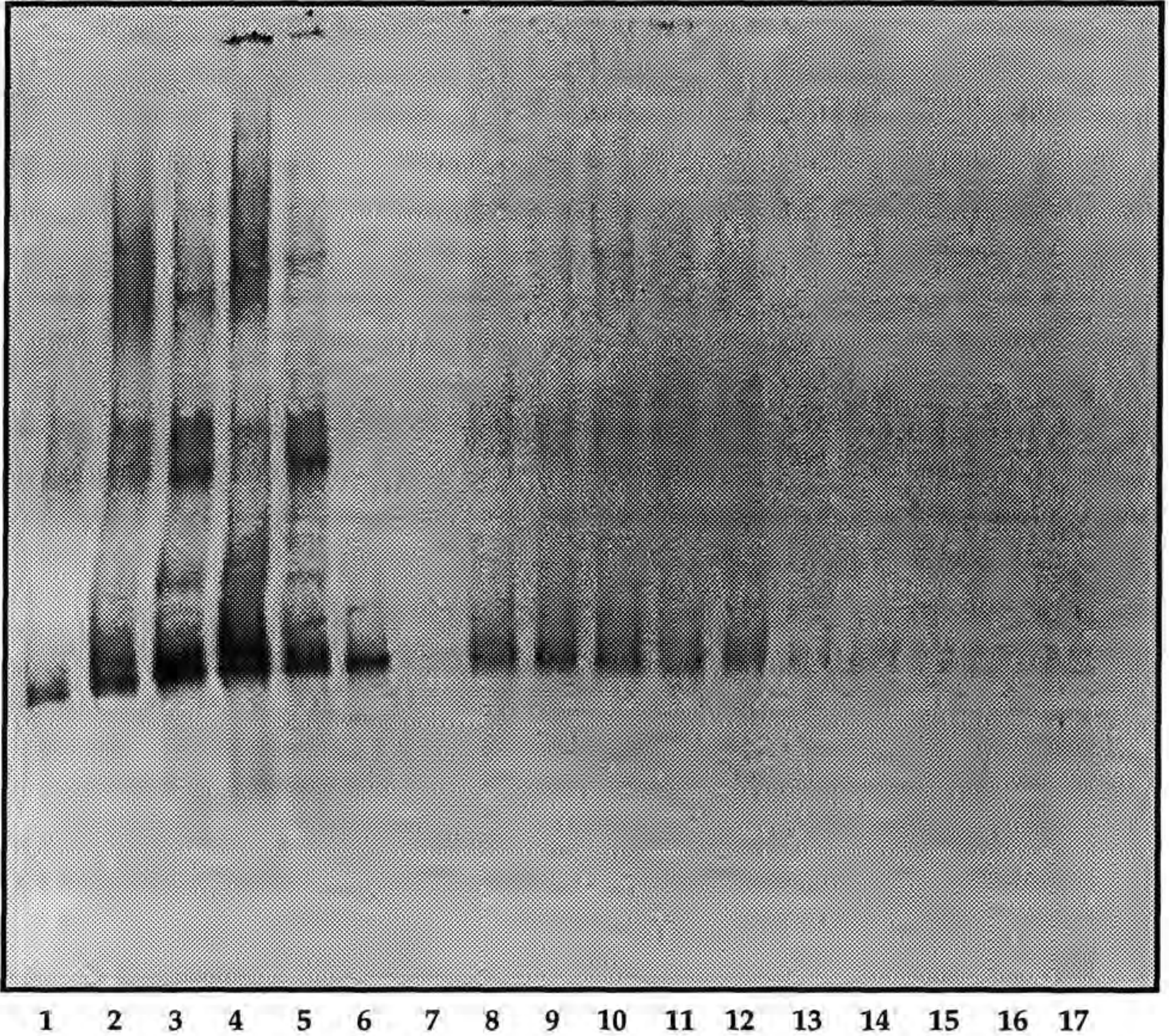

Fig. 2A. DGGE pattern of PCR-amplified 16S rDNA fragments isolated from Solar Lake cyanobacterial mat layers, and bacterial strains, hybridized with 16S rRNA probe 657. The mat samples were taken at 5°° am. Lanes 1-5, mat layers 1 - 5 m m (replicate core). Lane 6, *Desulfonema* enrichment of MPN dilution 10^4, on acetate, from the 0 - 2 mm surface layer of Solar Lake mat. Lane 7, *Desulfovibrio oxyclini*. Lanes 8 - 17, mat layers 1 - 10 mm.

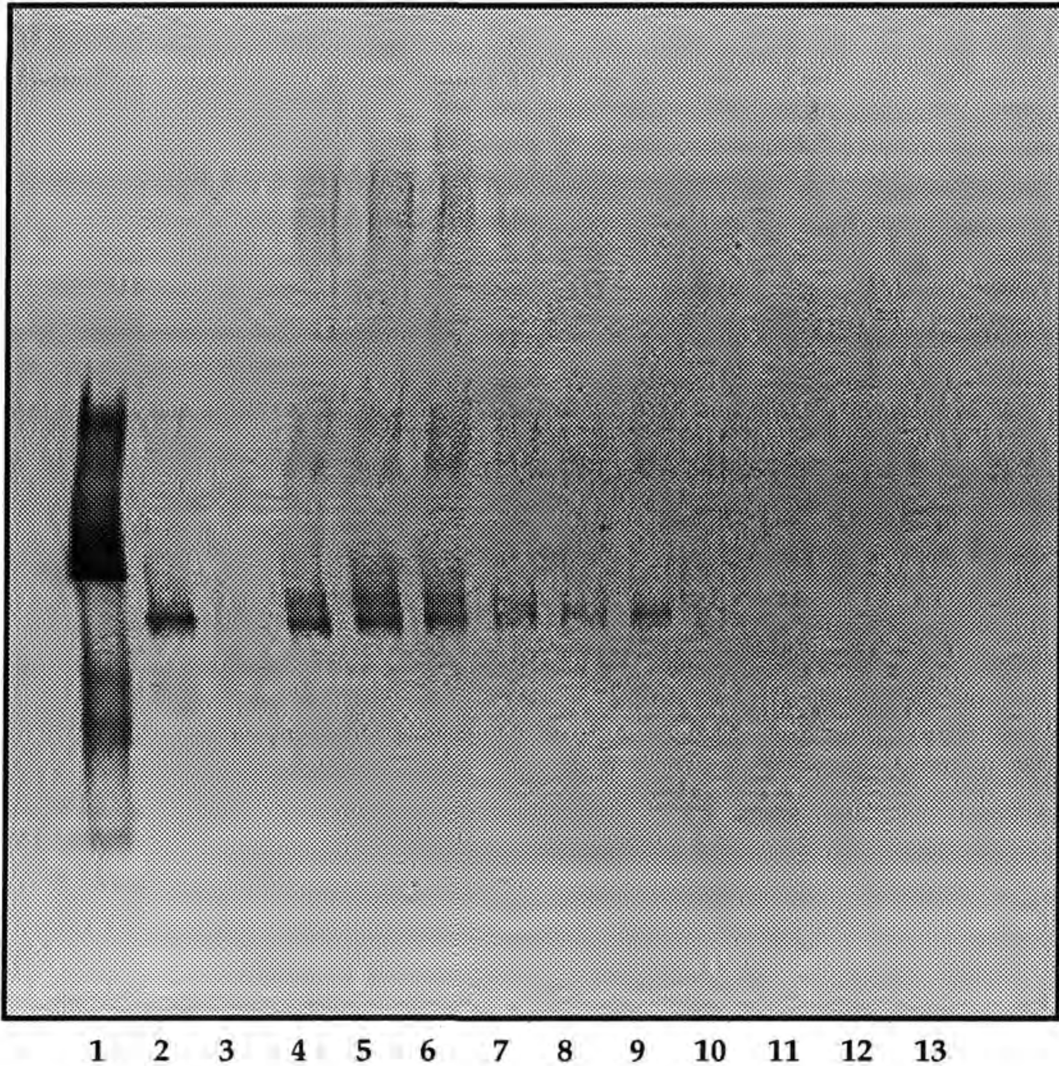

Fig. 2B. DGGE pattern of PCR-amplified 16S rDNA fragments isolated from Solar Lake cyanobacterial mat layers, and bacterial strains, hybridized with 16S rRNA probe 657. The mat samples were taken at 12^{00} noon. Lane 1, *Desulfonema ishimotoei*. Lane 2, *Desulfonema* enrichment of MPN dilution 10^4, on acetate, from the 0 - 2 mm surface layer of Solar Lake mat. Lane 3, *Desulfovibrio oxyclini*. Lane 4 - 13, mat layers 1 - 10 mm.

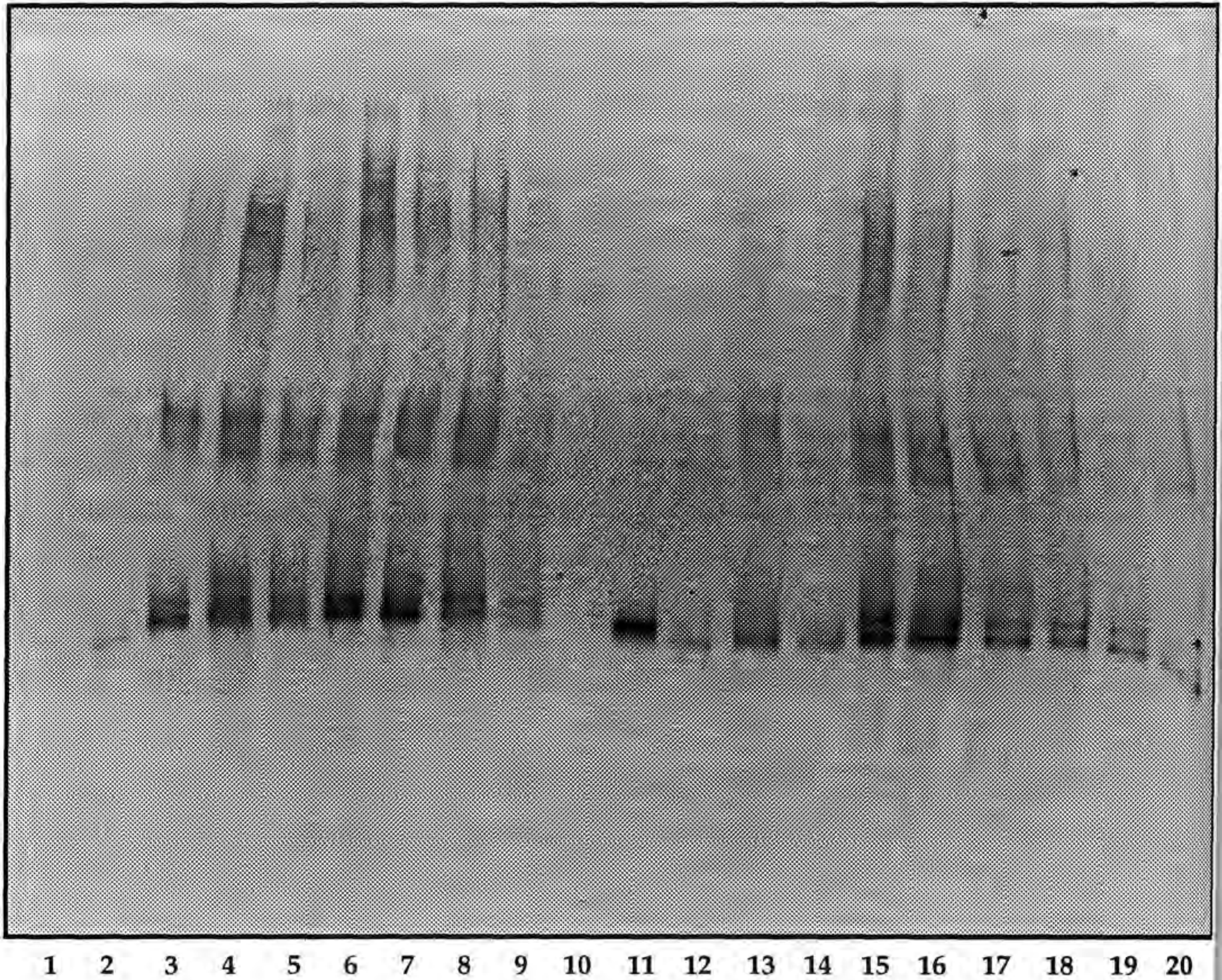

Fig. 2C. DGGE pattern of PCR-amplified 16S rDNA fragments isolated from Solar Lake cyanobacterial mat layers, and bacterial strains, hybridized with 16S rRNA probe 657. The mat samples were taken at 5^{00} pm. Lanes 1 - 9, mat layers 1 - 8 mm, 10 mm. Lane 10, *Desulfovibrio oxyclini*. Lane 11, *Desulfonema* enrichment of MPN dilution 10^4, on acetate, from the 0 - 2 mm surface layer of Solar Lake mat. Lanes 12 - 20, mat layers 1 - 8 mm, 10 mm (replicate core).

PCR-amplified 16S rDNA fragments from Solar Lake mat DNA extracted at 5^{oo} am, 12^{oo} noon, and 5^{oo} pm, from 2 mm and 4 mm depth, were compared directly and analysed side by side in the same DGGE experiment (Fig. 3). The bands, which hybridized with probe 657, had identical positions in all sample sets. Three bands, representing the three sampling times, were excised from the DGGE gel, reamplified and sequenced directly. In all cases, the same sequence was obtained, confirming the identity of the band in all sample sets. The hybridization pattern with probe 657 gave positive hybridizations signals, besides the Solar lake samples, also for *Desulfonema limicola*, *Desulfonema magnum*, *Desulfococcus multivorans* and *Desulfosarcina variabilis*. The latter two species, and the Solar Lake band, had one sequence mismatch to probe 657.

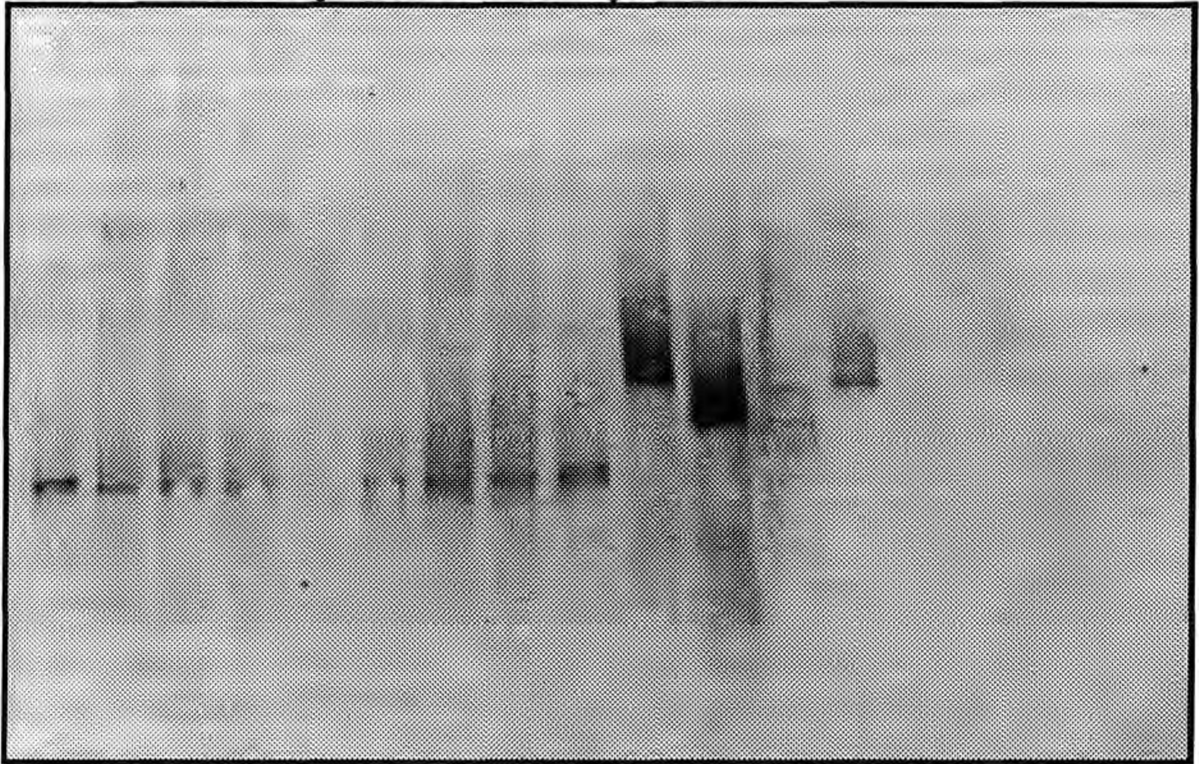

Fig. 3. DGGE pattern of PCR-amplified 16S rDNA fragments isolated from Solar Lake cyanobacterial mat layers, and bacterial strains, hybridized with 16S rRNA probe 657. Lane 1 and 9, *Desulfonema* enrichment of MPN dilution 10^4, on acetate, from the 0 - 2 mm surface layer of Solar Lake mat. Lane 2, mat sample 2 mm, 5^{oo}am. Lane 3, mat sample 2 mm, 5^{oo} am (replica core). Lane 4, mat sample 2 mm, 12^{oo} am. Lane 5, mat sample 2 mm, 5^{oo}pm. Lane 6, mat sample 2 mm, 5^{oo}pm (replica core). Lane 7, mat sample 4 mm, 5^{oo} pm. Lane 8, mat sample 4 mm, 5^{oo} pm (replica core). Lane 10, *Desulfonema limicola*. Lane 11, *Desulfonema magnum*. Lane 12, *Desulfococcus multivorans*. Lane 13, *Desulfosarcina variabilis*. Lane 14, *Desulfobotulus sapovorans*. Lane 15, *Desulfobacter postgatei*. Lane 16, *Desulfobacterium autotrophicum*. Lane 17, *Desulfovibrio salexigens*.

The hybridization experiments with probe 636, specific for *Desulfovibrio oxyclini*, showed a less consistent pattern. Besides the positive controls with *Desulfovibrio oxyclini* 16S rDNA amplificates, two very weak hybridization signals were found in the Solar lake samples, in the 1 mm surface sample of one of the 5^{oo} pm DGGE, and in the 5 mm sample of the 12^{oo} noon DGGE. However, these data demonstrate the presence of *Desulfovibrio oxyclini* within the mat and in the mat surface layer, and complement microbiological evidence for approx. 10^7 *Desulfovibrio oxyclini* cells per ml in the upper 3 mat millimeters (KREKELER ET AL. 1995).

Phylogenetic position. The molecular isolate from the Solar Lake mat surface layers, which hybridized with probe 657, falls into the *Desulfonema-Desulfococcus-Desulfosarcina-Desulfobotulus* cluster (Fig. 4). *Desulfonema*, the genus of filamentous sulfate reducers, has recently been included in this phylogenetic group (FUKUI ET AL. 1995). The Solar Lake surface layer organism does not belong to the *Desulfobacter-Desulfobacterium* lineage, the second major phylogenetic group of completely oxidizing sulfate reducing bacteria.

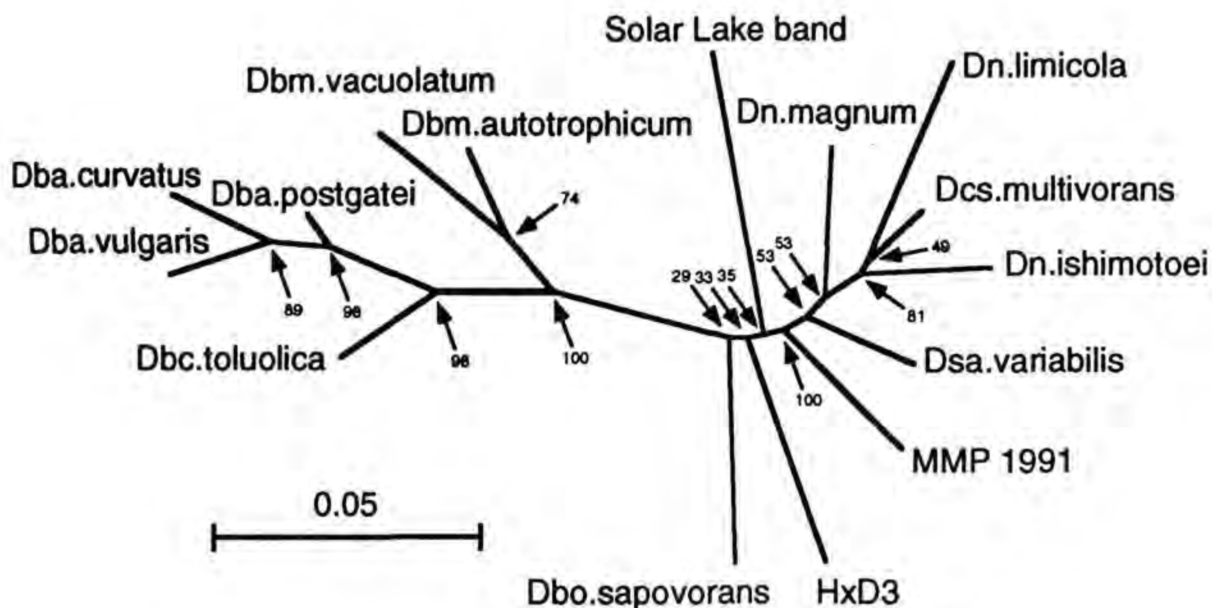

Fig. 4. 16S rRNA distance tree of completely oxidizing sulfate reducing bacteria of the delta proteobacteria subdivision, and the molecular isolate from the surface layer of Solar Lake cyanobacterial mat. The tree is based on 16S rRNA sequence positions 420-454 and 478-907, the range of the Solar Lake sequence (*E.coli* numbering). The scale bar corresponds to 0.05 mutations per nucleotide position. Bootstrap values from distance analyses (100 replicates) refer to species distal to the associated node. Sequences used in preparing this figure were derived from the Ribosomal Database Project (RDP) on the anonymous ftp server at the University of Illinois in Urbana, Illinois, updated on June 19th 1994 (MAIDAK ET AL. 1994). Abbreviations: Dn., *Desulfonema*; Dcs., *Desulfococcus*; Dsa., *Desulfosarcina*; Dbo., *Desulfobotulus*; Dbm., *Desulfobacterium*; Dbc., *Desulfobacula*; Dba., *Desulfobacter*; MMP1991, iron sulfide containing magnetotactic bacterium clone MMP1991 (DeLONG ET AL. 1993); HxD3, hexadecane-oxidizing sulfate reducing bacterium (AECKERSBERG & WIDDEL 1991). The partial sequence of the Solar Lake molecular isolate has Genbank accession number .

DISCUSSION

Molecular and classical-microbiological results indicate that sulfate-reducing bacteria of the *Desulfonema-Desulfococcus-Desulfosarcina-Desulfobotulus* group represent a dominant population of sulfate-reducing bacteria within the surface layers of Solar lake cyanobacterial mats, including the chemocline layer (2-4 mm) and the photosynthetic surface layer which turns oxic at daytime (0-2 mm). Most-probable-Number dilution series, in no way expected to account for *Desulfonema*, frequently yielded *Desulfonema* enrichments in the highest positive dilutions. Although the 12°° and 24°° lactate and acetate MPN series had always been inoculated with the same mat homogenate, *Desulfonema* numbers remained generally lower in the acetate series than in the - more incomplete - lactate series, where *Desulfonema* was probably growing on the products of incomplete lactate oxidation by lactate-utilizing sulfate-reducing bacteria. More fastidious substrate requirements of *Desulfonema*, which grow to higher numbers on nutritionally complex spent lactate medium, than on pure acetate, could explain this observation. It might therefore be justified to regard the consistent acetate counts of *Desulfonema* as the minimum base line, and to view the more incomplete, but higher lactate counts as indicators for potentially higher numbers. Following this line, the maximal cell density of Desulfonema within the upper 7 mm of the mat can be estimated as approx. 10^6 cells per ml, with peaks of 10^7 within the chemocline. In the 0 - 2 mm surface layer, *Desulfonema* was found in the 10^5 and 10^6 dilutions of the lactate MPNs. Towards deeper layers, between 7 and 13 mm, numbers of *Desulfonema* decreased to 10^4 - 10^5 cells per ml. Direct microscopical observations und in-situ-Fluorescence hybridizations with fresh Solar Lake mat, showing very high densities of non-cyanobacterial filaments which are in all likelyhood *Desulfonema* (COHEN, unpublished), reinforce these results.

It remains, at least at the time of writing this manuscript, an intriguing question whether the molecular isolate, which is found in the surface layers of the Solar Lake Cyanobacterial mat, is a *Desulfonema*. For comparison, high dilution Solar Lake *Desulfonema* have to be analysed by DGGE and sequencing. Although selected Solar Lake MPN *Desulfonema* enrichments yielded, in DGGE analysis, bands of a very similar position as the molecular isolate (Fig. 3A, B, C), sequencing of the promising candidates proved nonidentity. However, pure culture *Desulfonema* from the highest dilutions, 10^6 and 10^7, have not been examined yet.

A molecular study of cyanobacterial mats similar to those of Solar Lake, of Guerrero Negro in Baja California, Mexico, yielded congruent results (RISATTI ET AL. 1994). Bacterial rRNA, extracted directly from the mat, was blotted on

membranes and hybridized with different probes for sulfate reducing bacteria (DEVEREUX ET AL. 1992). In the 1-2 mm surface layer, rRNA which hybridized with probe 814 represented the most abundant fraction of delta subdivision sulfate reducer rRNA, and accounted for 3% of the total rRNA. Probe 814 covers in its target range *Desulfococcus, Desulfosarcina, Desulfobotulus*, and, with a single mismatch, *Desulfonema limicola* and *Desulfonema ishimotoei*. *Desulfonema magnum* shows two mismatches to probe 814 (Table 3). Since single mismatch discrimination in membrane blotting is problematical, ribosomal RNA of *Desulfonema* or a related organism probably contributed to the hybridization signal with probe 814 (D. Stahl, personal communication).

	Probe 657	Probe 814
Probe sequence	5'-TTCCGYTTCCCTCTCCCATA-3'	5'-ACCTAGTGATCAACGTTT-3'
Target sequence	UAUGGGAGAGGGAARCGGAA	AAACGUUGAUCACUAGGU
Dn.magnum	UAUGGGAGAGGGAAGCGGAA	AAACGGUGAUCACUAGGU
Dn.limicola	UAUGGGAGAGGGAAGCGGAA	AAACGUUGAUCACCAGGU
Dn.ishimotoei	UAUGGGAGAGGGAAACGGAA	AAACGGUGAUCACUAGGU
Solar Lake Band	UAUGGGAGAGGGNAGUGGAA	AAACGGUGNUNACUAGNU
Dcs.multivorans	UAUGGGAGAGGNNAGUGGNA	AAACGUUGAUCACUAGGU
Dsa.variablilis	UAUGGGAGAGGGAAGUGGAA	AAACGUUGAUCACUAGGU
Dbo.sapovorans	UAUGGCAGAGGAAAGCGGAA	AAACGUUGAUCACUAGGU
Strain HxD3	UAUGGGAGAGGGAAGUGGAA	AAACGUUGAUCACUAGGU
clone MMP91	UAUGGGAGAGGGAAGUGGAA	AAACGUUGAACACUAGGU
Dbm.autotr.	UACGGUNGAGGAAAGGGGNA	AAACGUUGUAUACUAGGU
Dbc.toluolica	UACGGGAGAGGAAAGCGGAA	AAACGAUGUACACUAGGU
Dba.vulgaris	UACGGGAGAGGAGAGAGGAA	AAACGUUGUACACUCGGU

Table 3. 16S rRNA target regions complementary to *Desulfonema* probe 657 (FUKUI ET AL. 1995) and to *Desulfococcus-Desulfosarcina-Desulfobotulus* probe 814 (DEVEREUX ET AL. 1992).

The addition of acetate has been found to stimulate sulfate reduction rates in hypersaline cyanobacterial mats, equally within the photosynthetically active, cyanobacterial layer (0-3 mm), and within the chemocline layer, which harbors anoxygenic phototrophic bacteria (3 - 5 mm), but not in the permanently reduced layer (5 - 12 mm). Lactate and ethanol, the typical substrates of *Desulfovibrio*, have no stimulating effect within the surface layer (FRÜND & COHEN 1992). Acetate-oxidizing sulfate reducing bacteria of the *Desulfonema-Desulfococcus-Desulfosarcina* cluster rationalize this dataset, which had remained unintelligible as long as *Desulfovibrio* had been considered the most likely candidate to dominate a surface-associated, oxygen-tolerant sulfate reducing population.

Desulfonema can grow on acetate, and some *Desulfonema* isolates obtained from the 10^6 MPN dilution of the 0 - 2 mm Solar Lake surface layer grow very well on acetate as sole substrate (TESKE, unpublished observations). Most members of the *Desulfonema-Desulfococcus-Desulfosarcina-Desulfobotulus* cluster, with the exception of *Desulfobotulus*, can oxidize acetate, and have a very diversified substrate range. The high metabolic diversity within this bacterial group precludes specific assumptions about the most likely physiological capabilities of the *Desulfonema*-related Solar Lake molecular isolate. This cluster contains also a hexadecane-oxidizing sulfate reducing bacterium, strain HxD3 (AECKERSBERG & WIDDEL 1991), and an uncultured magnetotactic bacterium of the iron sulfide type (DeLONG ET AL. 1993).
Comparisons of the total numbers of sulfate-reducing bacteria with the sulfate reduction rates of the Solar Lake mat, determined at the same time with the same sample material and identical sectioning of the mat (HABICHT, unpublished), indicated that the MPN estimates of total sulfate reducing bacterial numbers are at least tenfold underestimated, in the oxic as well as in the anoxic layers of the mat. Sulfate reduction rates between 1000 and 2200 nmol cm^{-3} d^{-1} within the oxic surface layer were obtained (HABICHT, unpublished). Total MPN estimates of sulfate reducing bacteria within this layer are between 2 - 5 x 10^6 SRB cm^{-3}. Specific sulfate reduction rates, determined for pure cultues of H_2, lactate-, and pyruvate-utilizing sulfate reducing bacteria, were found to range from 0.2 to 50 x 10^{-6} nmol $SO_4{}^{2-}$ SRB^{-1} d^{-1} (JØRGENSEN 1978b). With these rates, the observed numbers of sulfate reducing bacteria could account only for sulfate reduction rates of at best 100 - 250 nmol cm^{-3} d^{-1}, 10% of what was actually found. Similar calculations apply to the chemocline and the permanently anoxic layers of the mat, with a consistent discrepancy of at least factor ten between observed sulfate reduction rates and counted bacterial numbers. Some counts of acetate-oxidizing sulfate reducers, such as *Desulfonema*, are therefore likely to be systematic underestimates, due to inhomogeneous spatial distribution, filamentous morphology and clumping of these populations. Last not least, unknown types of sulfate reducing bacteria, like those represented by the *Desulfonema*-related molecular isolate, are still awaiting their isolation and proper quantification.

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4.4. Distribution of sulfate-reducing bacteria in a stratified fjord (Mariager Fjord, Denmark) as evaluated by Most-Probable-Number counts and denaturing gradient gel electrophoresis of PCR-amplified ribosomal DNA fragments

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ABSTRACT

The sulfate-reducing bacterial populations of a stratified marine water column, Mariagerfjord, Denmark, were investigated with molecular and culture-dependent approaches in parallel. Denaturing gradient gel electrophoresis (DGGE) of PCR-amplified 16S rRNA and rDNA, isolated from the water column, indicated specific bacterial populations in different water column layers, and revealed a highly differentiated pattern of rRNA-derived vs. rDNA-derived PCR amplificates, probably reflecting active and resting bacterial populations. Hybridization of DGGE patterns with rRNA probes indicated an increased presence and activity of sulfate-reducing bacteria within and below the chemocline by at least one order of magnitude. Parallel to this molecular approach, a similar distribution of cultivable sulfate-reducing bacteria in the water column of Mariagerfjord was found by most-probable-number (MPN) counts, with app. 25 cells per ml above and 250 cells per ml below the chemocline. *Desulfovibrio* and *Desulfobulbus*-related strains occured in the oxic zone. DGGE bands from highest positive MPN cultures were compared to those obtained from nucleic acids extractable from water column samples, and sequenced. The MPN isolates were phylogenetically affiliated to the proteobacteria delta-subdivision sulfate reducers *Desulfovibrio*, *Desulfobulbus*, and *Desulfobacter*, whereas the molecular isolates constituted an independent lineage of the delta-subdivision proteobacteria. DGGE of PCR-amplified nucleic acids, using general eubacterial PCR-primers, represented conceptually the general bacterial population, whereas the use of culture media selected for cultivable sulfate reducing bacteria.
A parallel study of Mariagerfjord biogeochemistry, bacterial acitivity and bacterial counts complementing this investigation is presented by RAMSING ET AL. (1995).

INTRODUCTION

Oxygen-deficient marine basins show characteristic chemical zones due to the gradual depletion of oxygen and other oxidized chemical species in the water column (EMERSON ET AL. 1979). The redox gradient in the water column is mirrored by different modes of bacterial respiration, from oxic respiration, through denitrification, manganese and iron reduction, sulfate reduction to methanogenesis (JØRGENSEN 1989a). According to the classic picture, oxidative processes occur in the oxygenated water above the chemocline; whereas reductive processes, *i.e.* denitrification and sulfate reduction, are confined to the anoxic zone. The confinement of sulfate reduction to anoxic water layers has, however,

been questioned. Sulfate reduction was observed in the presence of oxygen in and above the chemocline of the Cariaco trench (HASTINGS & EMERSON 1988). Particle-associated methanogenic and sulfate-reducing bacteria were enriched from oxic surface water as well as from pycnocline and bottom water of Chesapeake Bay (SIEBURTH 1993).

Mariagerfjord (Denmark) is an especially suitable marine environment to investigate the potential overlap of aerobic and anaerobic bacterial populations and their metabolic processes in a stratified water column. This fjord is connected to the sea by a long, narrow threshold of maximum 4 m depth, but widens and deepens in its inner part to a depth of 25 - 30 m. The threshold limits the exchange of oxygenated water and leaves the inner basin of the fjord anoxic, from a depth of approximately 14 m to the bottom (MILJØKONTORNE I ÅRHUS 1990). This polyphasic study of the distribution of sulfate-reducing bacteria in Mariagerfjord used cultivation-dependent and molecular approaches in parallel, to investigate the bacterial ecosystem as well as the strengths and biases inherent in the methods. A geochemical evaluation of Mariagerfjord and direct bacterial counts using fluorescent *in situ* hybridization were performed in parallel to this study, at the same location and time, using the same samples, as a framework and complement of this investigation (RAMSING ET AL. 1995). DGGE of enzymatically amplified 16S rDNA was used as a molecular approach to analyse the microbial community of Mariagerfjord. DGGE can separate PCR products all of the same length on the basis of melting domain structure and nucleotide composition (FISCHER & LERMAN 1983, MYERS ET AL. 1987, UITTERLINDEN & VIJG 1989). This technique has recently been introduced into molecular microbial ecology to determine the genetic diversity of natural microbial communities (MUYZER ET AL. 1993), and to identify dominant community members whose phylogenetic affiliation was subsequently determined by sequencing of the DGGE fragment (MUYZER & DE WAAL 1994, MUYZER ET AL. 1995). Bacterial DNA and total RNA were extracted from water column samples; rRNA was transcribed into ribosomal cDNA (rcDNA), and subsequently amplified by PCR. Since metabolically active cells contain more rRNA than resting or dormant cells (LEE & KEMP 1994, POULSEN ET AL. 1993, ROSSET ET AL. 1966), the DGGE patterns of PCR-amplified rRNA conceptually represent the metabolically active, rRNA-rich bacterial populations. The DGGE patterns derived from amplification of the ribosomal genes represent the presence of bacterial populations, without taking metabolic activity into account. Both DGGE patterns differ significantly. In addition, they reveal how the composition of the bacterial community changes throughout the water column. DGGE gels were analysed by blotting, and by re-amplification and sequencing of individual DGGE

bands. The resulting picture was evaluated with reference to physical parameters of the water column, total bacterial counts and counts of sulfate-reducing bacteria by MPN series.

We performed MPN counts of sulfate reducing bacteria in the water column, to assess the congruence of classical microbiological and molecular, rRNA-related approaches in the detection and quantification of sulfate-reducing bacterial populations. Special attention was paid to the enumeration and identification of sulfate-reducing bacteria from the oxic part of the water column. MPN cultures of sulfate-reducing bacteria from the oxic part of the water column were analysed phylogenetically to evaluate the existence of a possible distinct group of oxygen-tolerant sulfate-reducing bacteria.

MATERIALS AND METHODS

Sampling of Bacteria. Water samples were taken from Station Dybet M3, approximately 1 km north of the harbour of Mariager (lat. 56° 39' 66" N, long. 09° 58' 56" E), on August 19th and 20th 1993. After determining the position of the chemocline with a Conductivity-Temperature-Depth (CTD) sensor, measuring pressure, temperature, conductivity, oxygen (two independent sensors), incident light, and fluorescence. The sampling scheme was devised to obtain water samples from the upper, oxic water layer, from the chemocline and from the anoxic bottom water. The chemocline was distinguished by the simultaneous presence of both O_2 and H_2S in µmolar concentrations (RAMSING ET AL. 1995, Fig. 3A). Water samples for nucleic acid extraction and MPN counts were taken from the oxic water layer at 8 m depth, from the upper, central and lower chemocline at 13 m, 14.5 m, and 16 m depth, from the anoxic water layer at 20 m depth. For nucleic acid extraction, bacteria were collected from 200 ml sea water and from 20 ml sediment. Water- and bottom sediment samples were filtered through Durapore filters (0.22 µm-pore-size, 25 mm in diameter, Millipore) using a syringe filtration device, to capture bacteria and biomass on the filter for subsequent nucleic acid extraction. The very soft sediment samples were concentrated by the filtration from 20 ml to 2 ml total volume. Filters with captured bacteria and biomass were then frozen immediately and stored in liquid nitrogen until nucleic acid extraction.

MPN counts of sulfate reducing bacteria. Numbers of viable sulfate-reducing bacteria were estimated with most-probable-number (MPN) dilutions in liquid medium (AMERICAN PUBLIC HEALTH ASSOCIATION 1969). Multipurpose medium for sulfate-reducing bacteria defined was used, with non-chelated trace

element mixture No. 1 and sea water (WIDDEL & BAK 1991). MPN counts were performed on lactate (20 mM), acetate (20 mM), and hydrogen plus 2 mM acetate to account for non-autotrophic bacteria. The media were prepared anaerobically in a pressure-proof modified Erlenmeyer flask, and dispensed into culture vials (Kimex). After dispensing the medium, each MPN vial was immediately gassed with a gassing syringe according to the Hungate technique, and sealed with a butyl stopper. Lactate and acetate MPN vials were gassed with a mixture of 90% (vol/vol) N_2 and 10% (vol/vol) CO_2. The head spaces of the hydrogen MPN vials were gassed anoxically with a mixture of 90% (vol/vol) H_2 and 10% (vol/vol) CO_2.
Sulfate reducers were counted by threefold MPN dilution series at 8 m, 13 m, 14.5 m, 16 m, 20 m depth and in the bottom sediment. MPN dilution series were inoculated with 1 ml of water or sediment sample, respectively, and subsequently diluted in eight 1:10 dilution steps.

Nucleic Acid Extraction. Nucleic acids were extracted from the filters by the method of OELMÜLLER ET AL. (1990) adapted as follows: the filters were thawed and washed twice with 1 ml icecold AE buffer (20 mM Na-Acetate, pH 5.5, 1 mM EDTA). Each filter and buffer were added to 6 ml hot TE-buffered phenol-chloroform-isoamylalcohol (25:24:1, pH 8) and 60 µl 25% (w/vol) SDS. After 5 min of incubation at 60°C the solution was cooled on ice and then centrifuged for 5 min at 4000 *g*.. 250 µl of 2 M Na-acetate, pH 5.2, were added to the aequous phase. Proteins and lipids were removed by twofold extraction of the aequous phase with 5 ml TE-buffered phenol-chloroform-isoamylalcohol (25:24:1, pH 8). Nucleic acids were precipitated with 2.5 vol 96% (vol/vol) ethanol for 3 hours at -20°C, followed by 60 min centrifugation at 4000*g*. The pellet was washed with 75% (vol/vol) ethanol, dried for 20 min under vacuum and redissolved in 50 µl DEPC-treated water. Nucleic acid preparations including rRNA were tested in 2% (wt/vol) agarose gels using MOPS-buffer (SAMBROOK ET AL. 1989). Total amounts of nucleic acids extracted from the 200 ml water column samples were between 5 and 10 µg. Several different protocols for nucleic acid extraction were evaluated in test experiments with Mariager fjord sediments and water samples. However, only the protocol described above proved successful in isolating rRNA. The DNA yield of this method were equivalent to yields of methods optimized for DNA extraction (FUHRMAN ET AL. 1988). We therefore only applied a single nucleic acid procedure to obtain both RNA and DNA. The DNA and rRNA bands of the gel picture (Fig. 1) were scanned, to quantify DNA and rRNA separately, and the staining intensities integrated using the software program Photoshop 3.0 (Adobe). The nucleic acid extracts were used for amplification of rDNA. 1 µl of

each water column extract, diluted 1:10, and 1 µl of the sediment extract, diluted 1:100 to account for the higher concentration, were used for PCR amplification (10 - 20 ng DNA input).
DNA was extracted from MPN cultures (0.5 ml sample volume) by a proteinase K/SDS digestion step followed by phenol extraction and ethanol precipitation (SAMBROOK ET AL. 1989).

Preparation of ribosomal cDNA. 17 µl of the above undiluted nucleic acid extract were mixed with 2 µl 10x DNAse-buffer (400 mM Tris-HCl pH 7.5, 60 mM $MgCl_2$, 20 mM $CaCl_2$) and 1 µl DNAse (10U/µl, Boehringer Mannheim), and incubated for 30 minutes at 37°C. The DNAse was removed by phenol-chloroform extraction. After ethanol precipitation and centrifugation, the resulting RNA pellet was dissolved in 20 µl water. 4 µl of each RNA preparation was checked on a MOPS gel for contamination with genomic DNA. The RNA preparations were subsequently transcribed into cDNA. Transcription was carried out with 10 µl rRNA solution. The solution was denatured at 70°C for 10 minutes. Subsequently, 1 µl hexanucleotides (Boehringer Mannheim, 10x conc., diluted 1:50), 2 µl 10x PCR buffer, 2 µl 0.1 M DTT and 4 µl 2.5 mM dNTP were added. The mixture was incubated for two minutes at 37°C, before 1 µl of SuperscriptTM Reverse Transcriptase (200U/µl, Gibco BRL Life Technologies Inc.) was added. The samples were incubated at 37°C for 1 hour, and subsequently heated to 95°C for 5 minutes to stop the reaction. The cDNA preparations were diluted 1:1000 with water. Of these diluted preparations, 1 µl was used in the PCR (50 µl volume). Random priming of cDNA synthesis by a hexanucleotide mix prevents premature termination of 16S rcDNA synthesis due to modified nucleotides, such as in positions 966 and 967 of the 16S rRNA (WELLER ET AL. 1991). Random hexamers are consistently more efficient for cDNA synthesis than target site-directed primers, as shown by cDNA-PCR (KAWASAKI 1990).

PCR amplification of rDNA fragments. The 16S rRNA genes from mixed bacterial DNA or cDNA were amplified by the polymerase chain reaction (PCR). The primer combination GM5F-GC clamp and DS907R amplified a 550 base pair fragments of the 16S rRNA, suitable for subsequent DGGE analysis, membrane hybridization and sequencing (Table 1). This PCR amplificate yields sufficient sequence information for phylogenetic analysis (MUYZER ET AL. 1995b). PCR amplifications were performed with a Techne PHC-3 Temperature Cycler (Techne, Cambridge, United Kingdom) as follows: 10-20 ng of target DNA, or 1 µl 1:1000 diluted cDNA, respectively, were mixed with 25 pmol of each of the appropriate primers, 5 µl of 10x PCR buffer (i.e. 100 mM Tris-HCl [pH 9], 15 mM

$MgCl_2$, 500 mM KCl, 0.1% [wt/vol] gelatin, 1% [vl/vol] Triton X-100), 4 µl 2.5 mM deoxyribonucleoside triphosphate solution, and sterile water in a final volume of 50 µl, and overlaid in a 0.5 ml vial with 2 drops of mineral oil (Sigma Chemicals Co Ltd). To minimize non-specific annealing of the primers to non-target DNA, 0.5 U SuperTaq DNA polymerase (HT Biotechnology Ltd.) was added to the reaction mixture after the initial denaturing step (94°C, 5 min), at a temperature of 80°C. In addition, to increase the specificity of the amplification and to reduce the formation of spurious by-products a "touchdown" PCR (DON ET AL. 1991) was performed where the annealing temperature was set to 65°C , which is 10°C above the expected annealing temperature, and decreased by 1°C every second cycle until a touchdown of 55°C, at which temperature 9 additional cycles were carried out. The annealing temperature T_m(°C) was estimated as 81.5 + 16.6($log_{10}[cat^+]$) + 0.41(% G+C) - (600/N) (SAMBROOK ET AL. 1989). Denaturation was carried out at 95°C for 1 min, the annealing time was 2 minutes, primer extension was carried out at 72°C for 3 min. The ramp times were set to the fastest mode of the PHC3 Thermocycler. Aliquots of 5 µl of the amplification products were analyzed by electrophoresis in 1% (wt/vol) Nusieve agarose (FMC) gels containing 0.5 µg/ml ethidium bromide.

DGGE analysis. PCR products obtained with primer combination GM5F-GC clamp and DS907R were analysed by Denaturing Gradient Gel Electrophoresis. DGGE was performed using a BIO-RAD Protean II system, as described previously (MUYZER ET AL. 1993, MUYZER ET AL. 1995a). PCR products were prepared as follows: 300 µl of PCR product was precipitated with 30µl 5 M NaCl and 750µl ethanol at - 80°C for 1 hour. After centrifugation the pellet was washed with 70% (vol/vol) ethanol, dried under vacuum, dissolved in 15 µl water, and applied on the DGGE gel.

DGGE was performed with 6% (wt/vol) polyacrylamide gels in 0.5x TAE (20 mM Tris-acetate [pH 7.4], 10 mM acetate, 0.5 mM Na_2EDTA) with denaturing gradients ranging from 20-70 % denaturant (100% denaturant corresponds to 7 M urea and 40% [vol/vol] formamide, deionized with AG501-X8 mixed bed resin [BIO-RAD Laboratories, Inc]). Gels were made with 6% (wt/vol) acrylamide stock solutions (5.694 g acrylamide and 0.306 g Bis-acrylylcistamin, 20:1, per 100 ml solution) of 20% and 70% denaturant, using a BIO-RAD Econo pump and BIO-RAD Model 385 gradient former. DGGE gels were allowed to polymerize for at least 12 hours. Electrophoresis was performed for four hours at a constant voltage of 200 Volt and a temperature of 60°C. After electrophoresis the gels were incubated for 15 min in Milli-Q water containing ethidium bromide (0.5 mg/l), rinsed for 10 min in deionized water, and photographed on a UV transillumination table (302 nm)

with a Cybertech CS1 digital camera (Cybertech, Berlin).
Small pieces of selected DGGE bands were punched from the DGGE gel before the blotting procedure. The PCR products of the cutouts were eluted, and reamplified as described (MUYZER ET AL. 1995a). The cutout positions are indicated as small circles in figures 2A, 2B, 2C and 5A, 5B. Subsequently, the holes punched out of the DGGE gel served as position markers in aligning the DGGE ethidium bromide and hybridization pictures. Aligning the ethidium bromide-stained and the blotted DGGE pattern requires these position markers, since the blotting process distorts the proportions of the gel. Positions of the holes are marked with small circles in figures 2A, 2B, 2C and 5A, 5B. The alignment was done in the software program Photoshop 3.0 (Adobe).

Blotting and hybridization analysis of DGGE gels. DGGE-separated PCR products were analysed by blotting with rRNA-targeted probes (MUYZER ET AL. 1993, MUYZER ET AL. 1995a). The gels were equilibrated in 0.5x TBE (45 mM Tris-borate (pH 8), 45 mM boric acid, 1 mM Na_2EDTA) for 15 min. The DGGE-separated PCR products were transferred to a nylon membrane (Hybon-N+: Amersham, Amersham, UK) using a Trans-Blot SD semi Dry Transfer Cell (BIO-RAD Laboratories, Inc.) Electrotransfer was performed for about 1 hour at a constant amperage of 400 mA. The membrane was incubated for 15 min in 0.4 M NaOH, 0.6 mM NaCl solution to denature the DNA, subsequently neutralized by rinsing twice in a large volume of 2.5x SSC (1x SSC is 150 mM NaCl, 15 mM sodium citrate), and exposed for 45 s to 302 nm UV light to cross-link the DNA fragments to the membrane.
The 16S rRNA region amplified by primers GM5F and DS907R includes several target sites for general- and genus-specific oligonucleotide probes for sulfate-reducing bacteria. Three such probes (Table 1) were used to analyze the DGGE pattern for 16S rRNA amplificates of sulfate-reducing bacteria. Probes were labeled with digoxigenin, using the digoxigenin oligonucleotide labeling kit and protocols from manufacturer Boehringer Mannheim, Germany. Digoxigenin-labeled probes were detected by an antibody coupled with alkaline phosphatase, which gives a chemiluminescent reaction with CSPD (Serva Tropix). Hybridization was performed as described by MUYZER ET AL. (1995a). For probe 385, hybridization and washing temperature 50°C was used (MUYZER ET AL. 1993). At these hybridization conditions, probe 385 did not discriminate between different sulfate reducer populations on the basis of a possible single G/A mismatch in position 395 of the 16S rRNA sequence (*Desulfovibrio* and *Desulfobulbus* have a G at this position whereas *Desulfomicrobium* and *Desulfobacter* have an A). The probe was therefore targeted at the general delta-

subdivision sulfate reducing population. Discrimination against two mismatches was, however, confirmed by test blots with appropriate reference strains. Probe 804 was hybridized at 40°C and washed at 45°C (DEVEREUX ET AL. 1992). Probe 687 was hybridized at 40°C and washed at 45°C. The probes were removed from the membrane as described by MUYZER ET AL. (1995a) for rehybridization.

Table 1. Primers and Oligonucleotides used in this study

PRIMER	POSITION	SEQUENCE	
GM5F -GC-clamp*	341 - 357	5'- GC-clamp-CCTACGGGAGGCAGCAG -3'	primer for 16S rRNA gene DGGE-fragment amplification
DS907-rev**	907 - 928	5'- CCCCGTCAATTCCTTTGAGTTT -3'	primer for 16S rRNA gene amplification
Probe 804	804 - 821	5'- CAACGTTTACTGCGTGGA -3'	rRNA oligonucleotide probe for the genera *Desulfobacterium, Desulfobacter, Desulfosarcina variabilis, Desulfococcus multivorans,Desulfobotulus sapovorans* (DEVEREUX ET AL. 1992)
Probe 687	671 - 687	5'- TACGGATTTCACTCCT -3'	*Desulfovibrio* (DEVEREUX ET AL. 1992)
Probe 385	385 - 402	5'- CGGCGTTGCTGCGTCAGG -3'	*Desulfovibrio, Desulfobulbus* (AMANN ET AL. 1990) other delta-subdivision and grampositives (RAMSING ET AL. 1995)

*GC-clamp: 5'- CGCCCGCCGCGCGCGGCGGGCGGGGCGGGGGCACGGGGGG - 3'
**G/A mismatch at position 917 of the target site for *Desulfomicrobium escambium, Desulfovibrio desulfuricans, Desulfovibrio longreachii, Desulfobacterium*. In test PCRs, the primer mismatch did not affect the amplification under the conditions employed here.

Sequencing of PCR products. Re-amplified PCR products obtained from the excised DGGE bands were gel-purified as follows: a small well was cut into the agarose gel in front of the selected PCR-product. Electrophoresis was continued until the PCR product migrated into the buffer-filled well, from where it was taken up with a pipette and transferred into an Eppendorf vial, followed by ethanol precipitation. The purified PCR products were sequenced directly: 10-100 ng purified PCR-product were mixed with 2 µl 5x concentrated sequencing buffer (200 mM Tris-HCl pH 7.5, 100 mM $MgCl_2$, 250 mM NaCl), 1 µl 5% Nonidet P-40 (Sigma) aqueous solution, 2 pmol primer, bidest water in a final volume of 10 µl. The double stranded PCR-product was denatured by heating the 10 µl volume to 95°C for two 5 min periods, with a short centrifugation in between to recollect the 10 µl volume. The sequencing reaction was started by adding 1.0 µl 0.1 M DTT, 2.0

µl dNTP-solution (200 nM dGTP, dATP, dTTP), 0.5 µl ($a^{33}P$)-dCTP; (10µCi/µl; 3000 Ci/mmol), 2 µl (1Unit) Sequenase 2.0 (USB). After short centrifugation to collect and mix the sample volume (15.5 µl), the sample was incubated for 5 minutes at 37°C.
The sample volume was then divided into four 3.5 µl portions and added to dideoxynucleotide termination solutions (2.5 µl, containing 80 µM of each dGTP, dATP, cTTP, dCTP, and 8 µM dideoxynucleotide). After 5 min incubation at 37°C the reaction was terminated by adding 4 µl of a solution of 96% (vol/vol) formamide and 20 mM EDTA. The samples were heated for 5 min at 95°C and 2.5 µl of the samples were loaded onto a 0.25 to 0.40 mm wedge sequencing gel (6% [vol/vol] acryl/bisacrylamide [30:1] and 7M urea). After electrophoresis at constant power of 60 W for 4 hours, the gel was fixed, dried and exposed with an X-ray film (Kodak XAR5).

Sequence alignment and phylogenetic tree inference. The 16S rRNA sequences were aligned to those of other bacteria obtained from the Ribosomal Database Project (MAIDAK ET AL. 1994). The SIMILARITY_RANK tool of the RDP was used to search for close evolutionary relatives. Sequence alignments were prepared with the sequence alignment editor SEQAPP (GILBERT 1992). Distance matrixes were calculated with DNADIST as implemented in the software package PHYLIP (version 3.5) developed by FELSENSTEIN (1989), using the Jukes-Cantor model, which assumes independent change at all sites with equal probability (JUKES & CANTOR 1969). Phylogenetic trees were constructed from evolutionary distances, calculated with the algorithm of Fitch and Margoliash as implemented in the program FITCH in the software package PHYLIP (version 3.5). The sequences obtained in this study are available from Genbank under accession numbers L40767, L40783, L40789, L40784, L40785, L40786, L40787.

RESULTS

Extracted nucleic acids. Genomic DNA and ribosomal RNA were isolated from different water layers of Mariagerfjord, and equal volumes (5 µl) of the isolated nucleic acids (dissolved in 50 µl) were analysed on an ethidium bromide stained agarose gel (Fig. 1). Genomic DNA, in the upper position of the gel, and ribosomal RNAs, appearing as two distinct double bands, are the predominant nucleic acids visible in the agarose gel. Large Subunit rRNAs, eukaryotic 28S rRNA and prokaryotic 23S rRNA, form the upper double band. Small Subunit rRNAs, eukaryotic 18S rRNA and prokaryotic 16S rRNA, appear as lower double band. Eukaryotic 28S rRNA and 18S rRNA is only found in fully or partially oxygenated water layers above and in the chemocline until 14.5 m depth, whereas prokaryotic 23S rRNA and 16S rRNA is found in both oxic and anoxic water layers.

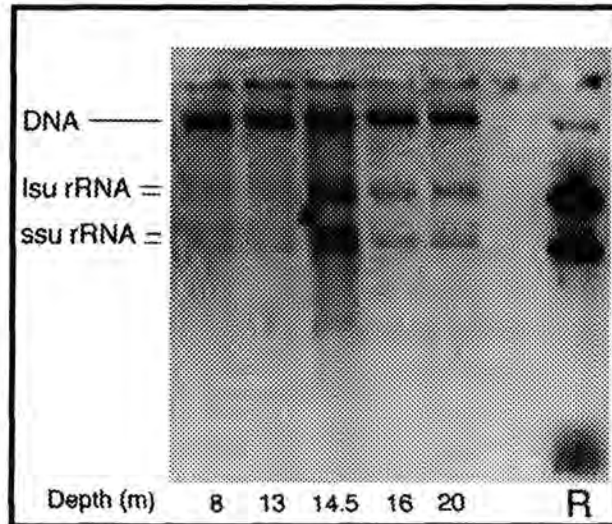

Fig. 1. Agarose gel of nucleic acids extracted from Mariagerfjord water samples, obtained from 8 m, 13 m, 14.5 m, 16 m and 20 m depth. 5 µl of each 50 µl sample were loaded on the gel. Total extracted nucleic acids were in the range of 5 - 10 µg. 1 µg RNA of *Desulfovibrio baculatus* (DSM 2555) was run as standard in lane R.

Total DNA, 16S rRNA and 18S rRNA were quantified by scanning and integrating staining intensities of the DNA and rRNA bands. The 18S rRNA disappeared below the chemocline. 16S rRNA showed a maximum abundance in the chemocline at 14.5 m depth, where the concentration increased by approximately factor four compared to other layers of the water column (Fig. 3A). Increased 16S rRNA content of cells in the chemocline was also evident from in situ fluorescence hybridization intensities with a general eubacterial 16S rRNA probe, shown in Fig. 6D of the accompanying study by RAMSING ET AL. (1995).

The DNA concentrations remained almost constant in all water layers of Mariagerfjord, except for a slight increase in the chemocline. The almost constant DNA concentrations correspond to uniform bacterial numbers obtained by general nucleic acid stains (RAMSING ET AL. 1995, Fig. 6A). Fluorescent in situ hybridizations and nucleic acid extractions were performed with bacteria sampled at the same time and location.

DGGE analysis of PCR-amplified rDNA fragments. 16S rDNA fragments, obtained after enzymatic amplification of nucleic acids from Mariagerfjord water column samples, were analysed by DGGE. The resulting pattern of bands is visualized by ethidium bromide staining (Fig. 2A). Different bands of the DGGE profile correspond to different PCR-amplified 16S rRNA fragments, obtained from different bacterial species or strains. The complex pattern of DGGE fragments is thus derived from the bacterial populations and their nucleic acids. Very small differences in the sequence of amplified 16S rRNA fragments, ranging from 1 - 10 nucleotides, are usually sufficient to separate similar PCR-products (UITTERLINDEN & VIJG 1989), although positional overlap of bands from two different species cannot be excluded. Aware of this caveat, we view the DGGE band pattern as an approximate representation of the bacterial species and strain diversity in the water column of Mariagerfjord. The DGGE pattern can be interpreted in two different ways. First, specific bands emerge and disappear in different water layers (Fig. 2A): Some bands in the DGGE profile occur in all layers of the water column (band No. 1), others are found in the oxic water layer only (No. 2), in the anoxic deeper water layers (No. 3), or specifically in the chemocline (No. 4). Supposing a constant PCR amplification bias for (or against) a specific bacterial rDNA or rcDNA sequence, the appearance and disappearance of a DGGE band reflects the increase and decrease of the corresponding bacterial population. Intensities of different DGGE bands, derived from different bacterial species, do not allow quantitative conclusions about the abundance of the respective bacteria, because of possible unknown PCR bias in amplification of different templates.
The second way to interpret the DGGE pattern is to compare DGGE bands which were derived from reverse-transcribed 16S rRNA with those from the corresponding genes, 16S rDNA. Since metabolically active cells are generally presumed to have a higher rRNA content and rRNA/DNA ratio than inactive, dormant cells cells (LEE & KEMP 1994, POULSEN ET AL. 1993, ROSSET ET AL. 1966), rRNA-derived DGGE bands could conceptually correspond to the metabolically active bacterial populations of Mariagerfjord, while rDNA-derived DGGE bands could reflect the numerically dominant populations. Limiting such comparisons to rcRNA- and rDNA-derived PCR products of identical mobility,

(presumably form the same bacterial species), to rule out PCR bias due to different template sequences, it is likely that such patterns point to actual differences between presence and expression of a particular 16S rRNA gene. DGGE patterns of PCR-amplified bacterial 16S rRNA genes differ markedly from that of reverse-transcribed, PCR-amplified 16S rRNA (Fig. 2A): Some bands are only visible in either the rRNA (No. 4) or rDNA-derived DGGE pattern (No. 2). The chemocline bands a and b appear dominant in the DGGE pattern obtained from transcribed rRNA, but are only faintly visible in the DGGE pattern of amplified 16S rRNA genes (Fig. 2A). All clearly defined DGGE bands were excised as shown on Fig. 2A. However, only bands which hybridized with probes for sulfate-reducing bacteria in subsequent blotting experiments were re-amplified and sequenced.

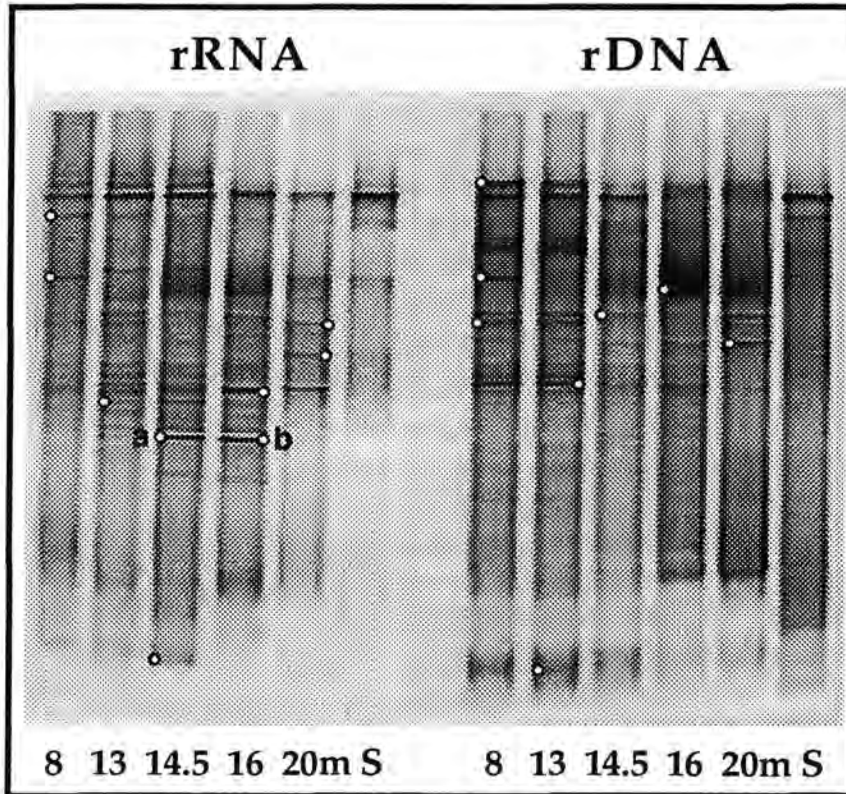

Fig. 2A. Ethidium bromide stained DGGE pattern of PCR products derived from rDNA and rRNA, extracted from Mariagerfjord water column and sediment samples. Lanes left: rRNA-derived DGGE bands from 8 m, 13 m, 14.5 m, 16 m, 20 m water column samples, and from 26 m sediment samples. Lanes right: the same for rDNA-derived DGGE bands. The encircled positions indicate excised gel portions, for reamplification and sequencing. Numbers 1 - 4 refer to DGGE bands discussed in the section 'DGGE analysis of PCR-amplified rDNA fragments. DGGE bands a and b are discussed in the section 'Hybridization analysis of DGGE patterns'.

Hybridization analysis of DGGE patterns. To identify bands of the DGGE patterns which are possibly derived from sulfate- reducing bacteria, the DGGE patterns were blotted onto a nylon membrane and hybridized with the DIG-labelled 16S rRNA probes 385 or with probe 804 (Table 1). Probe 385 was originally designed for Gram-negative sulfate-reducing bacteria of the Delta subdivision of the Proteobacteria (AMANN ET AL. 1990), and matches the target sequences of *Desulfobulbus* and *Desulfovibrio.* Acetate-oxidizing sulfate-reducing bacteria were monitored with probe 804, which is specific for the acetate-oxidizing sulfate-reducing genera and species *Desulfobacter, Desulfobacterium, Desulfosarcina variabilis, Desulfococcus multivorans* and *Desulfobotulus sapovorans* (DEVEREUX ET AL. 1992). The combination of probes 385 and 804 should therefore cover all Delta subdivision sulfate-reducing genera. Probe 687, specific for *Desulfovibrio,* was also applied. In DGGE blotting and hybridization experiments probe 687 gave weak hybridization signals with DGGE fragments from 16 and 20 m depth. In repeated membrane hybridization experiments with probe 687, unusually low hybridization signals were obtained in part also with positive controls, and therefore the probe was not used further.

The results of consecutive hybridization of the DGGE pattern with probes 385 and 804 are shown in Figs. 2B and 2C.

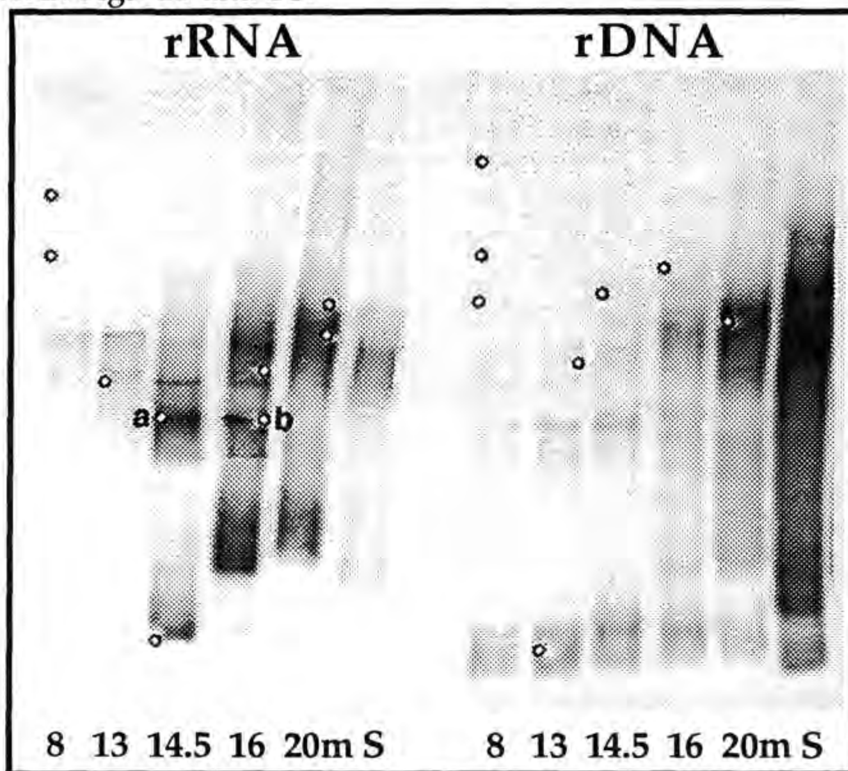

Fig. 2B. DGGE gel membrane-blotted and hybridized with rRNA probe 385 (AMANN ET AL. 1990). The encircled positions indicate excised gel portions, for reamplification and sequencing, which allow correllation to bands in the ethidium bromide stained DGGE gel (Fig. 2A). Bands a and b were sequenced.

Probe 385 only hybridized weakly with bands of rRNA-derived amplificates from the oxic water at 8 m and 13 m depth, but gave an intense hybridization signal with two rRNA-derived bands from the chemocline, at 14.5 and 16 m depth. Both bands, labelled a and b, show identical positions in the DGGE pattern (Fig. 2 A-C). The anoxic water sample from 20 m depth and the sediment sample from 26 m depth gave a strong, but diffuse hybridization signal with probe 385, which was probably derived from a multitude of different organisms which are not properly resolved in the DGGE pattern. A similarly diffuse hybridization signal of probe 385 was found for rDNA-derived amplificates from anoxic water and sediment. The strong and apparently dominant chemocline bands a and b are only faintly visible in the rDNA-derived DGGE pattern, and give a low hybridization signal with probe 385. This might indicate that the chemocline bacterium is far more significant by its activity than by its numerical presence.

Hybridization signals with probe 804 were only found below the chemocline, where it gave hybridization signals in both the rRNA and the rDNA derived DGGE pattern.

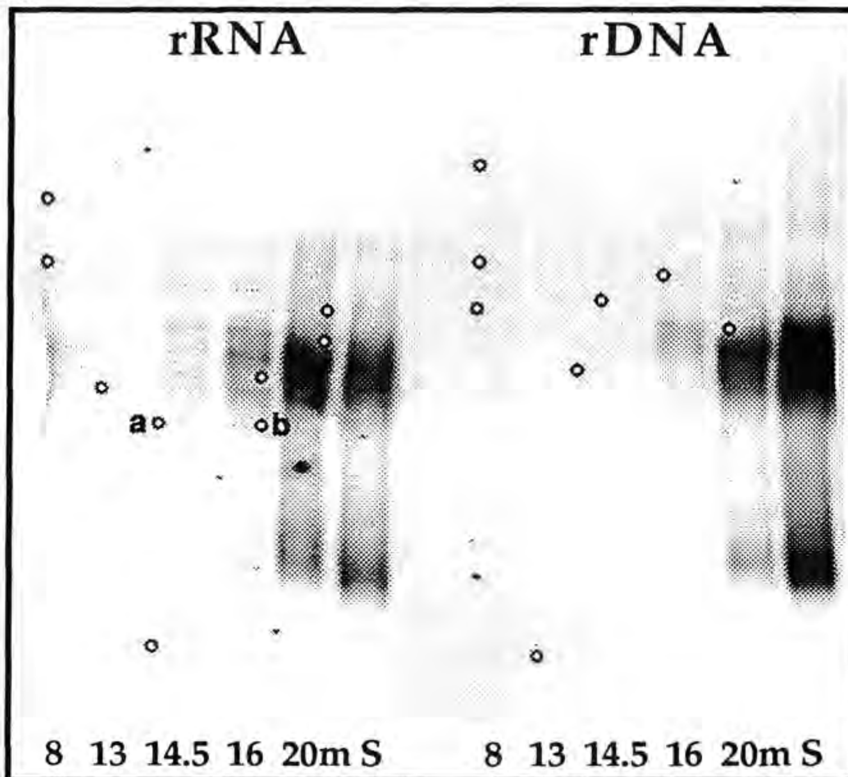

Fig. 2C. DGGE gel membrane-blotted and hybridized with rRNA probe 804, specific for *Desulfobacter, Desulfobacterium, Desulfosarcina variablilis, Desulfococcus multivorans* and *Desulfobotulus sapovorans* (DEVEREUX ET AL. 1992). The encircled positions indicate excised gel portions, for reamplification and sequencing, which allow correllation with bands in the ethidium bromide stained DGGE gel (Fig. 2A).

Hybridization intensities of probes 385 and 804 were integrated for both rRNA and for rDNA derived DGGE patterns. Integrated hybridization intensities of probes 385 and 804 in different water layers are given in Fig. 3B. The hybridization intensity of probe 385 generally increased with depth. In the rRNA-derived DGGE pattern, it reached a local maximum in the chemocline at 16 m. The hybridization intensity of probe 804 also increased with depth, in both rDNA and rRNA derived DGGE patterns (Fig. 3A). Interestingly, these results obtained with a qualitative method, PCR, reflect a trend which is defined by conceptually different, quantitative approaches: MPN counts indicate an increase of the sulfate reducing bacterial population at the chemocline of approx. factor 10 (Fig. 3C). Integrated fluorescent hybridization intensities of whole cells, obtained by direct hybridization of 16S rRNA with probe 385, without PCR-amplification, also increased by one order of magnitude (RAMSING ET AL. 1995, Fig. 7A). These population shifts took place against a background of almost constant bacterial cell density (RAMSING ET AL. 1995, Fig. 6A) and nucleic acid concentration (Fig. 3A) in the watercolumn.

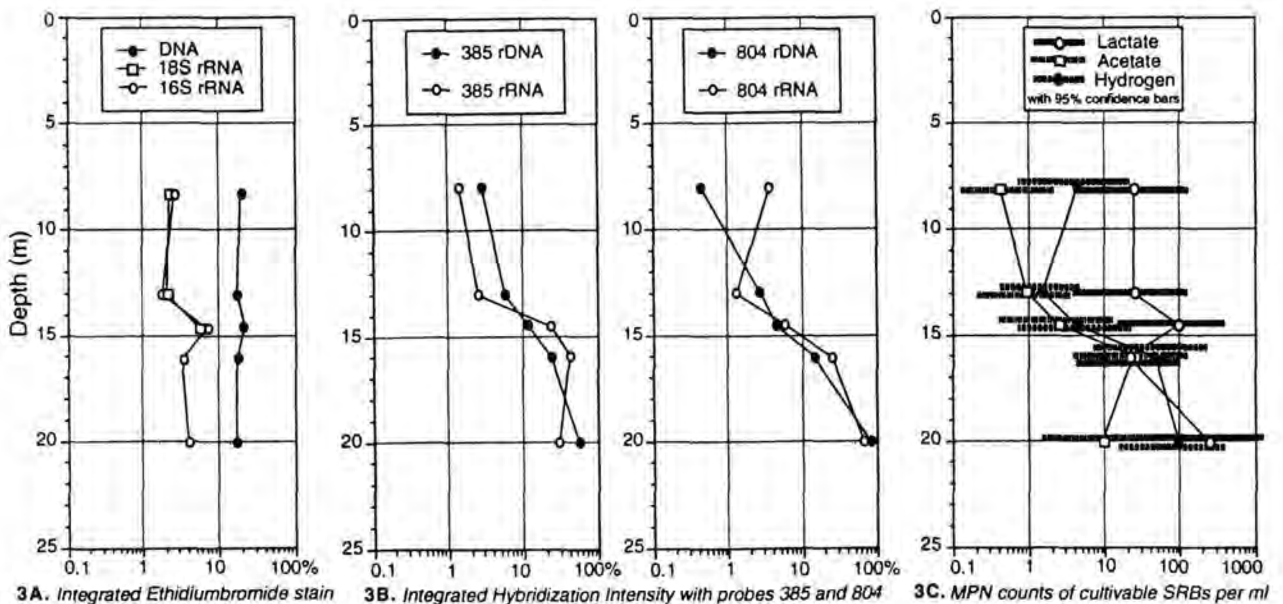

Fig. 3A. Ethidium bromide staining intensities of extracted ribosomal RNAs (bacterial 16S rRNA and eukaryotic 18S rRNA) and total extracted DNA from water column samples. **B.** Integrated hybridization intensities of rRNA- and rDNA-derived DGGE bands with probes 385 and 804 for each water layer, plotted in % of total integrated hybridization intensity. C. MPN counts of cultivable sulfate-reducing bacteria from water samples at depths of 8 m, 13 m, 14.5 m 16 m, 20 m. Hatched bars mark 95% confidence intervals.

Sequencing and identification of DGGE bands a and b. The rRNA chemocline bands a and b were re-amplified and sequenced. The sequences were compared to each other and analysed for their phylogenetic affiliation to known sulfate-reducing and Delta subdivision bacteria. The mere hybridization with probe 385 did not provide enough phylogentic resolution, as it for instance allowed crossreaction with the marine nitrifyer *Nitrospina gracilis* . When comparing the probe sequence with 16S rRNA reference sequences of the RDP database (MAIDAK ET AL. 1994), probe 385 matches at least six known non-sulfate-reducing Delta subdivision bacteria, *Bdellovibrio bacteriovorans,* four Myxobacteria (*Angiococcus disciformis, Archangium gephyra, Cystobacter fuscus, Corallococcus coralloides*) and *Nitrospina gracilis* (TESKE ET AL. 1994). Myxobacteria, typical soil bacteria, are unlikely organisms in marine habitats. However, *Nitrospina gracilis* is a marine nitrifyer which could occur in the Mariagerfjord chemocline and crossreact with probe 385, as furthermore nitrate and nitrite concentrations were highest in the upper chemocline (RAMSING ET AL. 1995, Fig. 3D). The gel positions of the *Nitrospina gracilis*-derived DGGE fragment and of the dominant rRNA bands a and b were compared in separate DGGE experiments, and found to be similar, but not identical.

Bands a and b had identical sequences, consistent with their identical position in the gel. The complete sequence of the DGGE fragment was determined and, by using the similarity check facilities of the RDP database (MAIDAK ET AL. 1994), identified as a Delta subdivision sequence. This was substantiated by checking the sequence for signature nucleotides of the Delta subdivision (WOESE 1987). The sequence was compared to sequences of sulfate-reducing and non-sulfate-reducing bacteria of the Delta subdivision, obtained from Genbank and the RDP database, but it could not be assigned to a known phylogenetic branch of the Delta subdivision, including recent molecular isolates (DeLONG ET AL. 1993, KANE ET AL. 1993, DEVEREUX & MUNDFROM 1994) and was not related to sequences obtained from MPN cultures. Instead, it appeared to be a new, distinct phylogenetic lineage within the Delta subdivision (Fig. 4). This sequence is positioned between the incompletely and the completely oxidizing sulfate reducing bacteria, in relative proximity to non-sulfate reducing members of the Delta subdivision, such as *Nitrospina gracilis, Geobacter metallireducens, Syntrophobacter wolinii,* the genera *Pelobacter, Desulfuromusa,* and *Desulfuromonas* (Fig. 4) The chemical conditions of the chemocline, especially the peak concentrations of elemental sulfur, oxidized iron and manganese, and nitrate (RAMSING ET AL. 1995, Fig. 3), allow several possible metabolic modes, i.e., iron-and manganese reduction (*Geobacter metallireducens, Pelobacter, Desulfuromonas*), fermentation of organic compounds (*Pelobacter*), sulfur

reduction (*Desulfuromusa, Desulfuromonas*), sulfur disproportionation, nitrification (*Nitrospina*), sulfate reduction (Delta subdivision sulfate reducers). A definite answer would require isolation of this bacterium in pure culture.

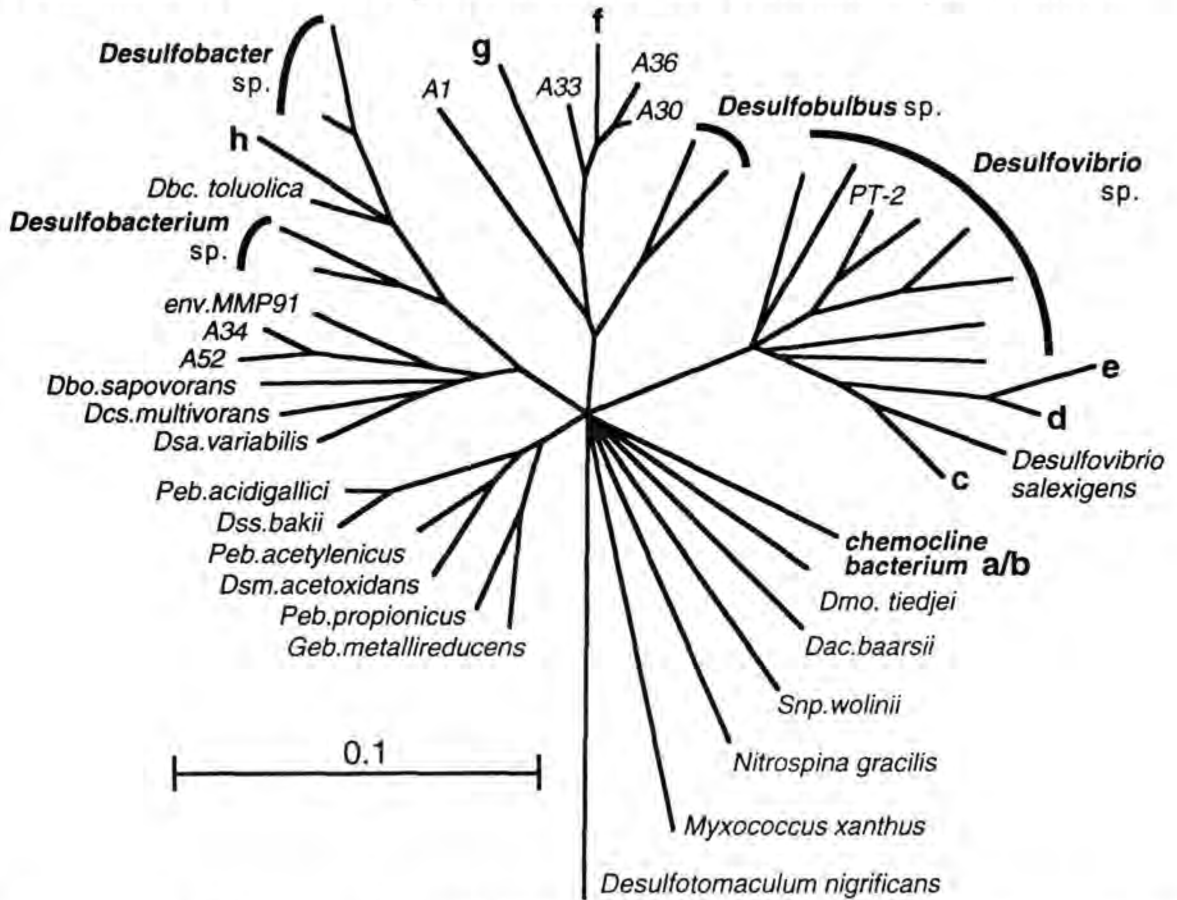

Fig. 4. 16S rRNA distance tree of Delta subdivision bacteria and sulfate reducers, used as an identification scheme and overview of the MPN isolates c, d, e, f, g, h from oxic waters and the rRNA-amplificate a/b from the chemocline of Mariagerfjord. The tree is based on 16S rRNA sequence positions 341-907 (*E.coli* numbering). The scale bar corresponds to 0.05 mutations per nucleotide position. The tree was rooted with the Gram positive sulfate reducer *Desulfotomaculum nigrificans* as outgroup. Multifurcations indicate lines of descent for which the relative branching order cannot be unambiguously determined, considering the different treeing methods and the limited amount of sequence information in a DGGE fragment. Sequences used in preparing this figure were derived from the Ribosomal Database Project (RDP) on the anonymous ftp server at the University of Illinois in Urbana, Illinois, updated on June 19th 1994 (MAIDAK ET AL. 1994). The new sequences from Mariagerfjord DGGEs and MPNs are available from Genbank under accession numbers L40767, L40783, L40789, L40784, L40785, L40786, L40787 (sequences a/b, c, d, e, f, g, h). Relevant molecular isolates from other sources: A1, A30, A33, A34, A36, A52 (DEVEREUX & MUNDFROM 1994), env.MMP91 (DeLONG ET AL. 1993), PT-2 (KANE ET AL. 1993).

MPN counts of sulfate-reducing bacteria. Cultivable sulfate-reducing bacteria of the water column of Mariagerfjord were counted by Most-Probable-Number (MPN) serial dilution. Lactate, acetate and hydrogen were used as substrates (Fig. 3C). Acetate-utilizing bacteria were found in low numbers (10-30 cells per ml) at 16 and 20 meter depth, in anoxic water. Even in the sediment we only found low numbers, 10^3-10^4 cells per ml. This might be a consequence of cell clumping, as often observed for non motile, marine acetate oxidizing sulfate reducers, i.e. *Desulfobacter* and *Desulfosarcina* (WIDDEL & PFENNIG 1981, 1984). MPN counts with lactate gave significantly higher numbers. Approximately 25 cultivable cells of sulfate-reducing bacteria per ml were counted in oxic water layers above the chemocline. Cell numbers increased below the chemocline to approximately 250 cells in the anoxic water layer at 20 m depth, and to 10^5-10^6 cells per ml in the bottom sediment. A similar trend was found when using hydrogen as substrate.

MPN cultures from high dilutions were PCR-amplified and analysed by DGGE. The DGGE patterns were blotted and hybridized with probes 385 and 804. As an example, Fig. 5A shows the ethidium bromide stained DGGE pattern of selected positive MPN dilutions, with lactate as substrate.

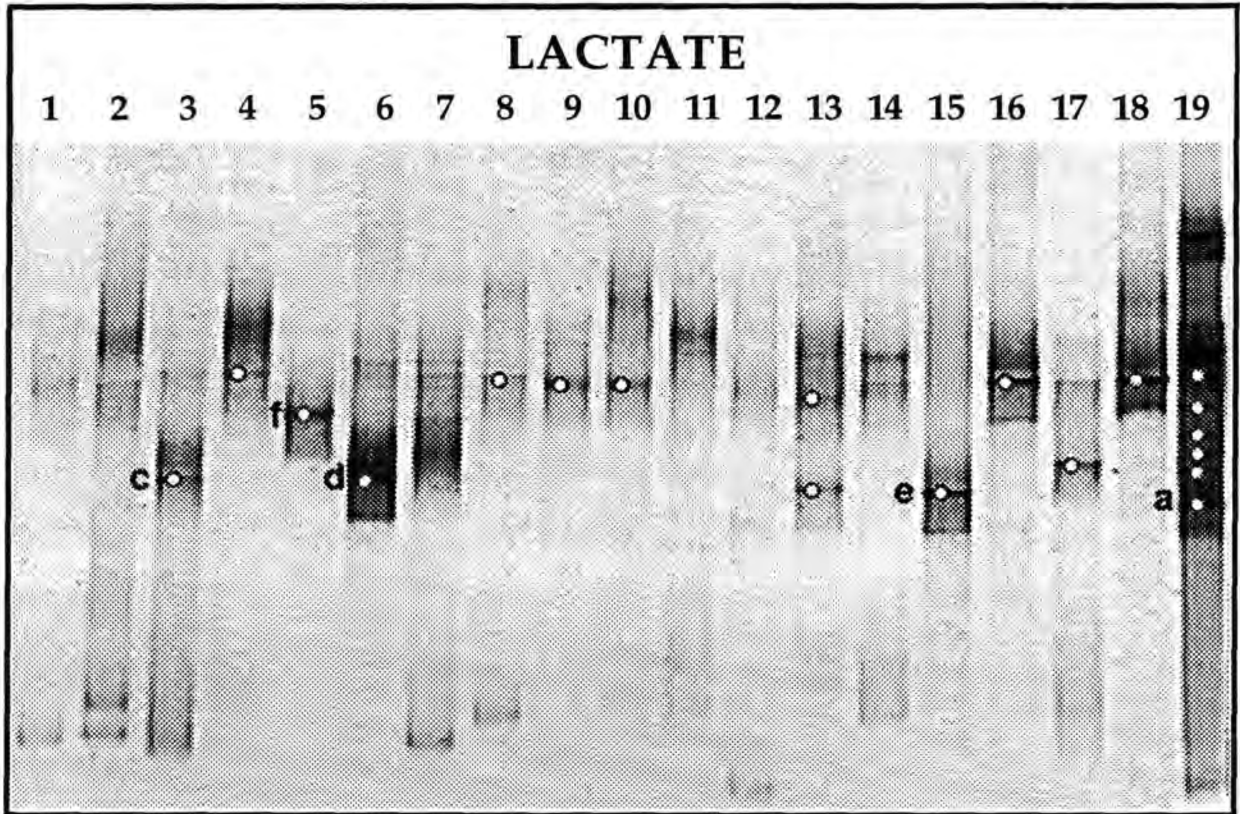

Fig. 5A. Ethidium bromide stained DGGE pattern of PCR products, derived from MPN cultures using lactate as substrate. MPN counts were done in triplicates. Each water layer is represented by three samples, numbered consecutively, from the highest positive MPN dilutions. Lanes 1-3: MPN cultures from the oxic zone at 8 m depth (dilutions 10^0, 10^{-1}, 10^{-1}). Lanes 4-6: MPN cultures from the upper chemocline at 13 m (dilutions 10^0, 10^{-1}, 10^{-1}). Lanes 7-9: MPN cultures from the central chemocline at 14.5 m (dilutions 10^{-1}, 10^{-1}, 10^{-1}). Lanes 10-12: MPN cultures from the lower chemocline at 16 m (dilutions 10^0, 10^{-1}, 10^{-1}). Lanes 13-15: MPN cultures from the anoxic water layer at 20 m (dilutions 10^{-2}, 10^{-2}, 10^{-1}). Lanes 16-18: MPN cultures from anoxic sediment samples at 26 m (dilutions 10^{-2}, 10^{-5}, 10^{-3}). Lane 19: DGGE bands derived from extracted rRNA of the central chemocline at 14.5 m, with DGGE band a, (cf. Fig. 2B), for comparison of this predominant chemocline band with MPN bands. The encircled positions indicate the punched out gel holes of DGGE band c, d, e, and f, which were re-amplified and sequenced.

The hybridization of this DGGE gel with probe 385 is shown in Fig. 5B. After hybridization with probe 385, the DGGE pattern of the MPN cultures was compared to the DGGE patterns of 16S rDNA fragments obtained from the extracted nucleic acids, i.e. DNA and rRNA, to identify bands of equal or similar gel position and hybridization signal. Such identical or similar DGGE bands, found in both DGGE patterns, could represent bacteria which were recovered both as molecular isolates and as MPN cultures. Whenever appropriate, similar MPN bands and water column bands were run on DGGE gels side by side to facilitate comparison, before being re-amplified and sequenced. Upon this closer examination, the investigated MPN bands and water column molecular isolates differed from each other.

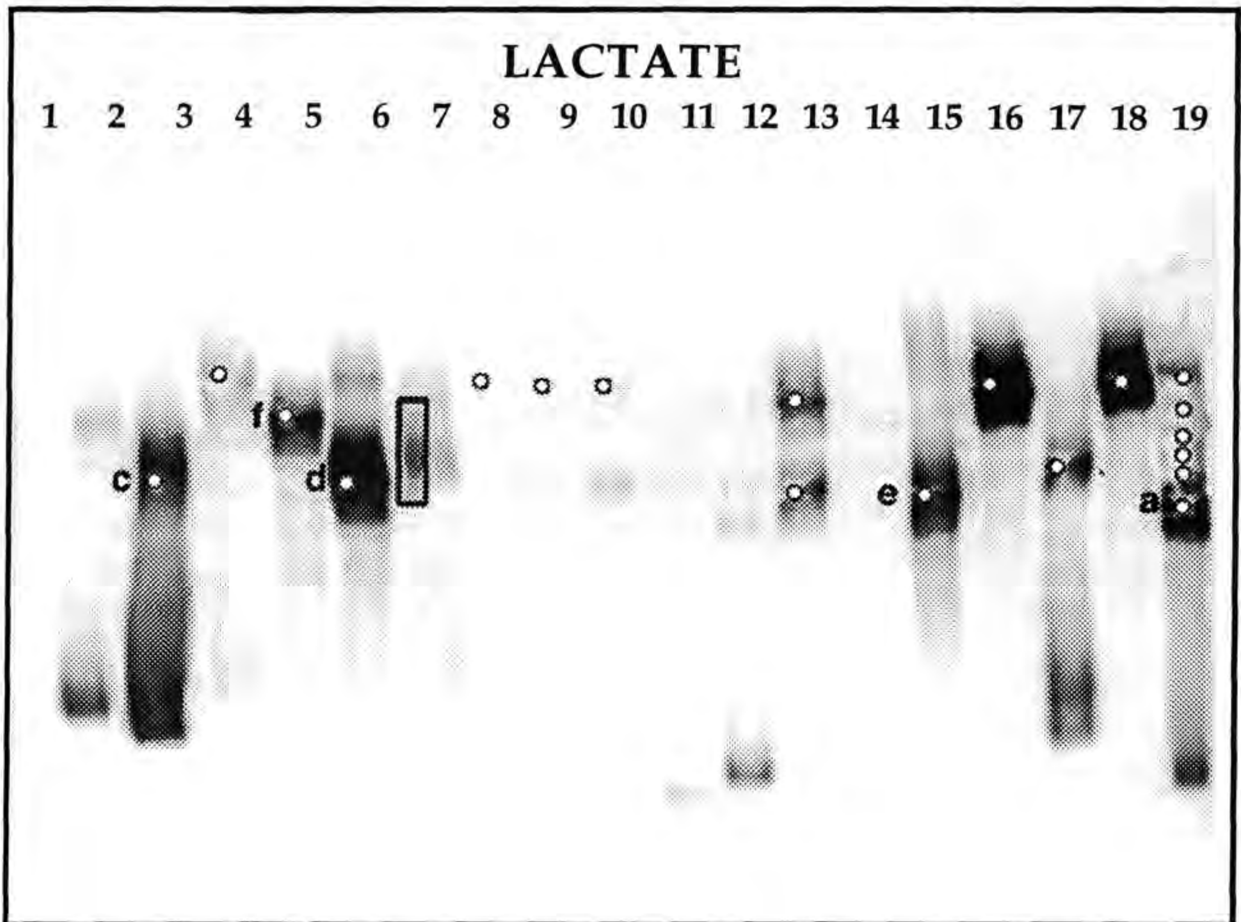

Fig. 5B. Blotted DGGE pattern and hybridized with rRNA probes 385. The encircled positions indicate the punched out gel holes of DGGE bands c, d, e, and f, which were re-amplified and sequenced. The small area in the frame is the only positive hybridization signal with probe 804.

Sulfate-reducing bacteria in the oxic layer of the water column. The existence of a specific physiological and phylogenetic group of oxygen-tolerant sulfate-reducing bacteria was considered, since high sulfate reduction rates and high counts of

sulfate-reducing bacteria have repeatedly been reported from oxic marine sediments and microbial mats (CANFIELD & DES MARAIS 1991, FRÜND & COHEN 1992, JØRGENSEN 1977a, JØRGENSEN & BAK 1991, RISATTI ET AL. 1994). To test this assumption, sulfate-reducing bacteria, growing in MPN cultures inoculated with oxic water samples from 8 m and 13 m depth, were identified by partial 16S rRNA sequences.
Four DGGE bands of the lactate MPN series, which were obtained in the dilution corresponding to 10 cells per ml, and which hybridized with probe 385, were sequenced: band c from 8 m, band d from 13m, band e from 20 m, and band f from 13 m depth (Fig. 5B). Band e was obtained from an MPN sample of the anoxic zone in 20 meter depth, but was included in the sequencing since its position in the DGGE gel was similar to those of bands c and d. DGGE bands c, d, and e turned out to be *Desulfovibrio* sequences, most closely related to the marine, salt-requiring species *Desulfovibrio salexigens*. Sequence f was distantly related to *Desulfobulbus*. Another *Desulfobulbus*-related sequence g was obtained from 8 m depth, in the first dilution step of the hydrogen MPN series. This sequence was closely resembling the sequence of a psychrotrophic, vacuolated, *Desulfobulbus*-related sulfate-reducing bacterium recently isolated from sediments of Kysing Fjord, Denmark (ISAKSEN & TESKE 1995). Sequences f and g clustered with four molecular isolates obtained from Florida marine sediments, A1, A30, A33, and A36 (DEVEREUX & MUNDFROM 1994), indicating a coherent, *Desulfobulbus*-related phylogenetic group of presumably sulfate-reducing bacteria. A *Desulfobacter-Desulfobacula*-like sequence h was obtained from 14.5 m depth, the central chemocline, in the second dilution step of the hydrogen MPN series, corresponding to 10 cells per ml. This sequence did not form a coherent phylogenetic group with related molecular isolates: Two of the Florida molecular isolates, A34 and A55 (DEVEREUX & MUNDFROM 1994), and the sequence of an uncultivable magnetotactic multicelled Prokayote (DeLONG ET AL. 1993), belonged to the *Desulfococcus-Desulfosarcina-Desulfobotulus* group (Fig. 4).
The investigated sulfate-reducing bacteria, obtained by MPN series from oxic water layers of Mariagerfjord, are phylogenetically affiliated to known sulfate reducing bacteria. *Desulfovibrio* and *Desulfobulbus*-related strains are feasable inhabitants of the oxic water layers of Mariagerfjord, since several species of these genera are capable of respiring various substrates aerobically. *Desulfovibrio* strains respire a wide variety of substrates, H_2, lactate, pyruvate, formate, ethanol, SO_3^{2-}, $S_2O_3^{2-}$, and H_2S (DANNENBERG ET AL. 1992). *Desulfobulbus propionicus* respires propionate, SO_3^{2-}, and H_2S. Interestingly, the *Desulfovibrio* sequences from Mariagerfjord were phylogenetically most closely related to the marine species *Desulfovibrio salexigens*, which showed almost no aerobic respiration at

all with the substrates tested (DANNENBERG ET AL. 1992), and to a recently isolated marine *Desulfovibrio* from Solar Lake, Egypt, which respired aerobically with various substrates (KREKELER ET AL. 1995). However, aerobic growth of oxygen-respiring sulfate-reducing bacteria, defined as more than one doubling of protein, has not been observed so far (MARSCHALL ET AL. 1993). It therefore seems unlikely that *Desulfovibrio* and *Desulfobulbus* strains of Mariagerfjord actually grow as free-living aerobes in the oxic water column, but are more likely to persevere in this environment by particle association or as components of marine snow (FUKUI & TAKII 1990, SIEBURTH 1993).

DISCUSSION

Integration of molecular, microbiological and biogeochemical approaches. Different approaches in the investigation of the Mariagerfjord bacterial ecosystem, such as fluorescent in situ hybridization and chemical analysis, DGGE analysis of PCR amplified 16S rDNA fragments, hybridization analysis with specific probes, DNA sequencing and MPN counts, result in different datasets which have to be crosschecked and reevaluated for a valid description of the bacterial populations within their natural habitat.

We have used DGGE analysis of PCR-amplified 16S rDNA fragments to infer an overall picture of the bacterial populations in different water layers of Mariagerfjord. DGGE analysis of DNA fragments obtained from amplification of rDNA and reverse-transcribed rRNA visualized the different patterns of bacterial activity and bacterial presence in the water column. DGGE patterns are of qualitative nature and importance, but have to be treated with caution when aiming for quantitative conclusions. The polymerase chain reaction, which predates the DGGE analysis, may selectively amplify specific members of a given nucleic acid population, resulting in a quantitatively distorted representation of the natural bacterial populations (MUYZER ET AL. 1993). Comparisons of rDNA- and rcDNA-derived DGGE bands with identical mobility and the ensuing qualitative conclusions about rRNA content and possibly metabolic activity of the corresponding bacterial population, rely on three assumptions: First, rcRNA yield by reverse transcription increases with rRNA content. Second, PCR amplification of genomic DNA sequences and the corresponding reverse-transcribed hexanucleotide-primed cDNAs are roughly equally efficient. Third, the amplification of a particular template should be largely unaffected by the presence of other templates. These assumptions are implied in referring to the bacterium of the rcDNA-derived chemocline DGGE band a/b as higly active. Supporting evidence is provided by pure culture and co-culture studies: PCR amplifications

of *Desulfovibrio* hydrogenase cDNA give the highest yield in the early logarithmic growth phase of bacterial cultures (WAWER & MUYZER, unpublished results). PCR amplification of hexanucleotide-primed 16S rcDNA and of genomic 16S rDNA sequences of a defined *Desulfovibrio-Marinobacter* co-culture both yielded the same set of 16S rRNA sequences (MUYZER ET AL., unpublished). PCR amplification of genomic DNA sequences and corresponding cDNAs did thus not miss a member of the model community. No chimeric artifacts were observed. The use of universal PCR primers in the amplification step, combined with effective separation of different PCR products, enables the DGGE to represent and to resolve a wide range of microbial diversity in a given microbial ecosystem, without the constraints of specific media or laborious sequencing of clone libraries. The novel highly active chemocline organism, representing a new phylogenetic lineage within the Delta proteobacterial subdivision, would probably not have been identified by any other molecular method. Cloning of PCR products could have yielded the bare sequence, but would have given little information about the high activity of this organism in the chemocline.

Although we have discussed DGGE of rDNA fragments as a predominantly qualitative method to describe the structure of microbial populations, it reflects quantitative trends to some degree, and is, in this investigation, congruent with explicitly quantitative methods. The DGGE pattern of the Delta subdivision sulfate-reducing bacteria, as obtained from membrane hybridizations with probes 385 and 804 (Fig. 2B and C), is congruent with the MPN counts of sulfate reducing bacteria (Fig. 3C). Both approaches indicate a maximum of sulfate-reducing bacteria in the anoxic water layer and in the bottom sediment of Mariagerfjord. MPN counts indicate an increase in the numbers of sulfate-reducing bacteria by approximately a factor of 10, from above to below the chemocline. The integrated intensity of the DGGE blot hybridization signals of probes 385 and 804, as shown in Fig. 3B, shows a similar increase of at least one order of magnitude. Interestingly, the same trend is reflected by whole-cell fluorescent hybridization intensities, as shown in Fig. 7D of the accompanying study (RAMSING ET AL. 1995), in spite of significant methodological differences: In situ hybridization of 16S rRNA with fluorophor-labelled oligonucleotides does not involve a PCR-amplification step, which is a prerequisite of the DGGE, and does not contain the possible amplification bias of the PCR.

In principle, MPN counts give a more stringent quantitative picture of the distribution of sulfate-reducing bacteria in the water column, since they count only viable, cultivable, actually sulfate-reducing cells, no matter how small this population might be in comparison to other bacterial populations. The

concentration of cultivable sulfate-reducing bacteria in the water column of Mariagerfjord, as determined by MPN counts, remained below 1300 cells per ml, which was the upper limit of the 95% confidence interval for the highest MPN count (Lactate, 20 m depth). This selectivity of MPN counts is reflected by the finding that MPN isolates did not match major bands of the DGGE pattern. The DGGE pattern of PCR-amplified nucleic acids conceptually represented the general bacterial population, since the PCR primers chosen for the reamplification step were targeted to universally conserved sites of the 16S rRNA sequence. The physiological selectivity of MPN counts is often regarded as an obstacle to the complete analysis of a microbial community, but should be viewed as a specific advantage and an important complement for rRNA-based molecular methods, which can by their nature give no direct information on physiological capabilities of bacterial populations. This information can sometimes be inferred from the 16S rRNA sequence, if a molecular isolate falls into a well known, phylogenetically and physiologically coherent bacterial group. On the other hand, molecular isolates without clear affinities to known bacterial genera remain, at best, ecophysiologically ambiguous: Sequencing and phylogenetic placement of the DGGE bands a and b, derived from an uncultured chemocline bacterium, thus gave no clear indication of its potential physiological capabilities.
Direct cell counts reveal other specific limitations of culture-dependent approaches. Cultivable sulfate-reducing bacteria in the water column, estimated by MPN counts as 2×10^1 - 3×10^2 cells per ml, represent only a minor fraction of the total bacterial number (Fig. 3C). Ethidium bromide staining indicated 2 - 4 x 10^6 cells per ml, and fluorescent *in situ* hybridization with a general eubacterial probe resulted in 1 - 2 x 10^6 cells per ml, as shown in figures 6A and 6B of the accompanying study. Fluorescent *in situ* hybridization counts with probe 385 indicated cell numbers in the range of 10^4 - 10^5 cells per ml, three orders of magnitude higher than the MPN counts of cultivable sulfate-reducing bacteria, as shown in figures 7A and 7B of the accompanying study. This discrepancy between total und cultivable cell numbers, the "Great Plate Count Anomaly" (STALEY & KONOPKA 1985), is particularly severe in marine habitats. In oligotrophic and mesotrophic marine habitats only 0.1% or less of the general bacterial population is generally recovered by cultivation-dependent approaches (FERGUSON ET AL. 1984, JANNASCH & JONES 1959, KOGURE ET AL. 1979, 1980, STALEY & KONOPKA 1985), which contrasts to higher values, 5-15%, in extremely eutrophic environments such as activated sludge (WAGNER ET AL. 1993). This discrepancy is usually attributed to the inadequacy of media used in cultivation-based approaches, but may also have other, more profound reasons. Populations of viable, but non-culturable cells account for much of the total bacterial

population and activity *in situ* (HOPPE 1978, OLIVER 1993). Such cells could still be viable in the given medium, but do not grow beyond a few cell divisions and are therefore not recognized in plate or MPN counts. From a soil model system, inoculated with a *Pseudomonas fluorescens* strain, after 40 days only 0.21% of the start population could be recovered as cultivable cells, but approx. 20% of the start population was found as viable, but non-culturable cells (BINNERUP ET AL. 1993). These cells were able to form microcolonies, but did not grow beyond a few cell divisions, and were only detectable by a combined microcolony-epifluorescence assay. Thus, the viable, cultivable population of the *Pseudomonas* strain corresponded to only 1% of the viable, but non-culturable population. A similar ratio of viable, culturable and viable, but non-culturable cells could apply to the Mariagerfjord sulfate reducers: Culturable sulfate-reducing bacteria in the anoxic water layers, estimated by MPN as 200-300 cells per ml, correspond to 0.2 - 0.3 % of 10^5 cells per ml, found by *in situ* hybridization with probe 385 (RAMSING ET AL. 1995).

The chemical conditions in the watercolumn, the position of the chemocline, the extent and overlap of oxidized and reduced water layers (RAMSING ET AL. 1995), are consistent with the results obtained in this molecular and microbiological investigation. The continuous gradients of chemical conditions in the watercolumn is mirrored by highly differentiated bacterial populations in the watercolum, as shown by DGGE (Fig. 2A). The availability of a wide range of electron donors and acceptors in the chemocline, such as oxygen, sulfide, elemental sulfur, sulfate, nitrate, ammonia, oxidized and reduced forms of manganese and iron (RAMSING ET AL. 1995), is connected with high bacterial activity and rRNA yield in the chemocline (Fig. 1). Numbers of cultivable sulfate reducers increased already within and not below the chemocline, evidenced by MPN counts, fluorescent in situ hybridization of individual cells and membrane hybridization of PCR-amplified 16S rRNA and rDNA fragments. This result suggests a potential of sulfate reducers to utilize alternative electron acceptors in the chemocline (DANNENBERG ET AL. 1992, LOVLEY & PHILIPPS 1994, RODEN & LOVLEY 1993, SEITZ & CYPIONKA 1986). Sulfur disproprotionation, which has been demonstrated for enrichments, pure cultures, and for the sulfate-reducing bacterium *Desulfobulbus propionicus*, is another possible process in the chemocline. Elemental sulfur was found in the chemocline in 0.5 µM concentrations, together with reduced iron and manganese for the concomitant reoxidation of sulfide produced by disproportionation (LOVLEY & PHILIPPS 1994, THAMDRUP ET AL. 1993). These reactions might be attributed to certain highly active delta-subdivision chemocline bacteria, such as the molecular isolate found by DGGE and sequencing in this study, and the long slim rod apparent by

fluorescent *in-situ* hybridization with probe 385, shown in the 16m sample of Figure 5 of the accompanying paper. Isolation of new biogeochemically significant bacterial types, combined with systematic probe development, is necessary to tie together microbiological and biogeochemical processes.

Summary. Different approaches of this polyphasic study allow to infer this overall picture of the sulfate-reducing bacterial populations of Mariagerfjord: Amounts of total bacterial nucleic acids remained almost constant throughout the water column of Mariagerfjord, with the exception of the chemocline. The bacterial population was continuously changing not in total mass, but in composition throughout the water column, and showed a highly differentiated pattern of active versus dormant subpopulations, as revealed by DGGE analysis of 16S rRNA- and 16S rDNA-derived PCR amplificates. A highly active subpopulation of novel Delta subdivision bacteria was found in the chemocline of Mariagerfjord, by DGGE analysis and subsequent sequencing of the individual bands. Delta subdivision sulfate-reducing bacteria have their maximum in the dysaerobic and anoxic waterlayers within and below the chemocline, a result substantiated by MPN counts, hybridization anlysis of the DGGE patterns, and fluorescent *in situ* hybridization of individual cells (RAMSING ET AL. 1995). Cultivation-dependent MPN counts of sulfate-reducing bacteria were consistently three orders of magnitude lower than direct counts by fluorescent *in situ* hybridization, but both approaches indicated an increase of numbers of cultivable sulfate-reducing bacteria in the chemocline by approximately one order of magnitude. This ratio also resulted from the integrated hybridization intensities of PCR-amplified rRNA and rDNA-derived DGGE bands with probes 385 and 804 (Fig. 3B). Although the maximum of sulfate-reducing bacteria occurred in and below the chemocline, cultivable sulfate-reducing bacteria were also quantified in the oxic part of the water column by MPN. Cultivable sulfate-reducing bacteria of the oxic water layers were by 16S rRNA sequencing shown to be affiliated to the genera *Desulfovibrio* and *Desulfobulbus*.

Biogeochemical, molecular and microbiological approaches contributed to this picture of the sulfate reducing bacteria and the microbial ecosystem of Mariagerfjord. Methodological limitations of each approach constrain the range over which valid conclusions can be drawn, and require a careful integration of these different perspectives to perceive the whole picture of a natural habitat. Unexpected mismatches between different datasets were found, such as the extremely different DGGE patterns derived from rRNA and rDNA, the nonidentity of molecular and culture isolates, or the gap between fluorescent and culture-dependent bacterial counts. Nethertheless, these discrepancies are in fact

highly valuable indicators of real phenomena: The highly differentiated pattern of bacterial activity, the abundance of uncultured bacteria, and the selectivity of our own approaches to study a microbial ecosystem.

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4.5. *Desulforhopalus vacuolatus* gen. nov. sp. nov, a new psychrotrophic sulfate-reducing bacterium with gas vacuoles isolated from a temperate estuarium, Kysing Fjord, Denmark

Mai Faurschou Isaksen, Andreas Teske

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ABSTRACT

A new type of gas vacuolated, sulfate-reducing bacterium was isolated at 10°C from anoxic, reduced mud from a temperate estuarium with thiosulfate and lactate as substrates. The strain was psychrotrophic with optimum growth at 18-19°C and temperature maximum on 24°C. Propionate, lactate and alcohols served as electron donors and carbon sources. No autotrophic growth was achieved on hydrogen as electron donor. Stoichiometric measurements revealed that propionate and lactate were incompletely oxidized to acetate, and that traces of lactate were fermented to propionate, CO_2 and possibly acetate in presence of sulfate. Pyruvate was utilized both with and without electron acceptor present. The strain did not contain desulfoviridin. The G + C mol % was 48.4. The 16S rRNA sequence differences of strain ltk10 from its next phylogenetic neighbour, *Desulfobulbus*, support the assignment of strain ltk10 to a new genus. Strain ltk10 is described as type strain of the new species and genus, *Desulforhopalus vacuolatus*.

INTRODUCTION

The important substrates for sulfate reduction are acetate and propionate, accounting for 50% and 10% of electron donors consumed by sulfate reducers (PARKES ET AL. 1989; SØRENSEN & JØRGENSEN 1981). Several genera are capable to oxidize propionate completely to CO_2 (WIDDEL & BAK 1991) whereas only the species of the genus *Desulfobulbus* carry out an incomplete oxidation of propionate to acetate and CO_2 (SAMAIN ET AL. 1984; WIDDEL & PFENNIG 1982). Until now, only a few incompletely propionate-oxidizing bacteria belonging to *Desulfobulbus* have been described (WIDDEL & BAK 1991). Despite this, PARKES ET AL. (1993) found the incomplete oxidation of propionate by sulfate reducers to dominate the degradation of propionate in marine sediment slurries. Further more, four new *Desulfobulbus*-related 16S rRNA sequences were found by PCR-amplification of nucleic acids extracted from marine sediment (DEVEREUX & MUNDFROM 1994). Two *Desulfobulbus*-related 16S rRNA sequences were obtained from Most-Probable-Number enrichments from the water column of Mariager Fjord, Denmark (TESKE ET AL. 1995). These results suggest high species diversity and ecological significance of *Desulfobulbus*-related propionate-oxidizing sulfate reducers.

The *Desulfobulbus* species described so far are all mesophilic with minimum growth temperature of 10°C or above. As the temperature in the marine environment generally is 5°C or below (HERBERT 1986), the incomplete

degradation there must be carried out by so far unknown species or genera or by new subspecies of known species. The present paper reports on the isolation and characterization of a new type of psychrotrophic incompletely propionate-oxidizing, anaerobic, gas-vacuole forming sulfate-reducing bacterium that was isolated at 10°C from a temperate marine environment.

MATERIALS AND METHODS

Source of organism. Sediment samples from the shallow but permanently water-covered coast of Kysing Fjord (JØRGENSEN & SØRENSEN 1985) were collected at 1 m water depth. The temperature at the sediment surface at this locality varies between 0° and 20°C throughout the year (JØRGENSEN & SØRENSEN 1985). Enrichments were inoculated with sediment taken from the uppermost part of the black reduced zone.

Media and Conditions of cultivation. The anaerobic sulfate-reducing bacteria were cultivated in a bicarbonate-buffered, sulfide-reduced mineral medium containing (g per liter): NaCl, 15.0; $MgCl_2 \cdot 6H_2O$, 2.0; KCl, 0.5; NH_4Cl, 0.25; KH_2PO_4, 0.2; $CaCl_2 \cdot 2H_2O$, 0.2; $NaHCO_3$, 1.75; $Na_2S \cdot 9H_2O$, 0.18; trace element solution SL 10a (Widdel and Bak 1991), 2 ml/l; vitamin solution (WIDDEL & PFENNIG 1981), 2 ml/l. All chemicals were of analytical grade. The pH was adjusted to 7.2. The medium was prepared under an O_2-free N_2 atmosphere as described by WIDDEL & BAK (1991). Substrates (electron donors and acceptors) were added before inoculation from sterile neutralized stock solutions to give the concentrations desired. Prior to inoculation, all cultures received sodium dithionite ($Na_2S_2O_4$,) as an additional reducing agent to a final concentration of about 5 µM. The sodium dithionite shortened the lag phase of bacteria growth in batch cultures, but was not required for growth.

Isolation. Pure cultures were obtained by repeated application of deep-agar dilutions as described by WIDDEL & BAK (1991). The method was modified to ensure that cells were kept constantly cold: a dilution series was made from an enrichment culture in medium cooled to 10°C. Each of the dilutions (6 ml) was then dispersed in 3 ml 3% agar of about 45°C and quickly transferred to a 0°C cooling bath. The gas phase was then exchanged with oxygen-free N_2/CO_2. The agar tubes were incubated at 10°C. Isolated strains were checked for purity under the microscope and by growth tests in complex medium. Stock cultures were kept at 6°C in the dark and transferred to fresh medium at monthly intervals.

Determination of growth rates. Batch cultures of strain ltk10 were grown at 10°C. Experiments were carried out with bacteria collected in late exponential growth phase. In all experiments, we used sulfate (10 mM) in combination with lactate (20 mM). Growth was followed by measuring the light attenuation at 500 nm in a spectrophotometer. The growth rate, r, of an exponentially growing culture was calculated from equation (1) by linear regression in a plot of ln(OD) as a function of time, t:

(1) $OD = OD_0 \exp(rt)$

where OD is the optical density at time t and OD_0 is the optical density at start of the incubation. There were at least 8 measurements for each regression.

Stoichiometric measurements and cell material determination. The stoichiometry of substrate oxidation was determined in 0.5 l bottles sealed with rubber stoppers; the gas phase above the culture was kept very small. Growth was measured as OD throughout the experiment. The concentration of sulfide, lactate, propionate, and acetate were measured at the start, twice in the exponential growth phase and at the end of the experiments. Cell biomass was determined at the start and at the end of the experiment on a HCN analyser (Carlo Erba Na 15000). 10.0 ml culture was filtered through GF/F filters (Whatman, glass microfibre filters), the filter was dried at 105°C for 24 h, where after the filter was analysed for C and N content. The results was converted to biomass as described by Isaksen and Jørgensen (unpublished).

Chemical determinations. H_2S was determined photometrically after the methylene blue methods described by CLINE (1969). Lactate, propionate, and acetate was determined by HPLC (Sykam, Gilching, Germany), column (HPX-87H) stored in a column oven at 60°C, and an UV-detector. The eluent was 0.01M H_2SO_4.

Electron Microscopy. Preparation of samples for electron microscopy was done as described by PEARL & SHIMP (1973). 0.1 - 2.0 ml of an early exponential growth culture were filtered down on nucleopore filters (0.7μm). The filters were fixed in 2% v/v glutaraldehyde for one hour at 4°C. After the fixation, synthetic sea water with decreasing salt concentration were passed through the filters (50%, 25%, and destilled water for 5 min each). The samples were here after dehydrated with ethanol of increasing concentration (10, 25, 50, 75, and 100% for 10 min each). Samples were left in 75% ethanol for up to one week, if critical point drying could not be done shortly after the last step.

Nucleic acid isolation and sequencing. Nucleic acids were isolated by Lysozyme/ ProteinaseK/SDS digest, followed by phenol extraction and ethanol precipitation as previously described (SAMBROOK ET AL. 1989). The 16S rRNA gene was amplified by PCR, using primers GM3 (5'-AGAGTTTGATC(A/C)TGGC-3', corresponding to E. coli positions 8-23) and GM4 (5'-TACCTTGTTACGACTT-3', corresponding to E. coli positions 1492-1507). Each of the 30 PCR cycles started with 1 minute denaturation at 95°C, followed by 2 minutes annealing at 40°C and ended with 3 minutes elongation at 71°C. The reaction mixture (total volume 50 µl) contained 50 mM KCl, 10 mM Tris-HCl pH 8.3, 1.5 mM $MgCl_2$, 0.01% (wt/vol) gelatine, 200 µM of each deoxynucleotide (dGTP, dATP, dTTP, and dCTP), 0.5-1.0 Units Taq DNA Polymerase (SuperTaq, HT Biotechnology limited), and 0.5 µM of each primer. The PCR-products were analyzed by electrophoresis on 1% horizontal agarose gels in TAE buffer using a defined 16S-PCR copy of 1.5 kb (*Desulfobulbus* sp.) as size marker. The PCR product was gel-purified as follows: A small well was cut into the agarose gel in front of the selected PCR-product. Electrophoresis was continued until the PCR product migrated into the buffer-filled well, from where it was taken up with a pipette and transferred into an Eppendorf vial. After precipitation one hour at -80°C with 0.1 volume 5 M NaCl and 2.5 volumes ethanol the PCR product was pelleted by centrifugation and redissolved in 50 µl distilled water.

PCR products were sequenced directly: 10-100 ng purified PCR-product were mixed with 2 µl sequencing buffer (200 mM Tris-HCl pH 7.5, 100 mM $MgCl_2$, 250 mM NaCl), 1 µl 5% Nonidet P-40 (Sigma) aqueous solution, 2 pmol primer, and adjusted with distilled water to a final volume of 10 µl. The double stranded PCR-product was denatured by heating the 10 µl volume to 95°C for two 5 min periods, with a short centrifugation in between. The sequencing reaction was started by adding 1.0 µl 0.1 M DTT, 2.0 µl dNTP-solution (200 nM dGTP, dATP, dTTP), 0.5 µl (alpha-33P)-dCTP; (10 µCi/µl; 3000 Ci/mmol), 2 µl 1 U Sequenase 2.0 (USB). After a short centrifugation to collect and mix the sample volume (15.5 µl), the sample was incubated for 5 minutes at 37°C. The sample volume was divided into four 3.5 µl portions and added to dideoxynucleotide termination solutions (2.5 µl, containing 80 µM of each dGTP, dATP, cTTP, dCTP, and 8 µM dideoxynucleotide). After 5 min incubation at 37°C the reaction was stopped by adding 4 µl of a solution of 96%-formamide and 20 mM EDTA.

Phylogenetic Tree Inference. The 16S rRNA sequence of strain ltk10 was compared to those of the Ribosomal Database Project (RDP) database (MAIDAK ET AL. 1994), using the SIMILARITY_RANK tool of the RDP database to search for close evolutionary relatives of strain ltk10. Near-complete 16S rRNA

sequences for the phylogenetic tree were obtained from the RDP database, including two *Desulfobulbus* sequences: *Desulfobulbus propionicus* strain Lindhorst 1pr3, a freshwater *Desulfobulbus*, and *Desulfobulbus marinus* strain 3pr10, a marine isolate (WIDDEL & BAK 1991; WIDDEL & PFENNIG 1982). Sequences of *Desulforhopalus*- and *Desulfobulbus*-related molecular isolates were obtained from Genbank. Phylogenetic trees were inferred using the distance matrix programs DNAdist and Fitch as implemented in the PHYLIP program package version 3.5c, by FELSENSTEIN (1993). In the program DNADIST, a matrix of evolutionary distances was computed from the sequence alingment, using the Jukes-Cantor model (JUKES & CANTOR 1969), which assumes independent change at all sites with equal probability. Phylogenetic trees were inferred from the distance matrix with the program FITCH, which uses the least squares algorithm of FITCH & MARGOLIASH (1967). The 16S rRNA sequence of *Desulforhopalus vacuolatus* is available from Genbank under accession number L42613.

DNA base composition and characterization of pigments. G+C content determination was carried out at the DSM, Braunschweig, Germany. DNA of strain ltk10 was hydrolyzed with P1 nuclease and the nucleotides dephosphorylized with bovine alkaline phosphatase (MESBAH ET AL. 1989). The resulting deoxyribonucleosides were analyzed by HPLC (TAMAOKA & KOMAGATA 1984). GC content was calculated from the ratio of deoxyguanosine (dG) and deoxyadenosine (dA) (MESBAH ET AL. 1989). For the detection of desulfoviridin, the fluorescence test of POSTGATE (1959) was applied.

RESULTS

Enrichment and Isolation. When culture media with lactate as sole electron donor and thiosulfate as sole electron acceptor was incubated with anoxic, reduced mud from Kysing Fjord at 10°C, sulfide developed within one to two weeks. The enrichment was dominated by gas vacuolated rod shaped cells of constant width but with variable length. Small oval to rod shaped bacteria of the *Desulfobacter* type were also present. Strain ltk10 was isolated in pure culture with lactate as sole electron donor and thiosulfate as sole electron acceptor. No colonies with bacteria of the *Desulfobacter* type developed in the high dilutions of the agar shakes.

Morphology. The cells of strain ltk10 were rod-shaped, 3.0 - 5.0 µm long and 1.5 - 1.8 µm wide, non-motile, and contained gas vacuoles. During exponential growth, cells were distributed evenly in the culture volume, whereas in the stationary growth phase where long cells were observed, the cells formed a white to light-beige layer at the medium surface. The electron microscopy showed that strain ltk10 did not have any flagella. The cells of strain ltk10 stained Gram negative.

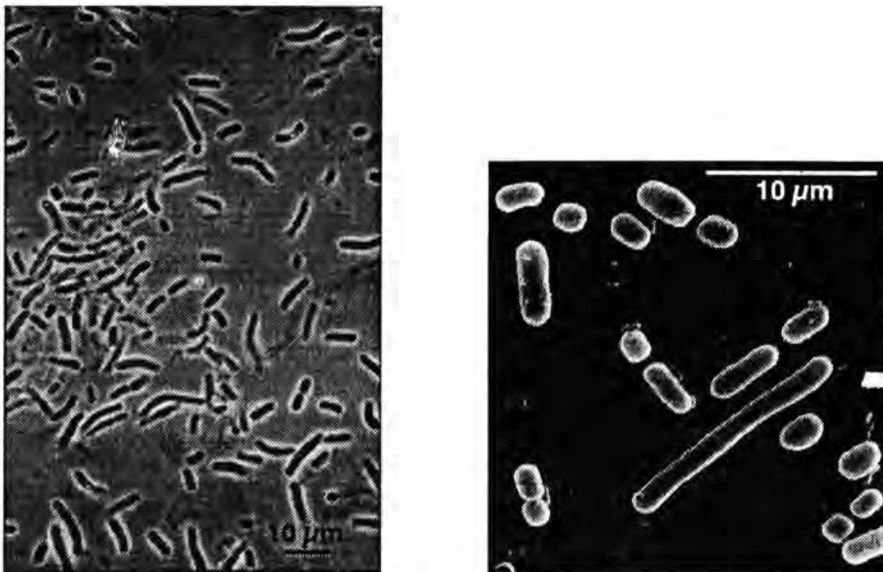


Fig. 1 (right): Phase contrast photomicrograph of the propionate-oxidizing sulfate reducer *Desulforhopalus vacuolatus* strain ltk10. Note the longer cells typical for cells from stationary growth phase. On this photo, only three cells have vacuoles.

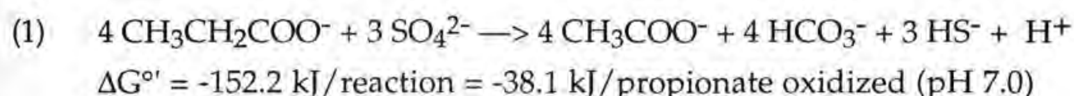
Fig. 2 (left): Transmission electron microscopy of *Desulforhopalus vacuolatus*. Scale bar represents 10 µm.

Growth conditions and nutrition. Strain ltk10 grew fastest with a salt concentration of 20‰, and it was able to grow in medium with a salt concentration from 5‰ to 50‰; with 80‰, no growth was observed. Below 15‰ and above 40‰, however, the terminal optical density was less than half of the optical density obtained with salt concentration from 20-30‰.
The optimal pH was at 6.8 to 7.2, and strain ltk10 was able to grow at pH from 5.7 to 8.0. At pH 8.2, no growth was observed. The terminal optical density of the culture was the same from pH 6.1 to 7.8; below and above, the terminal optical density was around the half of optimal. At pH from 5.3 to 5.7, the strain produced sulfide and grew slowly after one month of incubation.
Strain ltk10 was able to grow at temperatures from 0°C to 24°C with an optimum at 18 - 19°C. The doubling time with lactate and sulfate was 22.5 hours at 19°C. Even though the strain did not grow above 24°C, it was able to respire at temperatures up to 38°C. When exposed to these temperatures, the gas vacuoles disappeared.
With organic compounds other than lactate, growth and sulfide formation from sulfate were compared to blanks without added substrate. Best growth was obtained on lactate and propionate, but the strain was also able to grow on ethanol, propanol and on hydrogen with 2 mM acetate as C-source. Growth was also observed on pyruvate, but no sulfide was detected. No grow was observed on hydrogen without acetate, formate, acetate, butyrate, malate, fumarate, isopropanol, butanol, benzoate, 2,4,6-trimetoxybenzoate, glucose, or alanine. In the presence of propionate, growth was observed on sulfate, thiosulfate and sulfite. Strain ltk10 did not grow on elemental sulfur, fumarate, or nitrate. Nitrate was also tested in medium without sulfide, as sulfide can inhibit nitrate reduction (DALSGAARD & BAK 1994), but no growth was observed on this medium. Disproportionation on thiosulfate or on elemental sulfur with Fe^{2+} present did not support growth.
Tests for growth factor requirements revealed that of the nine vitamins originally added, the strain only required pyridoxamine dihydrogenchlorid, nicotinate, and 4-aminobenzoate.
Stock cultures of the sulfate reducers were kept at 2-5°C and transferred every month.

G+C content and pigments. Determination of the DNA base ratio of strain ltk10 yielded a content of 48.4 ± 0.3 mol % guanine + cytosine.

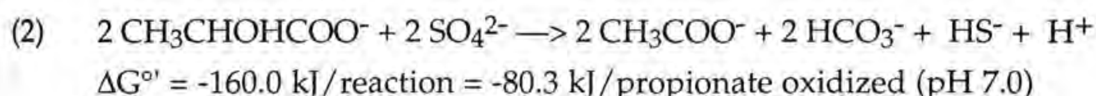
No Desulfoviridin was present.

Stoichiometry of propionate and lactate oxidation. When grown on propionate, strain ltk10 excreted acetate which could not be oxidized further. The results are listed in Table 1. To estimate the amount of propionate incorporated into biomass, it was assumed that propionate was assimilated together with CO_2 as described in the equation in Table 1. The molar ratios of propionate dissimilated, and sulfide and acetate excreted are in good agreement with the following equation:



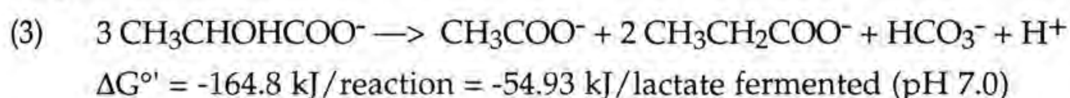
(1) $4\ CH_3CH_2COO^- + 3\ SO_4^{2-} \longrightarrow 4\ CH_3COO^- + 4\ HCO_3^- + 3\ HS^- + H^+$
$\Delta G^{\circ\prime} = -152.2$ kJ/reaction = -38.1 kJ/propionate oxidized (pH 7.0)

Strain ltk10 excreted acetate when grown on lactate. The molar ratios of lactate oxidized by sulfate reduction, and sulfide and acetate excreted are in good agreement (Table 2) with the incomplete oxidation of lactate to acetate:



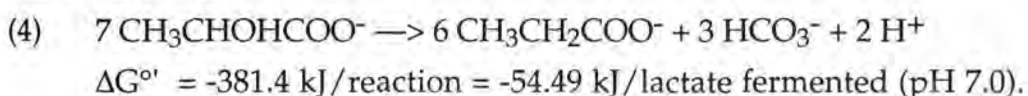
(2) $2\ CH_3CHOHCOO^- + 2\ SO_4^{2-} \longrightarrow 2\ CH_3COO^- + 2\ HCO_3^- + HS^- + H^+$
$\Delta G^{\circ\prime} = -160.0$ kJ/reaction = -80.3 kJ/propionate oxidized (pH 7.0)

In the late exponential growth phase, strain ltk10 also excreted small amounts of propionate. Acetate and Propionate are most likely produced by fermentation of lactate via a randomizing succinate pathway, as described for *Desulfobulbus propionicus* (STAMS ET AL. 1984). Two mol propionate, one mol acetate and CO_2 are formed from three mol lactate:



(3) $3\ CH_3CHOHCOO^- \longrightarrow CH_3COO^- + 2\ CH_3CH_2COO^- + HCO_3^- + H^+$
$\Delta G^{\circ\prime} = -164.8$ kJ/reaction = -54.93 kJ/lactate fermented (pH 7.0)

Since the enzyme activities of the succinate pathway remain high even in the presence of sulfate (STAMS ET AL. 1984), this fermentative pathway could still operate in the presence of sulfate, which is in agreement with the fermentative utilization of pyruvate by strain ltk10 in the presence of sulfate. It would be interesting to compare the contributions of sulfate-reducing and fermentative lactate utilization pathways under different sulfate concentrations.
Propionate can also be formed by fermentation of lactate to propionate and CO_2, with almost identical kj/lactate as in the fermentation of lactate to propionate, acetate and CO_2:



(4) $7\ CH_3CHOHCOO^- \longrightarrow 6\ CH_3CH_2COO^- + 3\ HCO_3^- + 2\ H^+$
$\Delta G^{\circ\prime} = -381.4$ kJ/reaction = -54.49 kJ/lactate fermented (pH 7.0).

All the $\Delta G^{\circ\prime}$ values were calculated from data of THAUER ET AL. (1977).

The stoichiometric amounts of lactate used (and acetate excreted) to form 0.73 mmol/l of propionate are shown as "fermentation" and the amounts are subtracted in the numbers presented in "respiration" in Table 2. The stoichiometry is based on equation (3). To estimate the amount of lactate incorporated into biomass, it was assumed that lactate was assimilated as described in the equation in Table 2.

The growth yield of strain ltk10 was found to be 3.86 g dry weight per mol propionate oxidized, similar to *Desulfobulbus propionicus* strain 1 pr 3, whose growth yield on propionate oxidation was found to be from 4.3 to 5.5 (WIDDEL & PFENNIG 1982). In a study of temperature influence on growth, respiration, and growth yield, Strain ltk10 was shown to have highest growth yield at temperatures below 12°C (ISAKSEN & JØRGENSEN, unpublished results). At 15°C, the growth yield was about 80% of maximal yield at 12°C. This can explain why the growth yield of strain ltk10 is lower than the yields of *D. propionicus*.

The growth yield of Strain ltk10 on lactate was higher than on propionate which primary was due to the difference in free energy from oxidation of lactate and propionate. *Desulfovibrio vulgaris* had a growth yield on 6.0 - 7.8 g cell weight per mole lactate assimilated (MAGEE ET AL. 1978; TRAORE ET AL. 1981), which is comparable with the growth yield found for Strain ltk10, whereas *Desulfovibrio gigas*, *Desulfovibrio desulfuricans*, and *Desulfovibrio africanus* was found to have rather low growth yield on 1.7 - 4.1 g dry weight per mole lactate assimilated (MAGEE ET AL. 1978; TRAORE ET AL. 1982). The low values found for the three last species could be due to nonoptimal growth conditions.

16S rRNA sequence analysis. Strain ltk10 is a member of the delta subdivision of the proteobacteria (Fig.3A), and is phylogenetically affiliated to *Desulfobulbus propionicus* and *D. marinus*. These two *Desulfobulbus* species are separated from each other by a 16S rRNA sequence distance of 0.086 (Jukes-Cantor distance: mutations per sequence position), but show distances in the range of 0.13 to strain ltk10.

Molecular isolates from Florida marine sandy sediments and from Mariagerfjord (Denmark) were also found to be related to *Desulfobulbus* and *Desulforhopalus vacuolatus*. Due to the shorter sequence stretches for the molecular isolates, a separate analysis and phylogenetic tree is given in Fig. 3B.

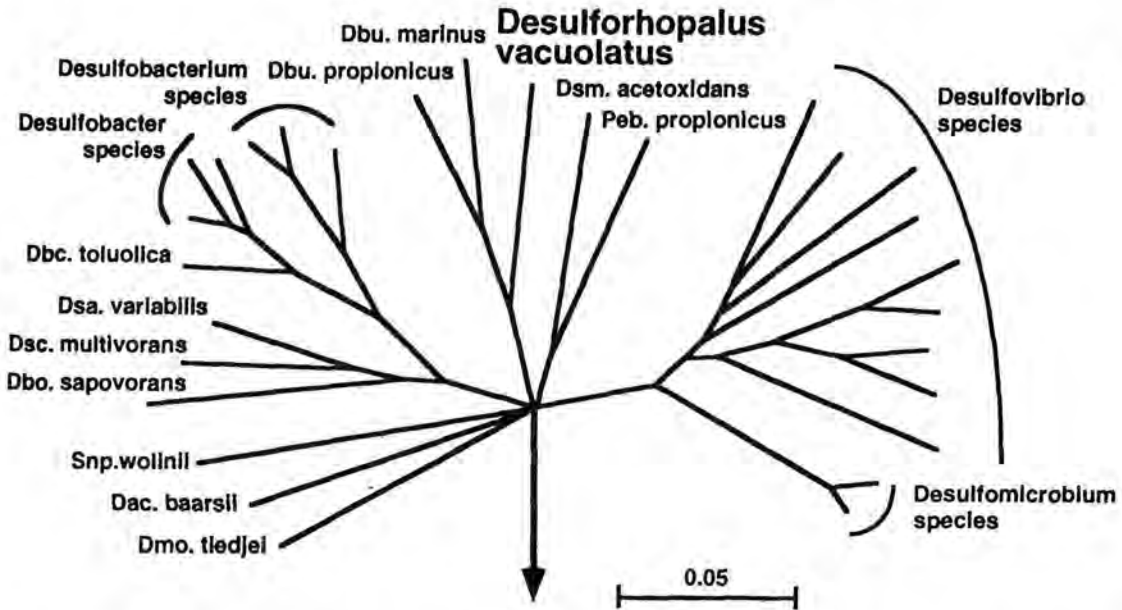
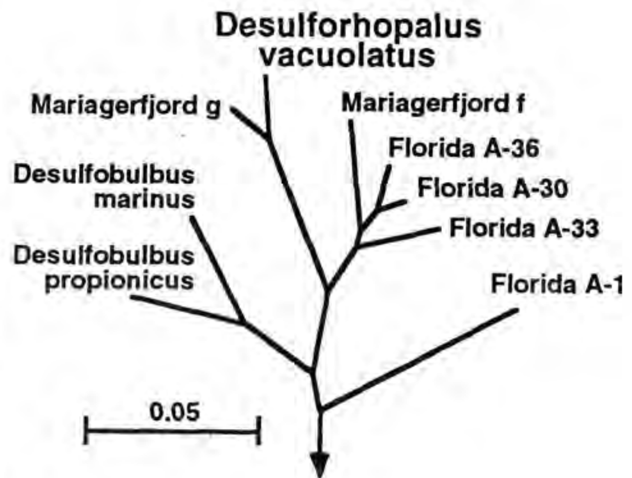


Fig. 3: 16S rRNA distance trees rooted with *Desulfotomaculum nigrificans* as outgroup. The scale bar corresponds to 0.05 mutations per nucleotide position. Sequences used in preparing the figures were derived from Genbank or from the Ribosomal Database Project (RDP) (MAIDAK ET AL. 1994).

A: The delta subdivision sulfate reducing bacteria, including *Desulforhopalus vacuolatus* strain ltk10, based on near-complete 16S rRNA sequences. Abbreviations: Dsv. = *Desulfovibrio*, Dsmi. = *Desulfomicrobium*, Dbu. = *Desulfobulbus*, Dbo. = *Desulfobotulus*, Dsc. = *Desulfococcus*, Dsa. = *Desulfosarcina*, Dbc.= *Desulfobacula*, Dbm. = *Desulfobacterium*, Dmo. = *Desulfomonile*, Dac. = *Desulfoarculus*, Dsm. = *Desulfuromonas*.

The 16S rRNA sequence of *Desulforhopalus vacuolatus* has Genbank accession number L42613.

B: *Desulforhopalus vacuolatus* strain ltk10, *Desulfobulbus propionicus*, *Desulfobulbus marinus*, and related molecular isolates A-1, A-30, A-33, A-36 from Florida marine sediments (DEVEREUX & MUNDFROM 1994), MPN isolates f and g from the water column of Mariager Fjord, Denmark (TESKE ET AL. 1995c). Genbank accession numbers: U08385 (A-1), U08387 (A-30), U08388 (A-33), U08391 (A-36), L40785 (f), L40786 (g). The tree is based on 16S rRNA nucleotide positions 384-896 (E.coli numbering).



DISCUSSION

Environment and temperature. Incompletely propionate oxidizing sulfate reducers related to *Desulfobulbus* are metabolic generalists, capable of growth by sulfate reduction and by fermentation, and catalyze the anaerobic turnover of propionate (and lactate) in marine environments (PARKES ET AL. 1993). Sulfate reducing bacteria of the genera *Desulfobulbus* and *Desulforhopalus* are widespread in marine sediments and in the marine water column, and were found as molecular isolates, or by Most-Probable-Number dilution series (DEVEREUX & MUNDFROM 1994, TESKE ET AL. 1995c). It seems surprising that only three *Desulfobulbus* species and one *D. propionicus*-related isolate have to our knowledge been described (TASAKI ET AL. 1990, WIDDEL & BAK 1991). The published *Desulfobulbus* species are not likely to be active at cool temperatures: *Desulfobulbus elongatus* did not grow below 20°C (SAMAIN ET AL. 1984) and *D. propionicus* and *D. marinus* had temperature minimum at 10°C (WIDDEL & PFENNIG 1982). These published species can therefore not be responsible for the incomplete oxidation of propionate in marine ecosystems, as the temperature in the majority of the marine environment is below 5°C through out the year (HERBERT 1986) and the rest is cold in the winter period.
Strain ltk10, isolated from Kysing Fjord, a shallow but permanently water covered estuarium, was able to grow at temperatures down to 0°C. It had a lower temperature optimum than the incompletely propionate oxidizing species described until now, combined with short-term tolerance against elevated temperatures. The seasonal temperature variations at this locality encompassed 0°C to 20°C, which is in the temperature range for growth of strain ltk10. Further more was strain ltk10 able to survive at temperatures above 25°C for 24 hours and thereby survive a warm sunny summer day where the water and sediment in the shallow estuarium can be further heated up.

Gas vacuoles. Bacteria from many different genera form gas vacuoles (reviewed in WALSBY 1994). The function for planktonic phototrophic bacteria is probably to maintain the buoyancy of cells and thereby ensure that they are placed optimally in the water column where light and nutrient are available. It is remarkable, however, that some strict anaerobic bacteria possess gas vacuoles (e.g. ZHILINA & ZAVARZIN 1987). Gas vacuoles on endospores can have a spreading function as the vacuoles can increase the possibility for transport out in the water phase, since spores are not harmed by oxygen. The function of gas vacuoles in vegetative cells is, however, less obvious. The gas vacuoles of strain ltk10 did apparently not have any effect on the buoyancy of the cells, as the cells were

evenly distributed in the medium bottle in exponential growth phase. At stationary growth phase, however, the cells formed clumps at the surface, which could result from the formation of longer cells in the stationary phase.
Interestingly, high numbers of bacteria with gas vacuoles were found in sea ice and in the water column of Antarctica (STALEY ET AL. 1989). A possible function of gas vacuoles in these cold environments could be to increase the cell surface without increasing the cell volume. This would increase transport of solutes over the cell membrane which could be an adaptation to low enzymatic activity at low temperature. Strain ltk10 was not isolated from Antarctica but from a temperate estuarium, but the temperature in these environments are below 10-15°C for most of the year. The ability to form gas vacuoles could also be an adaptation to low temperatures in this environment.
Strikingly, vacuolated types were the most abundant sulfate reducing bacteria in the surface layer (0-2 cm) of Kattegat sediment (JØRGENSEN & BAK 1991). Vacuolated sulfate reducers were isolated either from the highest dilutions of Most-Probable-Number counts, both with acetate and with hydrogen as electron acceptors, or from psychrophilic enrichments, when low incubation temperatures inhibited the growth of mesophilic competitors. Cell numbers were highest in the oxic surface layer (0-1 cm) and in the suboxic chemocline layer (1-2 cm), reaching 2 x 10^6 cells per ml. The low in-situ temperatures (5.5°C) within the sediment, possibly also the prevalence of oxic and suboxic conditions, could select for vacuolated types of sulfate reducing bacteria. Since *Desulforhopalus vacuolatus* was isolated from a similar marine environment, the shallow Kattegat tributary Kysing Fjord, it is a likely member of the psychrophilic vacuolated sulfate reducers which dominate sulfate reducing bacterial populations of Kattegat sediment surfaces.

Phylogenetic position of strain ltk10. By 16S rRNA analysis, strain ltk10 is a member of the delta subdivision of the proteobacteria, and is affiliated, although not closely, to the genus *Desulfobulbus*, represented by the 16S rRNA sequences of *Desulfobulbus propionicus* and *Desulfobulbus marinus* (Fig. 3A). Strain ltk10 is separated from *D. propionicus* and *D. marinus* by a 16S rRNA Junkes-Cantor distance of approx. 0.13, which is higher than the 16S rRNA distances within the genus *Desulfobulbus*, between *D. propionicus* and *D. marinus*. This supports to establish strain ltk10 as a new species and genus, *Desulforhopalus vacuolatus*, separately from *Desulfobulbus*. Within the delta-subdivision sulfate-reducing bacteria, 16S rRNA sequence distance in the range 0.10 to 0.15 have been discussed as upper limits for genus range (DEVEREUX ET AL. 1990).

Desulforhopalus vacuolatus is certainly not the only species of the genus. The 16S rRNA molecular isolates A-30, A-33, and A-36, obtained from sandy marine sediment in Florida (DEVEREUX & MUNDFROM 1994), form a monophyletic group together with *Desulforhopalus vacuolatus*, separate from *Desulfobulbus propionicus* and *Desulfobulbus marinus*. A 16S rRNA sequence obtained from Most-Probable-Number dilution cultures of the oxic layer of the stratified water column of Mariager Fjord, Denmark (Isolate f, 8 m depth, dilution 10^0 cells cm^{-3}), is closely affiliated to *Desulforhopalus vacuolatus*. A second sequence from the same source (Isolate g, 13 m depth, dilution 10^1 cells cm^{-3}) clusters with the Florida sediment molecular isolates (Fig 3B) (TESKE ET AL. 1995c). These findings indicate abundant, undescribed *Desulforhopalus* and *Desulfobulbus* strains and species in nature. The molecular isolates from Florida and Mariager-fjord, and *Desulforhopalus vacuolatus* do not share the 16S rRNA sequence motif CAGAGGGGAAAGUGGAAUUC (E.coli numbering 660-679), the target sequence of a *Desulfobulbus*-directed hybridization probe (DEVEREUX ET AL. 1992). This probe has been designed on the basis of the previously published 16S rRNA sequences of *D. propionicus* and *D. marinus*. A more complete collection of *Desulforhopalus* and *Desulfobulbus* species and 16S rRNA sequences will be required to provide a broader basis for probe design, and to recognize the actual ecophysiological diversity and phylogenetic depth of these genera.

Taxonomy. Strain ltk10 is a gram negative, non-spore forming sulfate reducing bacterium with respiratory/fermentative metabolism. It oxidizes propionate and other fermentation products incompletely to acetate and CO_2, a trait shared with the genus *Desulfobulbus*.

Contrary to *Desulfobulbus* species, strain ltk10 is psychrotrophic, able to grow at 0°C but not above 24°C, and differs morphologically by having large cells with gas vesicles and by forming long cells in stationary growth phase. Further more, strain ltk10 requires NaCl and two more vitamins than *Desulfobulbus propionicus*, the type species of *Desulfobulbus*. Genomic differences separate *Desulfobulbus* species and strain ltk10: The DNA base ratio of strain ltk10, 48.4±0.3 mol % G+C, is clearly lower than the base ratio of *D. propionicus* (60 mol % GC) and *Desulfobulbus elongatus* (59 mol % GC) (SAMAIN ET AL. 1984; WIDDEL & BAK 1991). The 16S rRNA sequence of ltk10 differs considerably from those of *Desulfobulbus* species (approx. 0.13 substitutions per site).

Considering these differences (Table 3), it appears justified to establish a new genus for the propionate-oxidizing, psychrotrophic, gas vacuolated sulfate reducers with strain ltk10 as type strain. Due to the morphology of the strain, we propose the name *Desulforhopalus vacuolatus* for the new genus and strain.

Genus *Desulforhopalus* gen. nov.

De.sul.fo.rho.pa.lus. L.pref. de from; L.n. sulfo sulfur; L.n. rhopalus cudgel; M. L. masc. n. *Desulforhopalus* cudgel-formed sulfate reducer.

Oval shaped, variable length, vacuolated, single or in chain. Nonmotile with no flagella. Gram negative.

Anaerobic chemoorganotroph, metabolism respiratory and fermentative. Sulfate and other sulfur compounds serve as electron acceptors and are reduced to sulfide. Propionate, lactate and alcohols are used as electron donors and carbon sources. Acetate is formed as end-product of incomplete oxidation.

The G + C content of the type species is 48.4 ± 0.3 mol %.

The type species is *Desulforhopalus vacuolatus* .

Desulforhopalus vacuolatus sp.nov.

va.cu.o.la'tus vacuolated due to the vacuolated morphology of the cells.

Rod-shaped with gas vacuoles, 3.0 - 5.0 by 1.5 - 1.8 µm with rounded ends. In stationary growth phase longer cells formed. Immotile. No spore formation. Gram negative.

Anaerobic chemoorganotroph. Propionate and other alcohols used as electron donors and carbon sources. Acetate is formed as end-product of incomplete oxidation. Sulfate, sulfite and thiosulfate serve as electron acceptor and are reduced to H_2S; elemental sulfur, nitrate, fumarate and oxygen are not utilized. Growth requires mineral medium with sulfide as reductant and not less than about 5 g NaCl and 1g $MgCl_2 \cdot 6\ H_2O$ per liter. Pyridoxamine dihydrogenchlorid, nicotinate, and 4-aminobenzoate are required as growth factors. Selective enrichment with thiosulfate and lactate or propionate at higher NaCl and $MgCl_2$ concentration and at temperature below 10°C.

pH-range: 5.7 - 8.0, optimum at 6.8 - 7.2. Temperature range: 0° - 24°C, optimum 18° C - 19°C. Desulfoviridin not present.

The G + C content of the type species is 48.4 ± 0.3 mol % (as determined by HPLC).

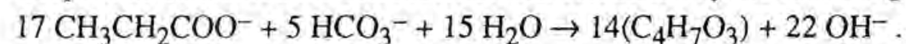
The type strain is DSM 9700 (= ltk10), deposited in the Deutsche Sammlung von Mikroorganismen und Zellkulturen, Braunschweig, Germany.

Acknowledgements. Financial support was provided by the Danish Natural Science Research Council (MFI) and by the Max Planck Society. We thank Jan Küver for critically reading and improving the manuscript.

Table 1. Results of stoichiometric measurements with ***Desulforhopalus vacuolatus*** strain LTK10 on propionate and sulfate as sole electron donor and acceptor, respectively. The data represent the difference between the amounts obtained in the start and at the end of the experiment.

Propionate utilized (mmol/l)	Acetate excreted (mmol/l)	H_2S formed (mmol/l)	Cell dry weight formed (mg/l)	Propionate consumed for cell material[1)] (mmol/l)	Propionate oxidized by sulfate reduction (mmol/l)	Molar ratio of propionate oxidation $\frac{\text{Acetate} / H_2S}{\text{propionate}}$	Growth yield: g dry weight per mol propionate oxidized
11.81	11.29	8.48	45.62	0.54	11.27	(1.00 / 0.75) / 1	3.86

1) Propionate consumed for cell material was calculated by the following equation:



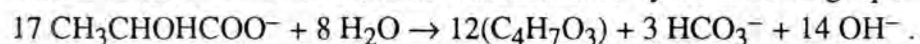
$17\ CH_3CH_2COO^- + 5\ HCO_3^- + 15\ H_2O \rightarrow 14(C_4H_7O_3) + 22\ OH^-$.

Thus, 0.0118 mmol propionate are required for 1.0 mg of cell dry weight.

Table 2. Results of stoichiometric measurements with *Desulforhopalus vacuolatus* strain LTK10 on lactate and sulfate as sole electron donor and acceptor, respectively. The data represent the difference between the amounts obtained in the beginning and at the end of the experiment.

	Lactate utilized (mmol/l)	Acetate excreted (mmol/l)	Propionate excreted (mmol/l)	H_2S formed (mmol/l)	Cell dry weight formed (mg/l)	lactate consumed for cell material[1] (mmol/l)	lactate oxidized by sulfate reduction (mmol/l)	Molar ratio of lactate oxidation $\frac{\text{Acetate} / H_2S}{\text{lactate}}$	Growth yield: g dry weight per mol lactate oxidized
Measured reactants	21.57	18.94	0.73	9.38	153.30	2.12			
respiration[2]	21.57	18.94	ignored	9.38	153.30	2.12	19.45	(0.97 / 0.48) / 1	7.11
fermentation[2]	1.10	0.37	0.73						
and respiration	20.47	18.58		9.38	153.30	2.12	18.35	(1.01 / 0.51) / 1	7.49

[1] Lactate consumed for cell material was calculated by the following equation:



$$17\ CH_3CHOHCOO^- + 8\ H_2O \rightarrow 12(C_4H_7O_3) + 3\ HCO_3^- + 14\ OH^-.$$

Thus, 0.0138 mmol lactate are required for 1.0 mg of cell dry weight.

[2] See the text for explanation.

Table 3. Morphological, physiological, and phylogenetic characteristics of *Desulforhopalus vacuolatus* strain LTK10 and three *Desulfobulbus* species.

Characteristics	*Desulforhopalus vacuolatus* Strain LTK10	*Desulfobulbus propionicus* Strain 1 pr 3 [1)]	*Desulfobulbus marinus* Strain 3 pr 10 [1)]	*Desulfobulbus elongatus* [2)]
Width x length (µm)	1.5 - 1.8 x 3.0 - 5.0	1.0 - 1.3 x 1.8 - 2.0	1.0 - 1.3 x 1.8 - 2.0	0.6 - 0.7 x 1.5 - 2.5
Flagellation	no flagellum, nonmotile	no flagellum, nonmotile	single polar, motile	single polar, motile
Vacuoles	yes	no	no	no
Gram stain	negative	negative	negative	negative
Growth factor requirement	4-aminobenzoic acid, pyridoxamine-2HCl, nicotinate	4-aminobenzoic acid	4-aminobenzoic acid	4-aminobenzonic acid
Growth temperature range (°C)	0 - 24	10 - 43	15 - 36	20 - 40
Temperature optimum (°C)	18 - 19	39	29	35
pH range of growth	5.7 - 8.0	6.0 - 8.6	6.6 - 8.1	6.0 - 7.8
pH optimum	6.8 - 7.2	7.1 - 7.5	7.4	7.0
salt requirement	NaCl: 5 g/l	no	NaCl: 15 g/l	no
Compounds tested as electron donors and carbon sources:				
$H_2 + CO_2$ + acetate	+	+	+	+
Formate + acetate	–	–	+	–
Acetate	–	–	–	–
Propionate	+	+	+	+
Butyrate	–	(+)	–	–
Ethanol	+	+	+	+
Propanol	+	+	+	+
Pyruvate	+	+	+	+
Lactate	+	+	+	+
Compounds tested and used as electron acceptors:	sulfate, thiosulfate, sulfite	sulfate, thiosulfate, sulfite, nitrate	n.d.	sulfate, thiosulfate, sulfite
Sulfur disproportionation	no	yes	n.d.	
GC mol %	48.4	59.9	n.d.	59.0

[1] Published in [WIDDEL, 1982]

[2] Published in [SAMAIN, 1984]

4.6. Phylogeny of *Thioploca* and related filamentous sulfide-oxidizing bacteria

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ABSTRACT

The phylogenetic relationships of three *Thioploca* species, *T. araucae*, *T. chileae* and *T. ingrica*, as well as *Beggiatoa alba*, *Beggiatoa* sp. str 1401-13, and *Thiothrix nivea* were determined by 16S rRNA sequence analysis. Fluorescent *in situ* hybridization with *Thioploca*-specific oligonucleotide probes was used to substantiate the sequences of *T. araucae* and *T. chileae*. All three *Thioploca* species form a monophyletic group. They are affiliated with *Beggiatoa alba* and *Beggiatoa* sp. str 1401-13. *Thioploca* and *Beggiatoa* constitute a new phylogenetic lineage within the gamma-subdivision of the proteobacteria, showing similar genetic diversity as other major groups of gamma- or beta-subdivision sulfide oxidizers. *Thiothrix nivea* is a member of the gamma proteobacteria, but does not form a monophyletic lineage with *Beggiatoa* and *Thioploca* .

INTRODUCTION

Thioploca, Beggiatoa, and *Thiothrix* are viewed as a group of morphologically and physiologically related bacteria, the filamentous sulfide-oxidizing bacteria, and are placed into the family *Beggiatoaceae* (FJERDINGSTAD 1979, LARKIN & STROHL 1983, STROHL 1989). They share morphological and ecophysiological features, such as the formation of long, motile, gliding filaments, the deposition of internal sulfur granules within each cell, and the occurrence in and on marine and freshwater sediments.

Of *Beggiatoa, Thiothrix* and *Thioploca*, the latter forms the largest known bacterial mats in nature (GALLARDO 1977), but, in contrast to *Beggiatoa* and *Thiothrix*, has not been grown in pure culture. The lack of pure cultures has impeded physiological studies of *Thioploca* and has allowed only experiments with natural, freshly collected *Thioploca* material. These experiments indicate that *Thioploca* is a mixotrophic sulfide oxidizer (MAIER & GALLARDO 1984a). The differentiation of *Thioploca* from other filamentous sulfur-oxidizing bacteria relies on morphological features, i.e. *Thioploca* filaments aggregate in bundles, surrounded by a polysaccharide sheath. A single *Thioploca* filament can not be reliably differentiated from a *Beggiatoa* filament by light microscopy, which was noted in the original description of the genus *Thioploca* (LAUTERBORN 1907).

Morphological taxon definitions are also problematic with *Thioploca* at the species level. *Thioploca* species are defined by filament diameter, which form distinct, non-overlapping size classes (MAIER & GALLARDO 1984b, STROHL 1989). Ultrastructure comparisons have provided additional criteria for

Thioploca species discrimination (MAIER & MURRAY 1965, MAIER ET AL. 1990). On the other hand, the validity of *Thioploca* species has been questioned, because *Thioploca* filaments of different diameters may be observed in the same bundle. Such *Thioploca* bundles have been observed in marine (MAIER & GALLARDO 1984b) and in freshwater environments (KOPPE 1924), and have been discussed as a new species "*Thioploca mixta*" (KOPPE 1924). On the basis of morphological similarities, two mutually exclusive phylogenetic affiliations of *Thioploca* have been suggested, a position close to *Beggiatoa* (LAUTERBORN 1907, STROHL 1989), and an affiliation with Cyanobacteria, especially *Oscillatoria* and *Microcoleus* (LAUTERBORN 1907, REICHENBACH & DWORKIN 1981, VISLOUCH 1912).

A phylogenetic framework, based on 16S rRNA sequences, was created to resolve these ambiguities in taxonomy and phylogeny of *Thioploca.* Partial to almost complete 16S rRNA sequences were determined for three *Thioploca* species: *T. araucae, T.chileae,* and *T. ingrica* (MAIER & GALLARDO 1984b, VISLOUCH 1912). The sequences of *T. araucae* and *T. chileae* were verified by *in situ* hybridization with fluorescent oligonucleotide probes. In addition, 16S rRNA sequences of *Beggiatoa alba* and *Beggiatoa* sp. str. 1401-13 were determined in this study (MEZZINO ET AL. 1984, PRINGSHEIM 1964). *Thiothrix nivea* was resequenced, since the previously published 16S rRNA sequence of *Thiothrix nivea* showed substantial gaps (LANE ET AL. 1992). We describe the relevance of this phylogenetic framework for the often-noted morphological and physiological similarities of *Beggiatoa, Thiothrix* and *Thioploca* and suggest an evolutionary scenario for this bacterial group.

MATERIALS AND METHODS

Bacteria and samples. *T. araucae* and *T. chileae* were collected from marine sediment sampled in the Bay of Concepcion, Chile, at 30 m depth and from the continental shelf off Concepcion at 100 m depth. The sampling was performed during March 1994 from RV Vidal Gormaz as a joint research project between the Max Planck Institute for marine Microbiology, Bremen, and the Centro-EULA, University of Concepcion, Chile. *T. araucae* and *T. chileae* were sorted according to filament diameters: *T. araucae* filaments are 30-43 µm in diameter, *T. chileae* 12-20 µm (MAIER & GALLARDO 1984b). The bundles were placed on agar plates (1% Bacto-Agar, made with artificial seawater) and examined under the microscope (Zeiss-Axioplan) for filament diameter and homogeneity. Bundles of mixed filaments occurred frequently and were discarded. *Thioploca ingrica* samples (filament diameter 3-4 µm) from Randersfjord, Denmark, were

generously provided by Dr. Lars Peter Nielsen, Aarhus University, Aarhus, Denmark. *Beggiatoa alba* strain B15LD (MEZZINO ET AL. 1984) and *Thiothrix nivea* type str. JP2 (LARKIN & SHINABARGER 1983) were obtained from the DSM, Braunschweig, Germany. *Beggiatoa* sp. strain 1401-13 (PRINGSHEIM 1964) was obtained from the culture collection of the Institute of Plant Physiology, University of Göttingen, Germany (SCHLÖSSER 1982).

Table 1: Bacteria	sampling site/strain
Thioploca araucae	Pacific continental shelf off Concepcion, Chile
Thioploca chileae	Pacific continental shelf off Concepcion, Chile
Thioploca ingrica	Randers Fjord, Denmark
Beggiatoa alba DSM1416	strain B15LD, Louisiana, USA (MEZZINO ET AL. 1984, STROHL & LARKIN 1978)
Beggiatoa sp. strain 1401-13	Göttingen, Germany (PRINGSHEIM 1964, SCHLÖSSER 1982)
Thiothrix nivea DSM 5205^T	strain JP2, Louisiana, USA (LARKIN & SHINABURGER 1983)

DNA extraction from *Thioploca*. *Thioploca* bundles were rinsed in sterile artificial seawater. The bundles were then repeatedly pulled through 1% Bacto-Agar to remove adherent mud particles and bacteria (PRUFERT-BEBOUT & GARCIA-PICHEL 1994). The bundles were checked microscopically for contaminations. Approx. 50-100 µl cell material of *T.araucae* and *T. chileae* were collected for DNA extraction. Smaller volumes, 10-20 µl, were used for *T. ingrica*. Nucleic acids were isolated from *Thioploca* by the use of cetyl-trimethylammonium-bromide (CTAB), a polysaccharide-complexing reagent (WILSON 1990), to overcome problems with polysaccharide contamination. After 30 min proteinase K/SDS digest, CTAB was added. CTAB-polysaccharide complexes, proteins and lipids were removed in the subsequent phenol extraction. The phenol extraction was repeated until no white interphase material was visible. The purified nucleic acids were precipitated with ethanol or isopropanol. This method was preferred to previous standard procedures (SAMBROOK ET AL. 1989), which had yielded no PCR-amplifyable DNA from *T. araucae* and *T. chileae* : DNA might have bound to an unknown component and was either removed with the phenol extraction or inactivated in some other way. Instead of DNA, the standard phenol extraction protocol had yielded a white, fluffy material in the ethanol precipitations after phenol extractions. The sheath polysaccharides were suspected to be the main component of this contaminating material. Similar problems are known in connection with sheaths of *Geotoga* and *Petrotoga* species (DAVEY ET AL. 1993). However, even DNA isolated by the CTAB technique allowed only partial PCR amplification (positions 358-949) of the

16S rRNA genes of *T. araucae* and *T. chileae*. Full length amplification was not achieved for these species.
T. ingrica yielded PCR-amplifiable DNA and the complete 16S-rRNA gene could be sequenced. This can be attributed to the smaller size of *T. ingrica* cells, filaments and bundles, and consequently more 16S rRNA genes per volume of cell material. *T. araucae* and *T. chileae* may have extremely unfavorable ratios of DNA to cell-or sheath volume. The volume of a single *Thioploca araucae* cell, approx. 25000 um^3, exceeds typical bacterial cell volumes by approx. 5 orders of magnitude.
For *Beggiatoa* and *Thiothrix* cultures, DNA was isolated by ProteinaseK/SDS digest, followed by conventional phenol extraction and ethanol precipitation (SAMBROOK ET AL. 1989).

PCR amplification and sequencing. 16S rRNA sequences were determined by direct sequencing of PCR-amplified 16S rRNA genes. 16S rRNA genes were amplified with primers GM-3-forward (5'-AGAGTTTGATC(A/C)TGGC-3', E.coli positions 8-23), and GM-4-reverse (5'-TACCTTGTTACGACTT-3', positions 1492-1507). For partial amplification of the 16S rRNA gene of *T. araucae* and *T. chileae*, the primers GM-5-forward (5'-CCTACGGGAGGCAGCAG-3' , positions 341-357) and GM-10-reverse (5'-TCGAATTAAACCACA-3', positions 950-964) were used.
Each of the 30 PCR cycles started with 1 minute denaturation at 95°C, followed by 2 minutes annealing at 40°C and 3 minutes elongation at 71°C. The reaction mixture (total volume 50 µl) contained 50 mM KCl, 10 mM Tris-HCl pH 8.3, 1.5 mM $MgCl_2$, 0.01% (wt/vol) gelatin, 200 µM of each deoxynucleotide (dGTP, dATP, dTTP, and dCTP), 0.5-1.0 Units Taq DNA Polymerase (SuperTaq, HT Biotechnology limited), and 0.5 µM of each primer. The PCR-products were analyzed by electrophoresis on 1% horizontal agarose gels in TAE buffer using a defined doublestranded 16S-PCR copy of 1.5 kb (Desulfobulbus sp.) as size marker. The PCR product was gel-purified as follows: A small well was cut into the agarose gel in front of the selected PCR-product. Electrophoresis was continued until the PCR product migrated into the buffer-filled well, from where it was taken up with a pipette and transferred into an Eppendorf vial. After precipitation for one hour at -80°C with 0.1 x volume 5M NaCl and 2.5 x volumes ethanol, the PCR product was pelleted by centrifugation and redissolved in 50 µl distilled water.

PCR products were sequenced directly: 10-100 ng purified PCR-product were mixed with 2 µl sequencing buffer (200 mM Tris-HCl pH 7.5, 100 mM $MgCl_2$, 250 mM NaCl), 1 µl 5% Nonidet P-40 (Sigma) aqueous solution, 2 pmol primer, and adjusted with distilled water to a final volume of 10 µl. The doublestranded PCR-

product was denatured by heating the 10 µl volume to 95°C for 5 min twice, with a short centrifugation in between. The sequencing reaction was started by adding 1.0 µl 0.1 M DTT, 2.0 µl dNTP-solution (200 nM dGTP, dATP, dTTP), 0.5 µl (alpha-^{33}P)-dCTP; (10 µCi/µl; 3000 Ci/mmol), 2 µl 1 U Sequenase 2.0 (USB). After a short centrifugation to collect and mix the sample volume (15.5 µl), the sample was incubated for 5 minutes at 37°C.
The sample volume was then divided into four 3.5 µl portions and added to dideoxynucleotide termination solutions (2.5 µl, containing 80 µM of each dGTP, dATP, cTTP, dCTP, and 8 µM dideoxynucleotide). After 5 min incubation at 37°C the reaction was stopped by adding 4 µl of a solution of 96%-Formamide and 20 mM EDTA.

Alignment and phylogenetic tree inference. The sequences were aligned by secondary structure according to the RDP database alignment (MAIDAK ET AL. 1994). The SIMILARITY_RANK tool of the RDP database was used to search the RDP database for close evolutionary relatives of *Thioploca*, *Beggiatoa* and *Thiothrix*. Additional 16S rRNA sequences to those determined in this study were obtained from the RDP database (MAIDAK ET AL. 1994). Sequence alignments were viewed and edited in the sequence alignment window SeqApp (Version 1.9a169) (GILBERT 1992). Phylogenetic trees were inferred with the programs DNADIST and FITCH as included in Felsensteins PHYLIP program package Version 3.5c (FELSENSTEIN 1989). DNADIST calculates a matrix of evolutionary distances from nucleotide sequence mismatches, based on the Jukes-Cantor model for independent nucleotide changes at all sites with equal probability. FITCH fits the phylogenetic tree to distance matrices by the Fitch-Margoliash least-square method. This phylogenetic tree was derived from analysis of the sequence positions 358 - 949, due to the limitations of the *T. araucae* and *T. chileae* sequences. The tree was checked by bootstrap resampling (100x) using SEQBOOT, a general bootstrapping tool included in PHYLIP 3.5c. The bootstrap values obtained from resampling sequence positions 358 - 949 were compared to bootstrap values from analysis of near-complete 16S rRNA sequences (*T. araucae* and *T. chileae* excluded), to evaluate the stability of the tree topology. Relevant branchings of the phylogenetic tree (Fig. 3) are annotated with both bootstrap numbers. Shifting branches and alternative phylogenetic positions for the deep-branching gamma-proteobacteria *Thiothrix nivea* and *Thiobacillus ferrooxidans* strain m-1, and corresponding bootstrap results, are discussed in the text.

Genbank accession numbers. The 16S rRNA sequences of *Thioploca ingrica, Thioploca chileae* and *Thioploca araucae* are available from Genbank under accession Nos. L40998, L40999 and L41043. The 16S rRNA sequences of *Beggiatoa alba* and *Beggiatoa* sp. strain 1401-13 are accessible under Nos. L40994 and L40997. The resequenced 16S rRNA sequence of *Thiothrix nivea* is available under accession number L40993.

Oligonucleotide design. 16S rRNA fluorescent oligonucleotide probes were developed to confirm, by fluorescent *in situ* hybridization, the sequences obtained from agar-cleaned environmental samples of *T. araucae* and *T. chileae*. Probe 829 was designed for 16S rRNA positions 829-849, a sequence motif shared by *T. araucae* and *T. chileae*. (Table 2). In addition, probe 462 was designed for positions 462-484 of *T. araucae* (Table 2). The CHECK_PROBE program of the RDP database (MAIDAK ET AL. 1994) was used to check the RDP database for specificity of the target sequences. The sequence signatures were not found in any of approx. 2000 16S rRNA sequences of the RDP database (updated June 19th 1994), even if two mismatches were allowed. Probe 463 was targeted at a sequence motif specific for *Beggiatoa alba*, and was used as negative control for *Thioploca*.

Table 2: rRNA-targeted probes used in this study

16S rRNA Probe	Position	Probe Sequence	
829-Thioploca	829-849	5'-GGATTAATTTCCCCCAACATC-3'	
462-Thioploca	462-484	5'-CGTCAAGACTTTAGAGTATTATC-3'	
463-Beg.alba	463-484	5'-CGTCAATTTCACATGGTATTAG-3'	
338-Eubacteria	338-355	5'-GCTGCCTCCCGTAGGAGT-3'	(AMANN ET AL. 1990)
385-delta-Proteobac.	385-402	5'-CGGCGT(T/C)GCTGCGTCAGG-3'	(AMANN ET AL. 1990)
ALF19b	19-35	5'-CGTTCG(T/C)TCTGAGCCAG-3'	(MANZ ET AL. 1992,
alpha-Proteobacteria			WAGNER ET AL. 1993)
23S rRNA Probe	**Position**	**Probe Sequence**	
BET42a	1027-1043	5'- GCCTTCCCACTTCGTTT-3'	(MANZ ET AL. 1992,
beta-Proteobacteria			WAGNER ET AL. 1993)
GAM42a	1027-1043	5'- GCCTTCCCACATCGTTT-3'	(MANZ ET AL. 1992,
gamma-Proteobacteria			WAGNER ET AL. 1993)

All probes labelled with Fluorescein-5-isothiocyanate (FITC) or tetramethylrhodamine isothiocyanate (TRITC) were purchased from Biometra GmbH, Göttingen, Germany. The labelled oligonucleotides were HPLC-purified and stored at -20°C before use.

Fluorescent *In Situ* Hybridization. Fresh samples of *T. araucae* and *T. chileae* were used for the hybridization experiments, which were performed at the Centro-EULA, University of Concepcion, Chile. Fixation, prehybridization, and hybridization were performed as described by RAMSING ET Al. (1993), except for the following modifications: 1 cm pieces of *Thioploca* bundles, fixed with 1.3 % paraformaldehyde, were laid out on gelatin-coated multi-well teflon slides, and consecutively dehydrated with 50%, 80% and 100% ethanol, prehybridized and hybridized with 20 µl per well of prehybridization mixture (5x SSC, 0.1% Triton-X, deionized Formamide 10-40%) and hybridization mixture, containing 5x SSC, 0.1% Triton-X, 0.5x Poly-A, 10x Denhardt, deionized Formamide 10-40% (SAMBROOK ET Al. 1989). Hybridization was continued for 3 hours at 37°C in a sealed Falcon tube, with a tissue paper placed under the multiwell slide, soaked with prehybridization solution to provide moisture and to prevent the hybridization mixture from drying out (RAMSING ET AL. 1993).
Hybridization conditions given in the literature for membrane hybridizations can often not be used for *in situ* hybridizations, due to the concentration dependence of the melting temperature of the template-oligonucleotide hybrid (RAMSING ET AL. 1993). Therefore test hybridizations with different stringencies (10%, 20%, 30% and 40% vol/vol Formamide) were performed to determine optimal conditions for *Thioploca* hybridization. The conditions were chosen as stringently as possible without significant reduction in signal intensity. The slides with hybridized *Thioploca* were mounted in Mowiol (Hoechst, Frankfurt, Germany) and examined through a Zeiss Axioplan microscope equipped with epifluorescence filtersets 0.1, 10 and 15 for UV excitation, blue excitation and green excitation respectively. Digital images were acquired with a high resolution Photometrics slow scan camera, and further analysed with NIH Image v. 1.56. The pictures in Fig. 1 were assembled using Photoshop 3.0 (Adobe).

RESULTS AND DISCUSSION

Fluorescent *In Situ* Hybridization. Fluorescent *in situ* hybridization of *Thioploca* is complicated by low fluorescent intensities for all probes used, as well as some background stain due to nonspecific binding of the probes to the polysaccharide sheaths. The low staining intensity is presumably caused by a low ribosome concentration in the large cell volume of *Thioploca*. The cells of *T. araucae* and *T. chileae* consist almost entirely of a liquid-filled vacuole, and the cytoplasm forms a thin film along the cell wall (MAIER & GALLARDO 1984). Ribosomes are confined to this cytoplasmic film and are thereby diluted over a large surface area (ca. 5000 μm^2 per cell for *T. araucae*, when cells are assumed as cylinders of 20 µm radius and 20 µm length). Consequently, fluorescence intensities of *Thioploca* cells are lower than those of normal-sized bacteria with surface areas in the range of a few μm^2. *Thioploca* bundles gave a more intense fluorescence signal than individual filaments, due to amplification of fluorescent light by overlaying filaments within a bundle. This effect is shown in Fig 1b, where a *T. chileae* bundle disintegrates partially into individual filaments. Fluorescence signals of individual *Thioploca* filaments were low and often only detectable by direct comparison to filaments hybridized with a negative probe.

The phylogenetic position of *Thioploca* was delineated in three hybridization experiments where we applied 16S rRNA probe pairs of stepwise narrowing phylogenetic range. In each experiment, *T. araucae* and *T. chileae* bundles were hybridized with a combination of rhodamine- and fluorescein-labelled probes. Both *Thioploca* species gave the same hybridization results, with *T. chileae* shown in Fig.1a and 1b, and *T. araucae* shown in Fig 1c. In the first experiment the general eubacterial probe 338 was used in combination with 385, a probe originally designed for the detection of delta subdivision sulfate reducers, but also staining other delta subdivision bacteria and several grampositives (AMANN ET AL. 1990). The positive fluorescence stain with the eubacterial probe 338 contrasts to the negative result with probe 385 (Fig. 1a). To clarify the position of *Thioploca* within the proteobacterial subdivisions, a second hybridization experiment was performed with two discriminative probes for the beta and gamma proteobacterial subdivisions, BET42a and GAM42a, respectively. Since these probes bind to the same site of the 23S rRNA, and differ at only one position, they should be applied in combination to ensure competitive discrimination (MANZ ET AL. 1992, WAGNER ET AL. 1993). *Thioploca* hybridized with GAM42a regardless of the attached fluorophore, and this probe gave a much stronger signal than either BET42a or ALF19b, thus visualizing the phylogenetic affiliation of *Thioploca* to the gamma subdivision. The third

hybridization experiment was performed with probe 829, targeted to a specific sequence motif of *T. araucae* and *T. chileae*, and with probe 463, targeted to a *Beggiatoa alba* sequence motif, and used here as a negative control (Fig. 1c). Probe 829 hybridized with *T. araucae* and *T. chileae*, thus substantiating that the sequences obtained by sequencing of PCR-amplified 16S rRNA genes actually belonged to *Thioploca*.

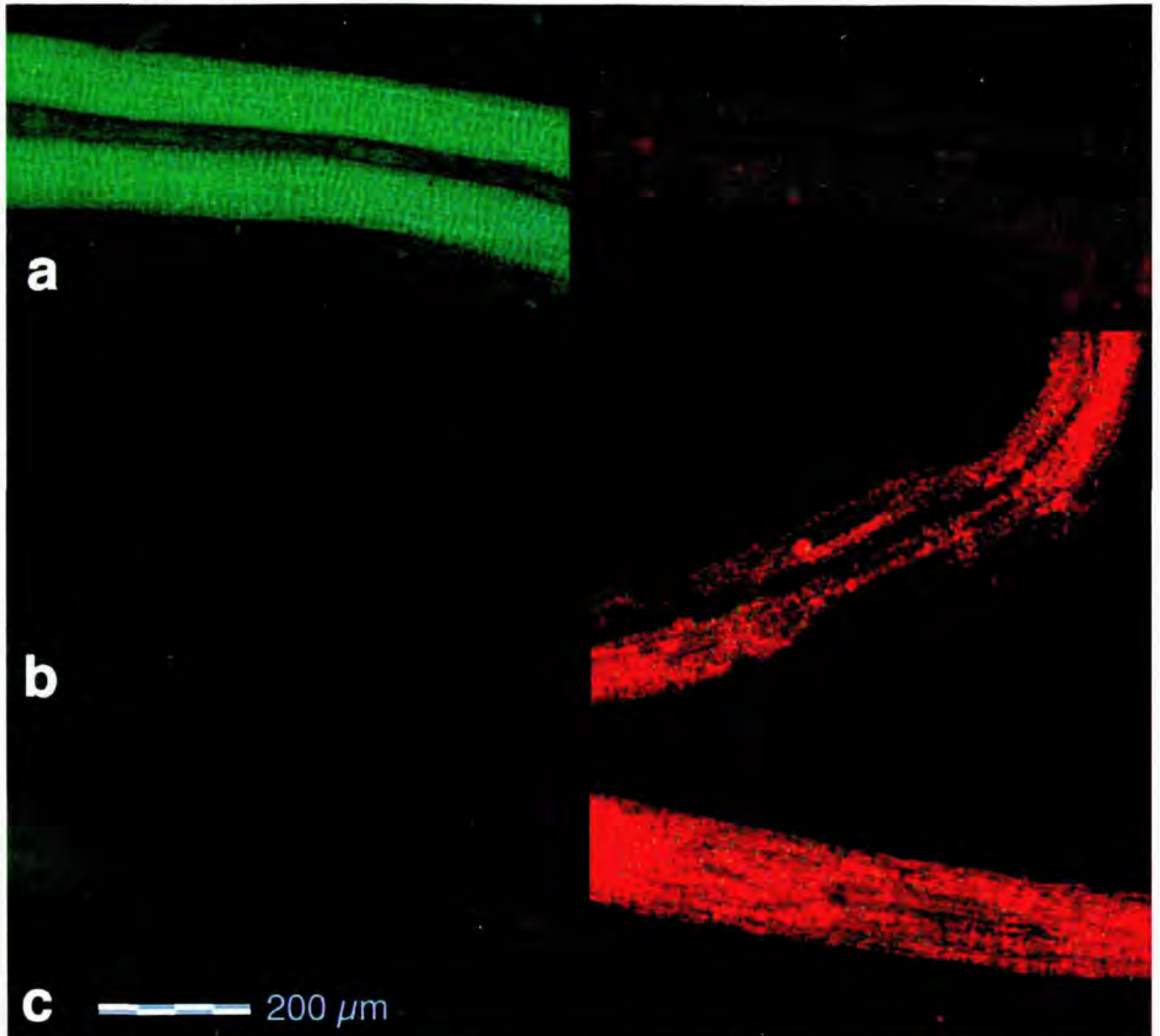

Fig. 1. *In situ* hybridization of *Thioploca* with fluorescent oligonucleotide probes of stepwise narrowing phylogenetic range. Hybridizations are carried out with three mixtures (a, b and c) of a rhodamine- and a fluorescein-labelled probe:
a) *T. chileae* hybridized with eubacterial probe 338 fluorescein and delta proteobacterial probe 385 rhodamine (35% Formamide). b) *T. chileae* hybridized with probes BET42a fluorescein and GAM42a rhodamine (30% Formamide). The intense fluorescence of the *Thioploca* bundle is accumulated from fluorescence signals of individual *Thioploca* filaments. c) *T. araucae* hybridized with probes Beggiatoa-463 fluorescein and Thioploca-829 rhodamine (30% Formamide). Scale bar = 200 μm.

Hybridization Blind Experiment. To obtain an objective assessment of the binding affinity of the *Thioploca* probe 829 a blind experiment was performed (RAMSING ET AL. 1993). Fresh *T. araucae* and *T. chileae* bundles were fixed, and hybridized in parallel at four formamide concentrations, 10%, 20%, 30% and 40%, using three different probe combinations: a) 829 rhodamine-labelled, and 463 fluorescein-labelled, b) 462 rhodamine-labelled, and 829 fluorescein-labelled, c) ALF19b rhodamine-labelled, and 385 fluorescein-labelled. Each combination was tested in duplicate, resulting in six hybridization slides. For comparison, we also hybridized mixtures of single filaments of *T. araucae* and *T. chileae*. Positive and negative stains were noted for each hybridization set after randomizing the samples. Results of the hybridization blind experiment are shown in Fig. 2.

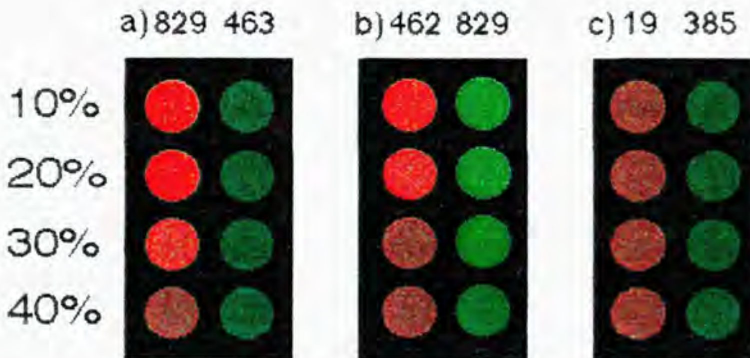

Fig. 2. Scheme of hybridization blind experiment results. Fluorescence signals of rhodamine- and fluorescein-labelled probes are represented by different intensities of red and green. Rhodamine and fluorescein intensities are drawn separately as red and green circles. Hybridizations were performed with three mixtures of a rhodamine- and a fluorescein-labelled probe: a) 829-rhodamine, 463-fluorescein. b) 462-rhodamine, 829-Fluorescein. c) ALF19b-rhodamine, 385-fluorescein. The slides were randomized and viewed with a fluorescence microscope (Zeiss Axioplan). % numbers indicate formamide vol%, increasing from 10 to 40 vol% formamide.

Positive stains of probe 829 were discriminated from negative stains of other probes in the randomized samples. The fluorescein- and the rhodamine-labelled version of probe 829 were tested and gave consistently positive fluorescence signals over a wide range of formamide concentrations. The most intense fluorescence was obtained at 10 and 20% formamide, decreasing slightly at 30%, and decreasing substantially at 40%. We used probe 829 mostly at 20% formamide concentration, to ensure high staining intensities, especially for staining small bundles or individual filaments. Probe 463, probe ALF19b and probe 385 have no

no target sequence on the known *Thioploca* 16S rRNA, and gave consistently negative hybridization results. Probe 462, complementary to sequence positions 462-484 (E.coli. numbering) of *T. araucae*, was designed as a second *Thioploca* probe specifically for *T. araucae*, but gave generally low fluorescent signals and was therefore not used further. The target sequence of probe 462 is likely to form a stable secondary structure, a short stem with a tetraloop (GAUAAUACU-CUAA-AGUCUUGAC), and remains therefore inaccessible for hybridization probes. Likewise, the probe could form a hairpin structure.

Phylogeny of *Thioploca* and related bacteria. Our results, combined with previous studies, demonstrated that *Thioploca* species, *Beggiatoa alba*, *Beggiatoa* sp. strain 1401-13 and *Thiothrix nivea* are members of the gamma subdivision of the proteobacteria. With the exception of *Thiothrix*, these filamentous sulfide oxidizers form a phylogenetically coherent group, of comparable genetic diversity as other major groups of H_2S-and sulfur-oxidizing bacteria, such as *Thiomicrospira*, *Thiobacillus*, *Chromatium* and *Ectothiorhodospira* (Fig.3).
T. araucae, *T. chileae* and *T. ingrica* constitute a monophyletic group, separated from the two *Beggiatoa* species. Bootstrap resampling of the sequences supported the*Thioploca* branch with 98 %. *Thioploca* is thus a phylogenetically coherent and valid genus, distinct from *Beggiatoa*. The three *Thioploca* species are separated from each other by 16S rRNA sequence distances in the range of approx. 0.02 - 0.07 substitutions per site (Jukes-Cantor), as far as possible to infer from the partial sequences obtained in this study. *T. araucae* and *T. chileae* show less 16S rRNA sequence differences to each other than than to *T. ingrica*, which agrees well with morphological and biogeographical evidence: *T. araucae* and *T. chileae*, both from the Chilean Pacific coast, have large cells which are filled almost completely by a central vacuole. *T. ingrica*, known from brackish water of the Baltic Sea and fresh water at Lake Erie, has smaller cells which contain predominantly cytoplasm and small vacuoles (MAIER & MURRAY 1965). *Thioploca* species, previously defined only by morphology, are substantiated as genospecies, and cannot be regarded as mere morphotypes.
Related to *Thioploca* are*Beggiatoa alba* and *Beggiatoa* sp. strain 1401-13. *Thioploca* and these two *Beggiatoa* species form a monophyletic group, supported by 79% bootstrap for the sequence ranges of *T. araucae* and *T. chileae*, and by 96% bootstrap for near-complete sequences of *T. ingrica*, *Beggiatoa alba* and *Beggiatoa* sp. strain 1401-13. This phylogenetic affiliation of *Beggiatoa* and *Thioploca* corresponds with their mutual morphological and physiological similarities. It appears justified to consider *Beggiatoa* and *Thioploca* as two evolutionary homologous genera, a bundle-forming and a non-bundle-forming

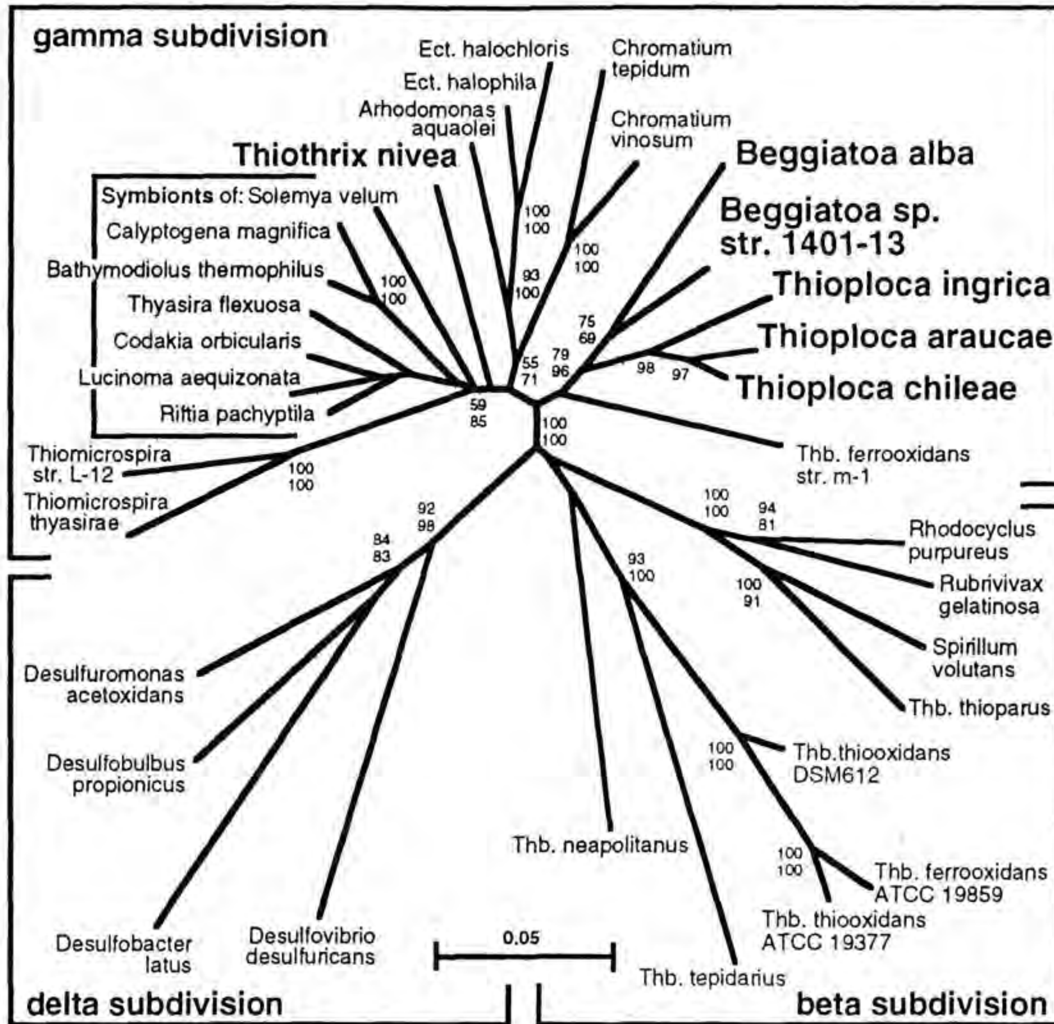

Fig. 3. 16S rRNA distance tree of *Thioploca, Beggiatoa* and representative H_2S- and sulfur-oxidizing bacteria of the proteobacterial beta- and gamma-subdivisions. Delta-subdivision sulfate-reducing bacteria are included as outgroup. The distances are based on 16S rRNA sequence positions 358-949 (E.coli positions), the sequence region determined for *T. araucae* and *T. chileae*. Primer sequences (positions 341-357 and 950-964) are excluded from the analysis. Bootstrap values for important branchings are shown at the nodes, in pairs: The upper number is the bootstrap value from analysis of sequence positions 358-949, determined for *T. araucae* and *T. chileae*. The number below is the corresponding value obtained from analysis of near-complete sequences, with *T. araucae* and *T. chileae* excluded. The scale bar corresponds to 0.05 mutations per nucleotide position. Sequences used in preparing this figure were derived from the Ribosomal Database Project (RDP) (MAIDAK ET AL. 1994). Abbreviations: Thb. = *Thiobacillus*, Ect. = *Ectothiorhodospira*. 16S rRNA Genbank accession numbers: *Thioploca ingrica, T. chileae* , *T. araucae*: L40998, L40999, L41043; *Beggiatoa alba, Beggiatoa* sp. strain 1401-13: L40994, L40997. *Thiothrix nivea* L40993.

genus of filamentous H_2S-oxidizers. The evolutionary distances of *Beggiatoa* to *Thioploca,* approx. 0.10-0.13 substitutions per site for near-complete sequences of *T. ingrica* and *Beggiatoa,* are in the appropriate range for two different genera (DEVEREUX ET AL. 1990). This picture of *Thioploca* and *Beggiatoa* phylogeny is not yet complete. The *Beggiatoa* species investigated here represent only a small fraction of natural *Beggiatoa* diversity. The DSM strain of *Beggiatoa alba,* which was investigated here, is one of 32 strains in the original publication (STROHL & LARKIN 1978). Likewise, *Beggiatoa* str. 1401-13 is one of 14 described freshwater and marine strains, with filament diameters of 1.25 - 3 µm, distinct in morphology, growth patterns and substrate utilization range (PRINGSHEIM ET AL. 1964). *Beggiatoa alba* B15DL and *Beggiatoa* str. 1401-13 are phenotypically quite similar (MEZZINO ET AL. 1984, PRINGSHEIM ET AL. 1964): They are freshwater strains, both capable of heterotrophic and mixotrophic growth, and have similar filament diameters, 2.8 - 3.2 µm (*B. alba* strain B15DL) and 2.5 µm (*Beggiatoa* sp. str. 1401-13). Nethertheless, their considerable 16S rRNA sequence distance (0.107 substitutions per site for the near-complete sequence) argues for high genetic diversity within the genus *Beggiatoa,* which is also indicated by different G+C % values of many *Beggiatoa* strains (MEZZINO ET AL. 1984, STROHL 1989). Marine *Beggiatoa* species with vacuolated cells and filaments diameters in the range of ca. 20 - 120 µm, strikingly reminiscent of *Thioploca araucae* and *Thioploca chileae,* would deserve special attention and could be closely related to these vacuolated marine *Thioploca* (NELSON ET AL. 1989).
Thiobacillus ferrooxidans str. m-1 is the closest non-filamentous relative of *Thioploca* and *Beggiatoa*. This organism is assigned to the genus *Thiobacillus,* but it is an atypical *Thiobacillus* and probably deserves reevaluation (HARRISON 1982). Strain m-1 differs from other strains of *Thiobacillus ferrooxidans* by its inability to use elemental sulfur as electron donor and by DNA homology (HARRISON 1982). Strain m-1 is the only *Thiobacillus* found within the gamma subdivision, whereas other *Thiobacillus* species fall into the beta-gamma transition range, into the beta- or alpha subdivisions of the Proteobacteria and are not closely related to *Thioploca* (LANE ET AL. 1992). The affiliation of this organism with the *Beggiatoa-Thioploca* lineage should be regarded as uncertain. By bootstrap analysis of near-complete 16S rRNA sequences, this organism could possibly be placed between the root of the *Beggiatoa-Thioploca* branch and the beta-subdivision (Bootstrap support 70%).
Thiothrix nivea is by 16S rRNA sequence not closely related to *Thioploca* and *Beggiatoa*. Initially, partial sequences suggested a placement with *Chromatium* , but this position was not supported by bootstrap (49%). On the contrary, analysis of the near-complete 16S rRNA sequence indicated that *Thiothrix nivea*

represents a deep-branching, distinct lineage of the gamma proteobacterial subdivision, at the root potentially affiliated with the symbiont cluster and *Thiomicrospira* (Bootstrap support 66%). *Thiothrix nivea* was placed into the phylogenetic tree (Fig. 3) according to the near-complete sequence, to illustrate this more likely position. If more *Thiothrix* sequences become available, a more definite phylogenetic placement of this genus should become possible. Genotypic, morphological and ecophysiological differences set *Thiothrix* apart from *Beggiatoa* and *Thioploca*. Rosette and gonidia formation are typical morphological features of *Thiothrix*, which are not found in *Beggiatoa* and *Thioploca*. *Thiothrix* is often found attached to reeds and plant material, above the sediment surface, while *Beggiatoa* mats grow on sediment surfaces (BAHR & SCHWARTZ 1956). *Thiothrix* prefers higher oxygen concentrations (appox. 10% air saturation) than *Beggiatoa* and *Thioploca*. Many *Thiothrix* strains are, in contrast to *Beggiatoa*, catalase positive (LARKIN & STROHL 1983, WILLIAMS & UNZ 1985). These morphological and ecophysiological features of *Thiothrix* justify removing *Thiothrix* from the family *Beggiatoaecae* and to place it into the family *Thiotrichaceae* , as has been suggested previously (PRINGSHEIM 1949, HAROLD & STANIER 1955).

The phylogeny of *Thioploca*, *Beggiatoa* and *Thiothrix* , as inferred by 16S rRNA analysis, disproves an interpretation of these bacteria as colourless, "apochlorotic" cyanobacteria (REICHENBACH & DWORKIN 1981). Cyanobacteria are phylogenetically not a subdivision of the proteobacteria, but a major, distinct bacterial phylum (WOESE 1987). Our results are consistent with a phylogenetic study of 5S rRNA sequences of *Thiothrix nivea* str. JP-2 and *Beggiatoa alba* type str.B18LD, which places them into the gamma subdivision of the proteobacteria (STAHL ET AL. 1987). Later analyses of partial 16S rRNA sequences of iron-and sulfur-oxidizing bacteria, which included *Thiothrix nivea* str. JP2, confirmed this placement (LANE ET AL. 1992). *Beggiatoa alba* str. OH-75-2a was assigned to the gamma subdivision by 16S-rRNA oligonucleotide analysis (WOESE ET AL. 1985). Protein data support the placement of *Beggiatoa* into the gamma-subdivision: several c-type cytochromes of *Beggiatoa alba*, which participate in the oxidation of sulfide to sulfur, have closely related counterparts in *Chromatium* species (SCHMIDT & DISPIRITO 1990).

Evolutionary significance of *Thioploca* and *Beggiatoa*. Although sulfide-and sulfur-oxidizing bacteria are known from all subdivisions of the proteobacteria (with the exception of the delta group), they are found in greatest physiological and ecological diversity within the beta-and gamma subdivisions. *Thioploca* and *Beggiatoa* represent a new branch of this evolutionary radiation of sulfide-

oxidizing bacteria. These sulfide-oxidizers include anoxygenic phototrophs such as *Chromatium*, *Ectothiorhodospira*, H_2S-oxidizing symbionts of marine invertebrates, and *Thiomicrospira* and *Thiobacillus*. Each of these groups presents a distinct physiological type: a) Anoxygenic photosynthesis with H_2S as electron donor for *Chromatium* and *Ectothiorhodospira*, b) H_2S-oxidizing chemolithotrophic symbionts of marine invertebrates, c) oxygen-respiring or denitrifying free-living bacteria like *Thiomicrospira* and *Thiobacillus*. *Thioploca* and *Beggiatoa* represent another variation of the latter theme, the motile filamentous sulfide oxidizers, either living in gradients of sulfide and oxygen or nitrate, as *Beggiatoa* or *Thiothrix*, or shuttling between spatially separated pools of sulfide and oxygen or nitrate, as *Thioploca* (FOSSING ET AL. 1995, JØRGENSEN 1987, SWEERTS ET AL. 1990).

Despite all their ecological and physiological peculiarities, these bacterial groups are viewed as descendants of an ancestral, H_2S-oxidizing phototroph (WOESE 1987), which subsequently evolved into different modes of sulfide oxidation. This evolutionary radiation, the emergence of microaerophilic and aerobic sulfide- and sulfur oxidizers, can be attributed to the stepwise oxidation of the early marine environment.

Although there is geochemical evidence for atmospheric oxygen already 2.5 billion years ago (HOLLAND 1984), the oxidation of the seas was delayed due to the buffer capacity of the ocean (KASTING 1993). If the beginning of the trace fossil record and the evolution of burrowing marine meio- and macrofauna is taken as an oxygen indicator, oxygen levels of these marine habitats - sea floors and sediment surfaces - reached the Pasteur point (0.2 % oxygen, or 1 % air saturation) as late as in the late Proteorozoic, ca. 600-800 m.a. (RHOADS & MORSE 1971). *Beggiatoa*, *Thiothrix* and *Thioploca* require a partially oxidized environment, they depend on gradients of sulfide with at least occasional presence of oxygen or nitrate. *Beggiatoa* mats, located in steep opponent gradients of O_2 and H_2S, prefer oxygen levels up to 1-5 % air saturation (2.4 - 12 μM) (MØLLER ET AL. 1985). Oxygen levels of sea water of the Peru-Chile Subsurface Countercurrent, the habitat of *Thioploca*, are typically around 1 % air saturation (GALLARDO 1977). *Thioploca* mats were found to underlie the oxygen minimum zone (< 5 μM O_2, i.e < 2 % air saturation) in the upwelling region on the chilean shelf near Concepcion (FOSSING ET AL. 1995). As a working hypothesis, we suggest that the evolutionary radiation of microoxic gradient bacteria, including the antecedents of modern *Thioploca*, *Beggiatoa* and *Thiothrix*, took place in the late proterozoic. By this measure they are distinctly modern bacteria. Compared to cyanobacteria, with their molecular and microfossil record of at least 1.5 to 2.7 b.a.bp. (OCHMAN & WILSON 1987, BUICK

1992, KNOLL & BAULD 1989), and to some ancient lineages of anoxygenic photosynthetic H_2S-oxididizing bacteria (WOESE 1987), *Thioploca, Beggiatoa* and *Thiothrix* are new characters in the evolutionary play, having emerged from the latest major evolutionary radiation of bacteria, the beta-and gamma subdivision proteobacteria (VAN DE PEER ET AL. 1994). Their mode of life, bound to or shuttling within oxidized-reduced gradients, reflects their evolutionary origin in a time of change from a reducing to an oxidizing marine biosphere.

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4.7. Phylogenetic relationships of *Thiomicrospira* species and their identification in deep-sea hydrothermal vent samples by denaturing gradient gel electrophoresis of 16S rDNA fragments.

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ABSTRACT

Denaturing gradient gel electrophoresis (DGGE) of PCR-amplified 16S rDNA fragments was used to explore the genetic diversity of hydrothermal vent microbial communities, specifically to determine the importance of sulfur-oxidizing bacteria therein. DGGE analysis of two different hydrothermal vent samples revealed one PCR-band for one sample and three PCR-bands for the other sample, which probably correspond to the dominant bacterial populations in these communities. Three of the four 16S rDNA fragments were sequenced. By comparison with 16S rRNA sequences of the Ribosomal Database Project, two of the DGGE-separated fragments were assigned to the genus *Thiomicrospira*. To identify these 'phylotypes' in more detail, a phylogenetic framework was created by determining the near-complete 16S rRNA gene sequence (approx. 1500 nucleotides) from three described *Thiomicrospira* species, viz., *T. crunogena*, *T. pelophila*, *T. denitrificans*, and from a new isolate *Thiomicrospira* sp. strain MA2-6. All *Thiomicrospira* species except *T. denitrificans* formed a monophyletic group within the gamma subdivision of the Proteobacteria. *T. denitrificans* was assigned as a member of the epsilon subdivision and was affiliated with *Thiovulum*, another sulfur-oxidizing bacterium. Sequences of two dominant 16S rDNA fragments obtained by DGGE analysis fell into the gamma subdivision *Thiomicrospira*. The sequence of one fragment was in all comparable positions identical to the 16S rRNA sequence of *T. crunogena*. Identifying a dominant molecular isolate as *T. crunogena* indicates that this species is a dominant community member of hydrothermal vent sites. Another 'phylotype' represented a new *Thiomicrospira* species, phylogenetically in an intermediate position between *T. crunogena* and *T. pelophila*. The third 'phylotype' was identified as a *Desulfovibrio*, indicating that sulfate-reducing bacteria, as sources of sulfide, may or could complement sulfur-and sulfide oxidizing bacteria ecologically in these sulfide-producing hydrothermal vents.

INTRODUCTION

The microbial communities of the hydrothermal vent ecosystems have been known for more than a decade (JANNASCH 1985) and continue to inspire searches for new bacterial and archeal species, undertakings of sometimes adventurous and unique character. Although several different bacteria have been isolated from these communities, little is known about the microbial diversity of these communities and about the relative abundance of their individual inhabitants. Sulfur-oxidizing bacteria of the genus *Thiomicrospira* have been

isolated frequently from deep-sea hydrothermal vent samples (RUBY ET AL. 1981; JANNASCH ET AL. 1985), which indicate their ecological importance in this habitat. In this study, we complemented cultivation-based studies of hydrothermal vent communities with a new molecular approach, denaturing gradient gel electrophoresis (DGGE) of 16S rDNA fragments, and focused on the congruences of both strategies.

DGGE of enzymatically amplified 16S rDNA has recently been introduced into molecular microbial ecology to determine the genetic diversity of natural microbial communities (MUYZER ET AL. 1993) and to identify the phylogenetic position of the community members (MUYZER & DE WAAL 1994). PCR amplifications from 16S rRNA genes of natural bacterial populations, using gene-specific primers, yield mixtures of products of identical length, but different in nucleotide sequence. DGGE can separate these PCR products on the basis of differences in melting behaviour in polyacrylamide gels containing a linear gradient of DNA denaturants, thereby giving a direct visualization of the dominant participants in the microbial populations. Subsequently, separated bands can be excised, re-amplified, and sequenced directly (MUYZER & DE WAAL 1994). Cloning of PCR products is circumvented and cloning biases in the analysis of PCR products are avoided.

Here we applied the DGGE approach to determine the genetic complexity of hydrothermal vent microbial communities and to assess the abundance of sulfur-oxidizing bacteria. 16S rDNA fragments obtained after enzymatic amplification of genomic DNA isolated from two deep-sea hydrothermal vent samples were analyzed by DGGE. The most dominant PCR fragments were sequenced and, after searching the Ribosomal Database Project for related sequences, identified as *Thiomicrospira* species. For a closer identification of our molecular isolates, we constructed a phylogenetic framework for all known *Thiomicrospira* species.

Nearly complete 16S rRNA sequences analyses have been published for *Thiomicrospira* sp. strain L-12 (RUBY & JANNASCH 1982) and *T. thyasirae* (WOOD & KELLY 1993). These species were grouped into the gamma subdivision of the Proteobacteria (LANE ET AL. 1992; DISTEL & WOOD 1992). Based on 5S rRNA sequences, *Thiomicrospira* sp. strain L-12 and *T. pelophila* were assigned to the gamma subdivision of the Proteobacteria (LANE ET AL. 1985). The phylogenetic relationships of *T. pelophila* (KUENEN & VELDKAMP 1972), *T. denitrificans* (TIMMER-TEN HOOR 1975), and *T. crunogena* (JANNASCH ET AL. 1985) based on 16S rRNA sequences have not yet been elucidated. We, therefore, determined near-complete sequences of 16S rRNA genes, comprising approx. 1500 nucleotides, of these three species and of a new isolate *Thiomicrospira* sp. strain MA2-6. Here we present the refined *Thiomicrospira* phylogeny, integrated

with our analysis of the dominant DGGE fragments. We illustrate the microbiological and ecological relevance of these molecular data and argue for closer connections between molecular- and cultivation-based microbial ecology.

MATERIALS AND METHODS

TABLE 1. Bacteria and hydrothermal vent samples used in this study

bacterium/sample	isolated/collected from	location	source
Thiomicrospira crunogena[1]	scrapings from outer side of tubeworm	East Pacific Rise	HWJ
Thiomicrospira denitrificans[2]	intertidal mud flat	Dutch Wadden Sea	DSM 1251
Thiomicrospira pelophila[3]	intertidal mud flat	Dutch Wadden Sea	DSM 1534
Thiomicrospira sp. str. MA2-6	slurp sample/ beehive smooker base	Mid-Atlantic Ridge	HWJ
sample #2613	outer side of a chimeny rock sample	Mid-Atlantic Ridge	HWJ
sample #2409	outer side of a chimeny rock sample	Mid-Atlantic Ridge	HWJ

[1]JANNASCH ET AL. 1985
[2]TIMMER TEN HOOR 1975
[3]KUENEN & VELDKAMP 1972

Table 1 gives a summary of the different bacteria and environmental samples used in this study. *Thiomicrospira pelophila* and *Thiomicrospira denitrificans* were obtained from the Deutsche Sammlung von Mikroorganismen und Zellkulturen (DSM, Braunschweig, Germany). *Thiomicrospira crunogena*, and *Thiomicrospira* sp. strain MA2-6 were provided by one of us (HWJ). The latter strain was isolated from a slurp sample of whitish material at the base of 'beehive' smooker at Snake Pit site, Mid-Atlantic Ridge, 23 degree 20"N, 44 degree 57"W. The temperature during sampling ranged from 17 to 35°C. A sample of a relict chimney (sample no. 2609) was collected at the TAG site at a depth of 3648 m. Another sample, i.e. a relict chimney sample (sample no. 2613) was collected from active site 1 at Moose site (Snake Pit, Mid-Atlantic Ridge, 23 degree 20"N, 44 degree 57"W) from a depth of 3523 m. Material scraped from the outer sides of these samples were used in this study.

DNA extraction. Genomic DNAs from bacterial cells and natural samples were obtained by using a modification of the method of ROCHELLE ET AL. (1992). One gram of hydrothermal vent sample was resuspended in 2 ml of 0.15 M NaCl, 0.1 M EDTA (pH 8). After the addition of 250 µl of lysozyme solution (80 mg/ml), the

samples were incubated for 60 min at 37°C, and mixed every 10 min. The samples were placed on ice and 2 ml of a solution containing 0.5 M Tris-HCl (pH 8), 0.1 M NaCl, and 10% (w/v) SDS was added. Subsequently, the samples were incubated in a mixture of ice and NaCl. A volume of 100 µl of Proteinase K (20 mg/ml) was added and the sample was incubated for 10 min at 55°C. The samples were placed on ice again and 4 ml of Tris-buffered phenol was added to the sample. After centrifugation at 6,000 x g for 10 min at 4°C, the aqueous phase was removed and placed in a clean tube and carefully mixed with an equal volume of phenol : chloroform : isoamyl alcohol (50:49:1) and centrifuged at 6,000 x g for 10 min at 4°C. This step was repeated until no protein precipitate was visible at the organic/inorganic interphase. A 0.1x volume of 5 M NaCl and a 2.5 x volume of 100% (v/v) ethanol was added to the aqueous phase, mixed, and incubated overnight at -20°C. The genomic DNA was precipitated by centrifugation at 6,000 g for 10 min at 4°C, dried under vacuum, and dissolved in TE-buffer.

PCR amplification of the 16S rRNA gene. The extracted DNA was used as target DNA in the polymerase chain reaction (SAIKI ET AL. 1988) to amplify the 16S ribosomal RNA coding regions (MEDLIN ET AL. 1988). Two primers, GM3F and GM4R (Table 2), were used to amplify the nearly complete 16S rRNA gene. Two other primers, GM5F and 907R, were used to amplify a 550 bp rDNA fragment, which was used for DGGE analysis. At the 5' end of the GM5F primer, an additional 40-nucleotide GC-rich sequence (GC-clamp) was added to obtain a stable melting behaviour of the DNA fragments in the DGGE (MUYZER ET AL. 1993). The sequences and the exact position of the primers are given in Table 2.

TABLE 2. Primer sequences and positions

primer[a]	position[b]	sequence
GM3F	8 - 24	5' - AGAGTTTGATCMTGGC - 3'
GM4r	1492 - 1507	5' - TACCTTGTTACGACTT - 3'
GM5F	341 - 357	5'- CCTACGGGAGGCAGCAG - 3'
907R	907-926	5' - CCGTCAATTCCTTTRAGTTT - 3'
GC-clamp[c]		5'-CGCCCGCCGCGCCCCGCGCCCGTCCCGCCGCCCCCGCCCG- 3'

[a] F is the forward primer; R is the reverse primer.

[b] Positions in the 16S rRNA of E.coli (BROSIUS ET AL. 1981)

[c] The GC-clamp is attached to the 5'- end of the GM5F primer

PCR amplifications were performed with a Techne PHC-3 Temperature Cycler (Techne, Cambridge, UK) as follows: 10-100 ng of target DNA, 25 pmol of each of the appropriate primers, 10 µmol of each deoxyribonucleoside triphosphate, and 10 µl of 10x PCR buffer [i.e. 100 mM Tris-HCl (pH 9), 15 mM $MgCl_2$, 500 mM KCl, 0.1% (w/v) gelatin, 1% (v/v) Triton X-100] were adjusted to a final volume of 50 µl with sterile water (Sigma, Germany), and overlaid with 2 drops of mineral oil (Sigma, Germany). SuperTaq DNA polymerase (HT Biotechnology, Ltd) was added to the reaction mixture after the initial denaturing step (94°C, 5 min), at a temperature of 80°C. In addition, to increase the specificity of the amplification and to reduce the formation of spurious by-products a "touchdown" PCR (DON ET AL. 1991) was performed, meaning that the annealing temperature was set 10°C above the expected annealing temperature (50°C for GM3F/GM4R, and 70°C for GM5F/907R) and decreased by 1°C every second cycle until a touchdown of 40°C or 60°C, at which temperature 9 additional cycles were carried out. Primer extension was carried out at 72°C for 3 min. The total number of cycles was 30.
Five µl aliquots of the amplification products were analyzed by electrophoresis in 2% (w/v) Nusieve agarose (FMC) gels containing 0.5 µg/ml ethidium bromide (SAMBROOK ET AL. 1989).

DGGE analysis. DGGE was performed using a Bio-Rad Protean II system, as described previously (MUYZER ET AL. 1993, 1995a). PCR samples were applied directly onto 6% (w/v) polyacrylamide gels in 0.5x TAE [20 mM Tris-acetate (pH 7.4), 10 mM acetate, 0.5 mM Na_2EDTA] with gradients formed with 6% (w/v) acrylamide stock solutions (acrylamide-N, N'-methylenebisacrylamide, 37 : 1) that contained 10% and 70% denaturant (100% denaturant is 7 M urea and 40% [v/v] formamide, deionized with AG501-X8 mixed bed resin [Bio-Rad]). Electrophoresis was performed at a constant voltage of 200 V and a temperature of 60°C. After electrophoresis, the gels were incubated for 15 min in Milli-Q water containing ethidium bromide (0.5 mg/l), rinsed for 10 min in Milli-Q water, and photographed with UV transillumination (302 nm) with Cybertech CS1 equipment.

Sequencing of PCR products. Amplified DNA fragments were separated from free PCR primers by electrophoresis in 2% (w/v) agarose gels using the Biometra E91 Electrophoresis Unit (Biometra, Göttingen, Germany). Electrophoresis was continued until the PCR product migrated into the buffer-filled well, from where it was taken up with a pipette and transferred into a clean 1.5 ml tube. After addition of 0.1x volume of 5 M NaCl and 2.5x volumes of 100% (v/v) ethanol and incubation for 1 h, at -80°C, the PCR product was pelleted by centrifugation.

The pellet was dried under vacuum and redissolved in 50 µl of sterile water.
The double-stranded PCR products were sequenced directly. One µl of purified PCR product (20-50 ng) was added to a 0.5 ml tube containing 2 µl 5x sequencing buffer [i.e. 200 mM Tris-HCl (pH 7.5), 100 mM $MgCl_2$, 250 mM NaCl], 1 µl of a 5% (v/v) Nonidet P-40 solution, and 1 µl of 'nested' sequencing primer (5 pmol/µl). The volume was adjusted to10 µl with sterile water. The mixture was incubated for 10 min at 95°C, with a short centrifugation step after 5 and 10 min to re-collect the sample in the bottom of the tube. After denaturation, the sequencing reaction was started by subsequently adding: 1.0 µl of 0.1 M DTT solution, 2.0 µl of dNTP-solution (200nM of each dGTP, dATP, dTTP), 0.5 µl of (a-^{33}P)-dCTP; (Amersham; 10 µCi/µl; 3000 Ci/mmol), and 1 µl (1 unit) of Sequenase (version 2.0; USB). The samples were incubated for 5 min at 37°C. Thereafter, 3.5 µl aliquots were added to 2.5 µl of the dideoxynucleotide termination solutions (80 µM dGTP, 80 µM dATP, 80 µM cTTP, 80 µM dCTP, and 8 µM of each of the dideoxynucleotides). After 5 min of incubation at 37°C, the reaction was stopped by adding 4 µl of 'stop solution' (i.e. 96% [v/v] formamide, 20 mM EDTA). The samples were heated for 5 min at 95°C and 2.5 µl of the samples were loaded onto a 0.25 to 0.40 mm wedge sequencing gel [6% (v/v) acryl/bisacrylamide (30:1) and 7M urea]. After electrophoresis at constant power of 60 W for 4 h, the gel was fixed, dried and exposed with an X-ray film (Kodak XAR5).

Databank accession numbers. The 16S rRNA sequences of *Thiomicrospira denitrificans, Thiomicrospira pelophila, Thiomicrospira crunogena* and Thiomicrospira sp. strain MA2-6 are available under Genbank accession numbers L40808, L40809, L40810 and L40811. The partial 16S rRNA sequences of DGGE fragments no. 1, 2 and 4 are available under Genbank accession numbers L40812, L40813 and L40814.

Phylogenetic analysis of the 16S rRNA sequences. The 16S rRNA sequences were aligned to those of other bacteria obtained from the Ribosomal Database Project (RDP) (MAIDAK ET AL. 1994). The SIMILARITY_RANK program of the Ribosomal Database Project was used to search this databank for close evolutionary relatives. Sequence alignments were prepared with the sequence alignment editor SEQAPP (GILBERT 1992). Matrices of evolutionary distances were computed from the sequence alignment, using the program DNADIST, implemented in the software package PHYLIP (version 3.5c) developed by FELSENSTEIN (1989). DNADIST calculates distance matrices according to the Jukes-Cantor model, which assumes independent mutations at all sites with equal probability (JUKES & CANTOR 1969). From these distance matrices,

phylogenetic trees were inferred with the program FITCH, which employs the least-square algorithm of FITCH and MARGOLIASH (1967) to match evolutionary distances with branch lenghts of the phylogenetic tree. To validate the reproducibility of the branching pattern, a bootstrap analysis was performed using the program SEQBOOT, also implemented in the software package PHYLIP 3.5c: A new data set is created by sampling N characters randomly with reolacement, so that the resulting dataset has the same size as the original, but some characters have been ledt out and others are duplicated. The random variation of the results from analyzing these bootstrapped data sets can be shown to be typical of the variation obtained by collecting new datasets. The percentages of the resamplings which support the branching pattern of the phylogenetic tree, the bootstrap values, are drawn into the phylogenetic trees (Figs. 1 and 3). Bootstrap value 100 indicates that a branching pattern was confirmed in all resamplings, whereas bootstrap value 50 indicates that the branching pattern was reproduced only in 50% of the resamplings. Bootstrap values, drawn into a phylogenetic tree, always refer to the distal branchings, i.e. those leading to the branch tips, not to the roots.

RESULTS

Phylogeny. The phylogeny of *Thiomicrospira* (Fig. 1) is based on analysis of near-complete 16S rDNA sequences, comprising approx. 1500 positions. All *Thiomicrospira* species and strains, with the exception of *T. denitrificans*, belong to the gamma subdivision of the Proteobacteria (Fig. 1). Within the gamma subdivision, *Thiomicro-spira* species form a distinct, monophyletic lineage. Their next relatives are obligately host-associated, symbiotic H_2S-oxidizing bacteria. Affiliated with the gamma-*Thiomicrospira* cluster are the H_2S-oxidizing symbionts of the bivalva families Lucinidae, Thyasiridae, Solemyidae, Vesicomyidae and Mytilidae, supported by 78 % of the bootstrap resamplings. These bivalve symbionts are divided into two distinct, monophyletic lineages, which correspond with the traditional systematic division of their respective hosts into the superfamily Lucinacea and the family Vesicomyidae (DISTEL ET AL. 1994). A specific affiliation of the *Thiomicrospira* group with one of these lineages is uncertain. The phylogenetic tree (Fig. 1) indicates that the gamma-*Thiomicrospira* species share, as a deep-branching lineage, their root with the Vesicomyidae- and Mytilidae-symbionts, but this is not significantly supported by bootstrap analysis (bootstrap value of 56%). *Thiomicrospira* shows no specific affiliation to any of the free-living H_2S- or sulfur-oxidizing bacteria of the gamma- and beta subdivisions, such as *Thiobacillus*, *Chromatium*, or *Ectothiorhodospira*.

The gamma subdivision *Thiomicrospira* species can be divided into two pairs of very closely related species: *T. thyasirae* and *T. pelophila* share very similar 16S rRNA sequences (6 nucleotides differences out of 1500 positions, i.e. less than 1% difference), as well as *T. crunogena* and *Thiomicrospira* sp. str. L-12 (12 nucleotide differences out of 1500 positions, i.e. less than 1% difference). *Thiomicrospira* sp. strain MA2-6 is related to the latter pair. This clustering, with a bootstrap value of 100%, might reveal the different habitats from which the bacteria were isolated. *T. thyasirae* was isolated from the gills of the bivalve *Thyasira flexuosa* collected from a marine sediment off Plymouth (WOOD & KELLY 1989), while *T. pelophila* was isolated from an intertidal mud flat in the Dutch Wadden Sea (KUENEN & VELDKAMP 1972). *Thiomicrospira* sp. strain L-12, *T. crunogena*, and *Thiomicrospira* sp. strain MA2-6 were all isolated from hydrothermal vent areas (JANNASCH ET AL. 1985, RUBY & JANNASCH 1982,).

T. denitrificans is a member of the epsilon subdivision of the Proteobacteria. The epsilon subdivision contains the genera *Campylobacter*, *Bacteroides*, *Wolinella*, *Thiovulum*, *Helicobacter*, and *Arcobacter* (VANDAMME ET AL. 1991). Although sulfur oxidation is a widespread feature in this subdivision, *T. denitrificans* is not

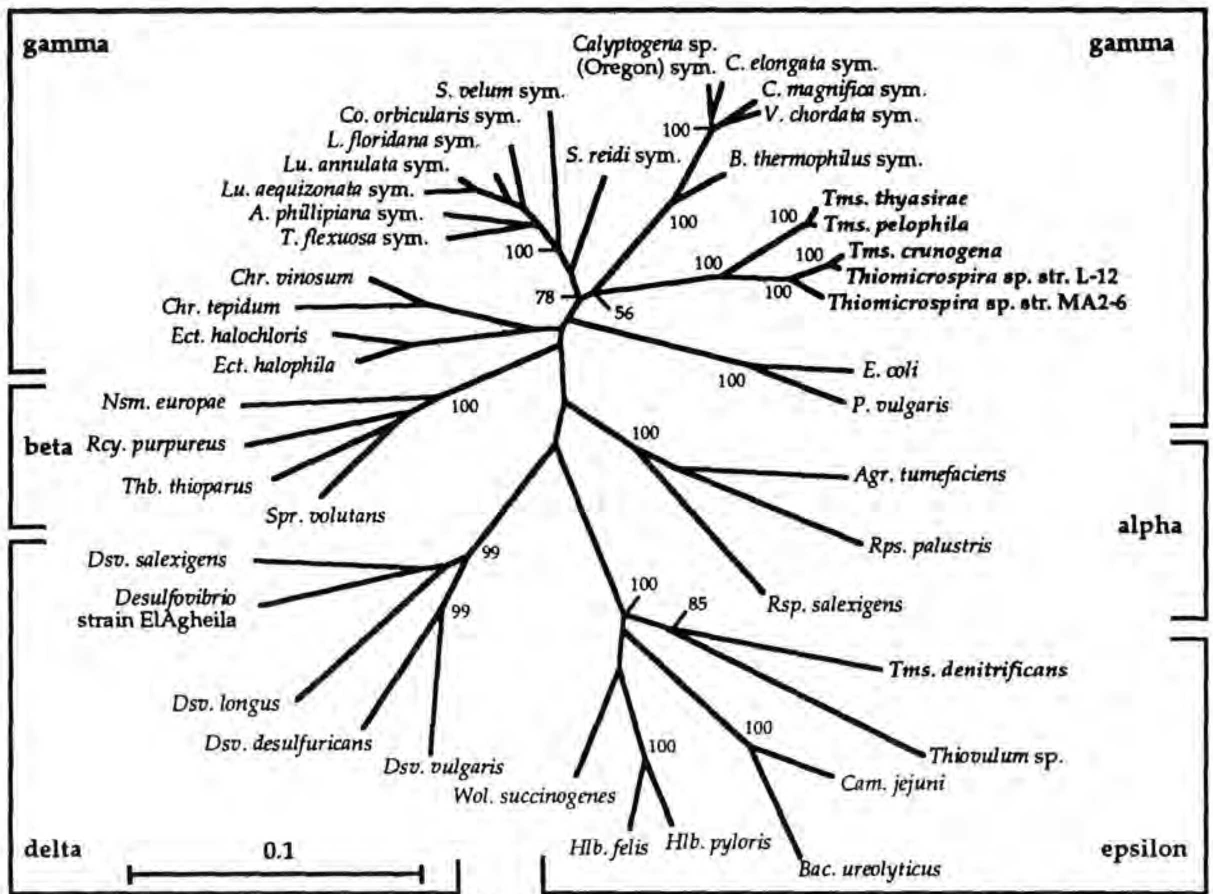

Fig. 1. Unrooted evolutionary distance tree based on nearly complete 16S rRNA sequences, showing the phylogenetic relationships of *Thiomicrospira thyasirae*, *Thiomicrospira pelophila*, *Thiomicrospira crunogena*, *Thiomicrospira* strains L-12 and MA2-6, and *Thiomicrospira denitrificans*. Sequences used in preparing this figure were derived from the Ribosomal Database Project (RDP) (MAIDAK ET AL. 1994). Bootstrap values from distance analyses (100 replicates) refer to species distal to the associated node. The scale bar represents 0.1 estimated nucleotide change per sequence position. The five different subdivisions of the Proteobacteria, i.e. alpha, beta, gamma, delta and epsilon (WOESE 1987; OLSEN ET AL. 1994) are indicated. Abbreviations of bacterial genera: *Agr.* = *Agrobacterium*, *Bac.* = *Bacteroides*, *Cam.* = *Campylobacter*, *Chr.* = *Chromatium*, *Dsv.* = *Desulfovibrio*, *E.* = *Escherichia*, *Ect.* = *Ectothiorhodospira*, *Hlb.* = *Helicobacter*, *Nsm.* = *Nitrosomonas*, *P.* = *Proteus*, *Rcy.* = *Rhodocyclus*, *Rps.* = *Rhodopseudomonas*, *Rsp.* = *Rhodospirillum*, *Spr.* = *Spirillum*, *Thb.* = *Thiobacillus*, *Tms.* = *Thiomicrospira*, *Wol.* = *Wolinella*. Abbreviations of bivalve genera: *A.* = *Anodontia*, *B.* = *Bathymodiolus*, *C.* = *Calyptogena*, *Co.* = *Codakia*, *L.* = *Lucina*, *Lc.* = *Lucinoma*, *S.* = *Solemya*, *T.* = *Thyasira*, *V.* = *Vesicomya*. The 16S rRNA sequences of *Thiomicrospira denitrificans*, *Thiomicrospira pelophila*, *Thiomicrospira crunogena* and *Thiomicrospira* sp. strain MA2-6 are available under Genbank accession numbers L40808, L40809, L40810 and L40811.

closely related to any of these genera. The least distant relative of this organism is the sulfur-oxidizing bacterium *Thiovulum* (LA RIVIERE & SCHMIDT 1991). The genetic distance of their 16S rRNA sequences (13% sequence difference) and the ecophysiological differences argue against a close connection of *T. denitrificans* and *Thiovulum*. Considering its isolated phylogenetic position, *T. denitrificans* deserves reclassification as a new genus. This will require a more detailed physiological comparison of *T. denitrificans* with other epsilon subdivision sulfide- or sulfur oxidizers.

DGGE analysis. DGGE analysis of PCR products, comprising approx. 550 nucleotides from position 357 to 907, revealed one dominant band for the hydrothermal vent sample no. 2609, and 3 bands for sample no. 2613 (Fig. 2). Three of the four bands were sequenced. The sequencing of a DGGE fragment requires elution of the fragment from the denaturant gel and subsequent re-amplification (MUYZER ET AL. 1995). This was unsuccessful with DGGE band No. 3, probably due to its low intensity, compared to DGGE bands No. 1, 2 and 4.
For sequence comparisons and phylogenetic analysis, alignments of DGGE fragment sequences and corresponding parts from complete 16S rRNA sequences were prepared (positions 357 - 907 Escherichia coli numbering). The phylogenetic affiliations of the DGGE fragments are shown in Figs. 3a and 3b.

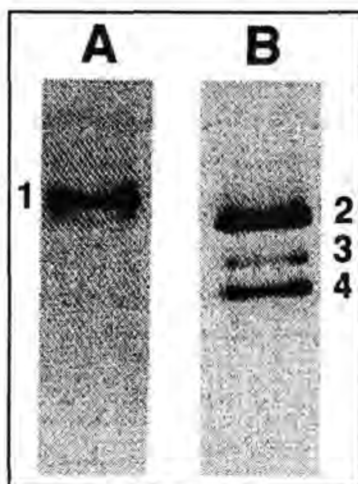

Fig. 2. Negative image of an ethidium bromide-stained DGGE separation pattern of PCR-amplified 16S rDNA fragments (positions 357 - 907) from two hydrothermal vent samples, Lane A no. 2609, and lane B no. 2613. One dominant band was found for sample no. 2609 (lane A), and 3 bands for sample no. 2613 (lane B). The denaturant gradient from gel top to bottom was 20% - 60%.

The sequence of DGGE band no. 1 was phylogenetically affiliated with the gamma-Thiomicrospira cluster (Fig. 3a). Here it held a phylogenetic position between the two branches of the gamma-subdivision *Thiomicrospira* species, with *T. thyasirae* and *T. pelophila* on one side and *T. crunogena, Thiomicrospira* sp. strain L-12, and *Thiomicrospira* sp. strain MA2-6 on the other. The phylogenetic position of this DGGE fragment strongly suggests that it is derived from a new *Thiomicrospira* species. The sequence of DGGE-fragment no. 2 was in all comparable positions identical to *T. crunogena* (Fig. 3a). "Comparable" means readable in the sequence gel; unreadable and therefore unknown nucleotides are not comparable. This DGGE fragment is therefore derived from *T. crunogena* or at least from a phylogenetically very closely related bacterium that shows no 16S rRNA sequence differences to *T. crunogena* over the range of the DGGE fragment. DGGE fragment no. 4 was not derived from *Thiomicrospira* species, but was a *Desulfovibrio* fragment, most closely related to *Desulfovibrio salexigens* (Fig. 3b).

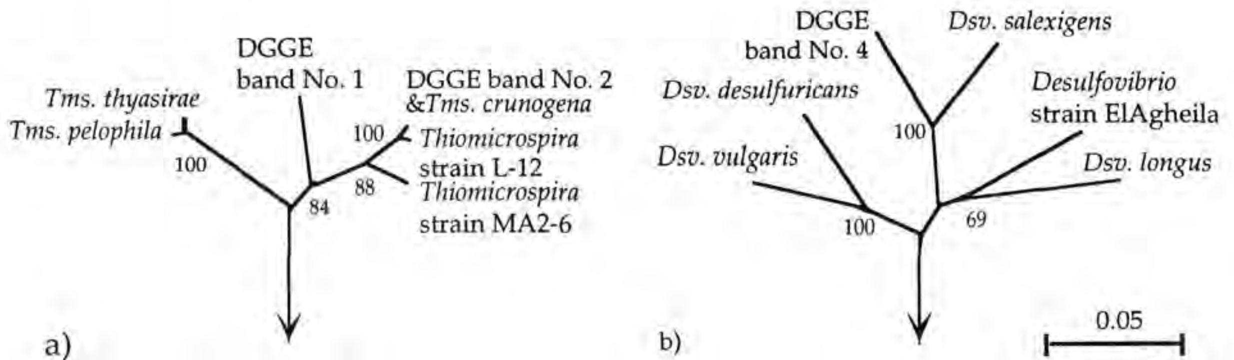

Fig. 3. Phylogenetic relationships of the sequenced 16S rRNA DGGE fragments, comprising positions 357 - 907 : A) *Thiomicrospira* cluster of the gamma subdivision of the Proteobacteria, with sequences from DGGE fragments no. 1 and 2, figure 2 b. B) *Desulfovibrio* species of the delta subdivision of the Proteobacteria, with the sequence of DGGE fragment no. 4. The trees are based on the evolutionary distance estimates obtained after comparision of positions 357 - 907 of aligned 16S rDNA sequences. The scale bar represents 0.05 estimated nucleotide change per sequence position. Bootstrap values indicate how many of 100 resamplings and reiterations of the phylogenetic analysis support the branching pattern. They always refer to the distal branchings, i.e. those leading to the branch tips, not to the roots. The sequences of DGGE fragments no. 1, 2 and 4 are available under Genbank accession numbers L40812, L40813 and L40814.

In former studies (MUYZER ET AL. 1993; MUYZER & DE WAAL 1994) two bacterial primers were used that amplified 16S rDNA fragments of about 200 bp, encompassing positions 341 to 534. However, the small size of these fragments limits an optimal phylogenetic analysis (MUYZER & DE WAAL 1994). In this study, two primers were used that amplify 16S rDNA fragments of about 550 bp (Table 2). These fragments are short enough to be separated well by DGGE (LESSA & APPLEBAUM 1993), and are long enough to give a reliable phylogenetic inference. We have compared phylogenetic trees based on nearly complete 16S rRNA sequences (approx. 1500 positions) and on DGGE fragment sequences (approx. 550 positions), and found ambiguities only on the root level of deeply branching groups, which are already difficult to resolve by analysis if complete 16S rRNA sequences (EVERS ET AL. 1993).

DISCUSSION

In this study, the phylogeny of *Thiomicrospira* was originally established as a framework for the identification of the molecular isolates, but has also shown more general implications. First, the phylogeny of *Thiomicrospira* is relevant for the evolution of "autotrophic animals", in most cases bivalves associated with symbiotic sulfide-oxidizing bacteria that provide the animal with autotrophically fixed carbon (CAVANAUGH ET AL. 1981; FELBECK ET AL. 1981). Two monophyletic lineages of obligately bivalve-associated symbiotic sulfide-oxidizers are phylogentically affiliated with the gamma subdivision *Thiomicrospira* species. The lineages of these free-living and host-associated sulfide-oxidizers are affiliated at their roots only. This supports an early origin of the host-symbiont relationship within each lineage and a subsequent associated evolution of host bivalvia and bacterial symbionts (DISTEL ET AL. 1994), independent from the evolution of free-living *Thiomicrospira* species. Consequently, these *Thiomicrospira* species are viewed as modern descendants of the last free-living ancestor of the obligate bivalve symbionts. This scenario allows a prediction: free-living bacteria, closely related to obligately host-associated bacteria of an ancient symbiosis, do not exist.
The phylogeny of *T. denitrificans* illustrates the limitation of the use of metabolic and morphological features as reliable taxonomic markers. This organism is assigned to the genus *Thiomicrospira* based on the basis of its morphology and the ability to grow as an obligate chemolithotroph by oxidation of sulfide and thiosulfate, although differences in G+C content have already been noted (TIMMER TEN HOOR 1975). The low G+C content of *T. denitrificans* (36%) separates this organism from other *Thiomicrospira* species with G+C contents

between 42% and 52% (KUENEN ET AL. 1991; WOOD & KELLY 1993). In addition, physiological differences between the different *Thiomicrospira* species did not support the unity of the genus. *T. pelophila* and *T. crunogena* are motile, can grow under oxic conditions, and do not denitrify, while *T. denitrificans* is not motile, grows strictly anaerobically, and is a denitrifier (TIMMER-TEN HOOR 1975; JANNASCH ET AL. 1985; KUENEN ET AL. 1991). Considering these distinctive traits of *T. denitrificans* together with its isolated phylogenetic position, distantly related to *Thiovulum*, reclassification of this organism as a new genus is recommended. Its morphology, size, and lack of motility distinguishes *T. denitrificans* significantly from *Thiovulum*. According to GARCIA-PICHEL (1989) *Thiovulum* is the fastest swimmer among all bacteria. By this ability, *Thiovulum* forms free-floating veils to create and maintain steep gradients of oxygen and hydrogen sulfide.

The low number of bands in the DGGE patterns for these two hydrothermal vent samples argues for a comparatively small number of dominant bacterial species, although this does not exclude the possibility of numerous other bacteria occuring in smaller numbers in these hydrothermal vent microbial communities. This finding is supported by the results of a recent study on the microbial diversity and community structure of an active, hydrothermal vent system located at Loihi Seamount, Hawaii (MOYER ET AL. 1994). By using a different molecular approach, restriction fragment length polymorphism (RFLP) analysis of cloned PCR-amplified 16S rRNA genes, these authors found two dominant operational taxonomic units (OTUs), which accounted for 72.9% of all of 48 16S rDNA clones. Unfortunately, the phylogenetic affiliation of these two dominant OTUs has not been determined. Bacterial populations more complex than those of hydrothermal vent sites were found by DGGE analysis of a permanently stratified marine watercolumn: These highly differentiated DGGE band patterns indicated a bacterial ecosystem with more diversified dominating bacterial populations (TESKE ET AL. 1995c).

After sequencing the re-amplified DGGE bands, we were able to determine the phylogenetic relationships of the dominant hydrothermal vent microbial community members. A phylogenetic framework of *Thiomicrospira* was created to allow a detailed identification of two of the separated PCR-amplified 16S rRNA gene fragments. DGGE fragment no. 2 from sample no. 2613 was derived from *T. crunogena* or a very closely related organism of identical 16S rRNA DGGE fragment sequence. DGGE fragment no. 1 from sample no. 2609 was related to the gamma-*Thiomicrospira* sequences. The phylogenetic position of this DGGE fragment, in the center of the gamma-*Thiomicrospira* cluster, strongly suggests that it is derived from a new *Thiomicrospira* species. If the physiological

properties of this species are not fundamentally different from those of other *Thiomicrospira* species, it should be possible to isolate this organism. This finding indicates that more *Thiomicrospira* species are waiting in nature to be discovered.
DGGE fragment no. 4 from sample no. 2613 was not derived from *Thiomicrospira* species, but was identified as a *Desulfovibrio* fragment, most closely related to that of *Desulfovibrio salexigens*. Sulfate-reducing bacteria have been isolated on various occasions from deep-sea hydrothermal vent sites (ELSGAARD ET AL. 1991), but have so far not been considered as a potentially dominant part of the hydrothermal vent bacterial community. A *Desulfovibrio* phylotype among the dominant DGGE fragments argues for a more important role of these sulfate reducers in the hydrothermal vent community.
Although molecular approaches avoid the bias that is unevitable in studies based on selective cultivation (WARD ET AL. 1992), it has been observed in many cases that the phylogenetic analysis of microbial communities using rRNA and PCR-based methods yield a variety of unknown sequences that can not be ascribed to known bacterial genera or species (LIESACK & STACKEBRANDT 1992). From this point of view, it appears impractical to forgo the use of pure cultures in microbial ecology altogether as sometimes suggested. The present study was meant to exemplify how molecular approaches can be used jointly with those of classical microbiology to acquire new capabilities, e.g. those of the DGGE approach, with the store of physiological and biochemical information that is available and still arises from studies of pure cultures.

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4.8 Late Proterozoic rise in atmospheric oxygen from phylogenetic and stable isotopic studies

Donald E. Canfield, Andreas Teske

Diese Arbeit wird zur Einreichung bei *Nature* vorbereitet.

ABSTRACT

Multicellular animal life (Metazoans) evolved late in Earth history. Amino acid sequence comparisons indicate an evolutionary radioation at between 0.8 to 1.0 Ga (billion years before present) (RUNNEGAR 1986)., though the Ediacaran fauna at 0.59 Ga represents the earliest well accepted fossil evidence (GLAESSNER 1971). The late emergence, and subsequent rapid radiation, of Metazoans through the terminal Proterozoic (0.544 Ga, BOWRING ET AL. 1993) is thought to have coincided with an increase in atmospheric oxygen to greater than 3-10% present atmospheric levels (PAL), amounts conducive for animal respiration (BERKNER & MARSHALL 1965, KNOLL 1992, RUNNEGAR 1991). Oxygenation of the Earth surface occurs as organic carbon is buried in sediments, releasing oxidized equivalents ultimately as ferric iron, sulfate and atmospheric oxygen (DES MARAIS ET AL. 1992, HOLLAND 1978). Stable carbon isotope studies indicate high organic carbon burial rates between 0.9 to 0.6 Ga (DES MARAIS ET AL. 1992, KNOLL ET AL. 1986), supportive of the proposed late Proterozoic rise in atmospheric oxygen. No additional evidence, however, has been available to verify this rise in oxygen. In this contribution we report a significant evolutionary radiation of nonphotosynthetic marine sulfide oxidizing bacteria, and a gramatic increase in sulfur isotope fractionation at between 0.67 to 1.05 Ga. We argue that both events were driven by oxygenation of the marine environment, consistent with an increase in atmospheric oxygen to 5 to 18% of present levels.

Bacterial Evolution: The phylogeny of the Prokaryotes, as revealed by 16S rRNA sequences, demonstrates not only genetic relationship, but also major evolutionary radiations of bacteria with similar metabolisms, or with similar environmetal niches (WOESE 1987). In general, major bacterial groups are viewed to originate through rapid, episodic evolution, imposed by environmental change (WOESE 1987). The oxygenation of bacterial habitats is such a change, which triggered the relatively late emergence of aerobic and microaerophilic bacteria throughout the Eubacterial kingdom (FOX ET AL. 1980, WOESE 1987). We focus here on the radiation of nonphotosynthetic sulfide-oxidizing bacteria of the gamma Proteobacterial subdivision, emerging near the basal transition of the gamma and the beta subdivisions (VAN DE PEER ET AL. 1994). This radiation produced the largest cluster of aerobic and microaerophilic sulfide oxidizers among the Eubacteria, the timing of which we thus argue constrains the oxygenation of sulfide-containing bacterial, most commonly marine, habitats. Sulfide oxidizers are of special interest because the occurrence of

obligate sulfide oxidizing bivalve sysmbionts, within the same Proteobacterial subdivision, allows us to date the radiation event.

We consider the microaerophilic filamentous sulfur bacteria, *Beggiatoa*, *Thiothrix* and *Thioploca*, and the aerobic to microaerophilic non-filamentous *Thiomicrospira* and *Thiobacillus*, which all oxidize sulfide or sulfur with either oxygen or nitrate as the electron acceptor. *Thiothrix* grows in tufts attached to pebbles, rocks and plant material, in sulfide and oxygen-containing flowing water (BLAND & STALEY 1987). *Beggiatoa*, a common constituent of modern microbial mats and sulfidic sediments, is typically found in steep opposing gradients of O_2 and H_2S, with the bacteria confined to the oxic-anoxic interface (JØRGENSEN 1982a). The denitrifying sulfide oxidizer *Thioploca* shuttles between spatially separated pools of sedimentary sulfide and seawater nitrate. *Thioploca* mats occur in low oxygen waters (<5µM O_2) of the Peru-Chile upwelling region (FOSSING ET AL. 1995) where nitrate is found at levels of 25 µM. Nitrate is formed from the aerobic decomposition of marine organic matter - approximately one mole nitrate produced for every 8.6 moles oxygen molecules used (REDFIELD ET AL. 1963) - and thus, the metabolism of *Thioploca* is indirectly coupled to an aerobic environment. *Thiomicrospira* sp. have been isolated from sulfidic marine sediments, hydrothermal vent waters and crusts, and the water column of the Black Sea (KUENEN ET AL. 1991, JANNASCH ET AL. 1991). *Thiomicrospira* sp. may grow in sulfide/oxygen gradients, but are not bound to such gradients. *Thiobacillus* sp. are also not bound to chemical gradients, and occur commonly in both water columns and sediments, using oxygen or nitrate in sulfide oxidation (KUENEN ET AL. 1991).

We use ribosomal RNAs (rRNA) to determine a time frame for the evolutionary radiation of these sulfide oxidizers. 16S rRNAs occur ubiquitously in all prokaryotes, and remain highly conserved in their primary sequence, secondary and tertiary structure, due to functional coupling with the evolution of the ribosome. Mutually independent mutations of the approximately 50 different secondary structure elements (WOESE ET AL. 1983) lead to a gradual accumulation over time of substitutions in the 16S rRNA sequence, rendering the molecule a potentially calibratable clock (OCHMAN & WILSON 1987). We calibrate our clock by utilizing obligately bivalve-hosted sulfide-oxidizing symbionts, which, like their free-living counterparts, *Beggiatoa, Thioploca, Thiothrix, Thiomicrospira,* and *Thiobacillus* (*T. thiooxidans, T. ferrooxidans, T. caldus*) represent deep-branching phylogenetic lineages emerging from near the root of the Proteobacterial gamma-subdivision (DISTEL ET AL. 1994, LANE ET AL. 1992, MUYZER ET AL. 1995b; POLZ ET AL. 1995; TESKE ET AL. 1995a). Between all these lineages, not uniform, but similar 16S rRNA sequence

distances have accumulated, averaging 15.9 ± 4.1% substitutions per site (Table 1). Similar sequence distances demonstrate similar substitution rates between freeliving and symbiotic sulfide oxidizers, minimizing complications imposed by large lineage to lineage variations in rate (WOESE 1987, YANG & WOESE 1989).

Table 1. 16S rRNA distances (Jukes-Cantor) ± standard deviations, between phylogenetic lineages of gamma-subdivision sulfur bacteria, incl. *Thiobacillus* of the beta-gamma transition. Average interlineage distance is 15.9 ± 4.1%.

	1.	2.	3.	4.	5.	6.
1. Endosymbionts Vesicomyidae-Mytilidae	---	13.11 ± 1.20	13.24 ± 0.32	14.52 ± 1.26	15.26 ± 0.92	20.33 ± 0.92
2. Endosymbionts Lucinacea-Solemyidae		---	12.26 ± 0.90	13.29 ± 1.51	13.82 ± 0.94	17.88 ± 1.18
3. Thiothrix			---	14.65 ± 0.94	16.54 ± 0.88	17.08 ± 0.79
4. Beggiatoa - Thioploca				---	17.58 ± 0.64	16.68 ± 1.37
5. Thiomicrospira					---	22.26 ± 0.59
6. Thiobacillus beta-gamma-Transition						---

16S rRNA sequence regions of ambiguous alignment, and incompletely sequenced 3' - and 5'- ends were excluded from the analysis. Distances were inferred using the Jukes-Cantor algorithm as implemented in the program DNADIST of the PHYLIP package 3.5c (FELSENSTEIN 1993, and references therein). The distance matrix was calculated from 16S rRNA positions 220-840, 846-1026, 1035-1134, 1140-1371 (E.coli numbering), total 1130 nucleotides. 16S rRNA sequences of the following organisms were used:

1.) Bacterial endosymbionts of *Vesicomyida chordata, Calyptogena magnifica, Calyptogena elongata, Calyptogena* species (Oregon), *Bathymodiolus thermophilus*

2.) Bacterial endosymbionts of *Thyasira flexuosa, Anodontia philippana, Codakia orbicularis, Lucina floridana, Lucinoma aequizonata, Lucinoma annullata*

3.) *Thiothrix nivea, Thiothrix ramosa*

4.) *Beggiatoa alba, Beggiatoa* species (Strain 1404-13 Göttingen), *Thioploca ingrica*

5.) *Thiomicrospira pelophila, Thiomicrospira crunogena, Thiomicrospira* species (hydrothermal vent strain MA2-6)

6.) *Thiobacillus caldus, Thiobacillus ferrooxidans* (strain N-Fe4), *Thiobacillus thiooxidans* (strain B-S3)

Sequence data from DISTEL ET AL. 1994 (obligate Bivalve endosymbionts), TESKE ET AL. 1995a (*Beggiatoa, Thioploca, Thiothrix nivea*), MUYZER ET AL. 1995b (*Thiomicrospira*), POLZ ET AL. 1995 (*Thiothrix ramosa*) and from the Ribosomal Database Project (MAIDAK ET AL. 1994).

To calculate substitution rates we assume congruent evolution between obligately bivalve-associated endosymbiotic bacteria and their host bivalvia *Solemyidae, Lucinidae, Thyasiridae, Vesicomyidae* (DISTEL ET AL. 1994). Supporting this association, bacterial symbiosis is argued to have provided the essential driving force for the evolution and radiation of the *Lucinidae* and *Thyasiridae*, their morphology, and their infaunal mode of life (REID & BRAND 1986). A long history of symbiosis is indicated for Lucinoid bivalves and *Solemya* by their association with fossil hydrothermal vents (GAILLARD ET AL. 1992, CAMPBELL & BOTTJER 1993). The dependence of the *Solemyidae* on their endosymbionts for nutrition, the reduction or total disappearance of mouth and gut, and their conserved morphology in the fossil record, support an early establishment of the symbiosis (CAVANAUGH 1994, POJETA 1988). Further, all members of the *Soleymidae, Lucinidae* and *Vesicomyidae* so far examined contain endosymbionts, arguing for symbiont-associated evolution of these families (CAVANAUGH 1994, DISTEL ET AL. 1994).

Thus, we compute the 16S rRNA sequence distances accumulated between the different symbiont species accociated with particular bivalve families, assuming that symbionts diverged in parallel with the evolution of their host bivalve family. Substitution rates are then obtained by combining these sequence distances with the palaeontological age of the respective bivalve families (Table 2). Substitution rates for endosymbionts from *Solemyidae, Lucinidae* and *Thyasiridae* average 1% per 47.7 ± 16 million years (ma), or 39.1 ± 14.3 ma including the unusually fast substitution rate of the *Vesicomyidae* symbionts (Tab. 2). These values compare to the general bacterial substitution rate, 1% per 50 to 60 ma, based on diversified bacterial lineages and environmental or physiological calibration events (OCHMAN & WILSON 1987), and the substitution rate of 1% per 25 - 50 ma for bacterial aphid symbionts (MORAN ET AL. 1993).

The symbiont-derived substitution rates, applied to the average interlineage distance of sulfide oxidizers (15.9 ± 4.1 %, Tab. 1), result in their evolutionary radiation at 758 ± 321 ma (without *Vesicomyidae*), or 621 ± 278 ma (with *Vesicomyidae*). Application of the generalized Eubacterial substitution rate, 1% per 50 to 60 ma, results in an age of 795 ± 205 to 954 ± 246 ma. All these data indicate a late Proterozoic origin of non-photosynthetic, sulfide-oxidizing bacteria of the Proteobacterial gamma subdivision. As argued previously, the requirements of these sulfide oxidizers for oxygen reflect their evolutionary origin in a time of increased oxygenation of sulfidic bacterial habitats, most common in the marine realm.

Table 2. 16S rRNA substitution rates of gamma subdivision sulfide oxidizing endosymbiotic bacteria.

Bivalve families with obligate endosymbionts	Symbionts of bivalve host species (DISTEL ET AL. 1994)	Paleontological age of host bivalve lineages	16S rRNA sequence distance of bivalve endosymbionts (average ± st. deviation)	16S rRNA substitution rate
1) Solemyidae	Solemya reidi Solemya velum	Llanvirn, 470 ma (COX ET AL. 1969, POJETA 1988)	7.25 %	1% / 64.8 ma
2) Lucinidae	Lucina floridana Codakia orbicularis Lucinoma aequizonata Anodontia philippiana	Middle or Upper Jurassic radiation, approx. 200 ma (COX ET AL. 1969, BRETSKY 1976)	4.93 ± 1.54 %	1% / 40.55 ± 12.69 ma
3) Thyasiridae	Thyasira flexuosa	Middle Triassic, Ladinian 235 ma (COX ET AL. 1969)	6.24 ± 0.75 %	1% / 37.65 ± 4.55 ma
4) Vesicomyidae	Calyptogena elongata Calyptogena magnifica Calyptogena sp. oregon Vesicomyida chordata	Oligocene, 35 ma (GOEDERT & SQUIRES 1993)	2.64 ± 0.39 %	1% / 13.25 ± 1.94 ma

The rates are calibrated by comparing the palaeontological age of host bivalves with the average mutual Jukes-Cantor-distance (%) of the respective endosymbionts. 16S rRNA sequence regions of ambiguous alignment, and incompletely sequenced 3'- and 5'- ends were excluded from the analysis. The distance matrix was calculated from 16S rRNA positions 220-840, 846-1026, 1035-1134, 1140-1371 (E.coli numbering), total 1130 nucleotides.

1) Comparison between S. reidi and S. velum symbiont.

2) Comparison between endosymbionts of Lucina floridana, Codakia orbicularis, Lucinoma aequizonata and Anodontia philippiana, representing four different lucinid phylogenetic lineages, of the jurassic radiation of the Lucinidae (BRETSKY 1976): Lucina, Codakia, Miltha and Anodontia. Lucinoma and Anodontia are oligocene and paleocene genera, but derived from jurassic antecedents, Miltha and Jagonoma (BRETSKY 1976).

3) Comparison of the thyasirid symbiont of Thyasira flexuosa with the four lucinid endosymbionts of 2), time-calibrated by middle triassic split of the Superfamily Lucinacea intro the families Thyasiridae and Lucinidae (COX ET AL. 1969). This calculation presupposes that the symbiosis was already well established within the superfamily Lucinacea at the time of the split (DISTEL ET AL. 1994, REID & BRAND 1986).

4) Comparison within the Vesicomyidae symbionts. Their unusually high substitution rate could either reflect rapid genomic evolution, in the consequence of a recent evolutionary radiation and speciation within the group, or it might be due to an underestimate of the age of the Vesicomyidae lineage.

Stable Isotopes: In modern marine sediments, and sulfide-rich euxinic water bodies (e.g. Black Sea), sulfide is produced by the bacterial reduction of sulfate. During this process, pure cultures of sulfate reducing bacteria produce sulfide depleted in the isotope ^{34}S by between 4 to 46 ‰ (Fig. 1) with an average of 18±10 ‰ (n=155). By contrast, the isotopic composition of marine sulfides exhibit much greater depletions in ^{34}S, with values ranging between 24 to 71 ‰, with an average of 51±10 ‰ (n=234) (Fig 1). These observations reinforce previous suggestions (KEMP & THODE 1968, JØRGENSEN 1990, CANFIELD & THAMDRUP 1994) that isotopic discrimination during bacterial sulfate reduction is unable to explain fully the isotopic composition of marine sulfides.

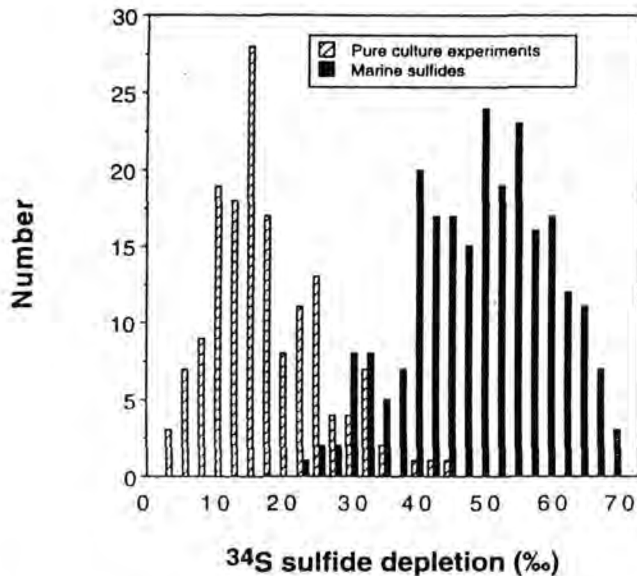


Fig. 1. Histogram of ^{34}S sulfide depletions (‰) for marine sedimentary sulfides and for sulfides generated by sulfate reducing bacteria. For sediments, ^{34}S depletions are taken as the difference between the isotopic composition of pore water sulfate and sedimentary sulfide. Isotopic values are generally from near the sediment-water interface where the isotopic composition of sulfate is near to the seawater value. Also, only one value per sediment is reported, which is the minimum measured. Minimum values should be most free of the „closed system" addition of ^{34}S-enriched sulfide (GOLDHABER & KAPLAN 1980) and hence best represent fractionations during the biological processing of sulfur. Experimental ^{34}S depletions are the difference between the isotopic composition of sulfate and the sulfide produced by pure cultures of sulfate reducing bacteria. All isotopic compositions were originally presented as ‰ (parts per thousand) differences relative to Canyon Diablo troilite (CDT), such that

$$\partial^{34}S_{sam} = [[(^{34}S/^{32}S)_{sam} - (^{34}S/^{32}S)_{CDT}] / (^{34}S/^{32}S)_{CDT}] \times 10^3$$

where sam is the sample, and $\partial^{34}S_{sam}$ is the original isotopic composition of the sample. List of references available on request.

The large ^{34}S depletions of modern sedimentary sulfides are argued to result from an initial fractionation by sulfate reducing bacteria (Fig. 1), followed by further ^{34}S-depletion during the oxidative part of the sulfur cycle (CANFIELD & THAMDRUP 1994, JØRGENSEN 1990). Results from modern, nonphotosynthetic, siliciclastic sediments, support this contention, where the depletion of sulfide in ^{34}S correlates with the extent of sulfide oxidation (Fig. 2A). Also, large ^{34}S-depletions of 62‰ and 53‰, respectively, are observed for sulfides in euxinic Black Sea and Cariaco Trench waters, far exceeding the 30‰ provided by sulfate reducers from the Black Sea water column (FRY ET AL. 1991). Substantial sulfide oxidation has been demonstrated in the Black Sea (JØRGENSEN ET AL. 1991). Modern photosynthetic microbial mats and marine sapropels provide an exception with less isotopic fractionation than anticipated (Fig. 2A). These isotopic systematics will be discussed below. Finally, we find no influence of sulfate reduction rate on the isotopic composition of sedimentary sulfides (Fig. 2B), as previously reported (GOLDHABER & KAPLAN 1975, OHMOTO ET AL. 1993).

Elemental sulfur has been implicated as an important reaction intermediate (CANFIELD & THAMDRUP 1994, JØRGENSEN ET AL. 1991, PYZIK & SOMMER 1981, ZHANG & MILLERO 1993), with the bacterial disproportionation of elemental sulfur to sulfate and sulfide, producing ^{34}S-depleted sulfur (CANFIELD & THAMDRUP 1994). Through repeated cycles of sulfide oxidation to elemental sulfur, followed by disproportionation, ^{34}S-depleted sedimentary sulfides can be generated, and, in general, the trends in Fig. 2 can be rationalized (CANFIELD & THAMDRUP 1994). Other parts of the oxidative sulfur cycle, such as thiosulfate formation and disproportionation, could also lead to ^{34}S-depleted sulfides (JØRGENSEN 1990), though these isotopic systematics have yet to be reported. In modern nonphotosynthetic sediments and euxinic water bodies, sulfide is oxidized either directly or indirectly with oxygen (Fig. 2) (CANFIELD ET AL. 1993, JØRGENSEN ET AL. 1991). These observations form the basis for our interpretation of the isotopic record of sedimentary sulfides.

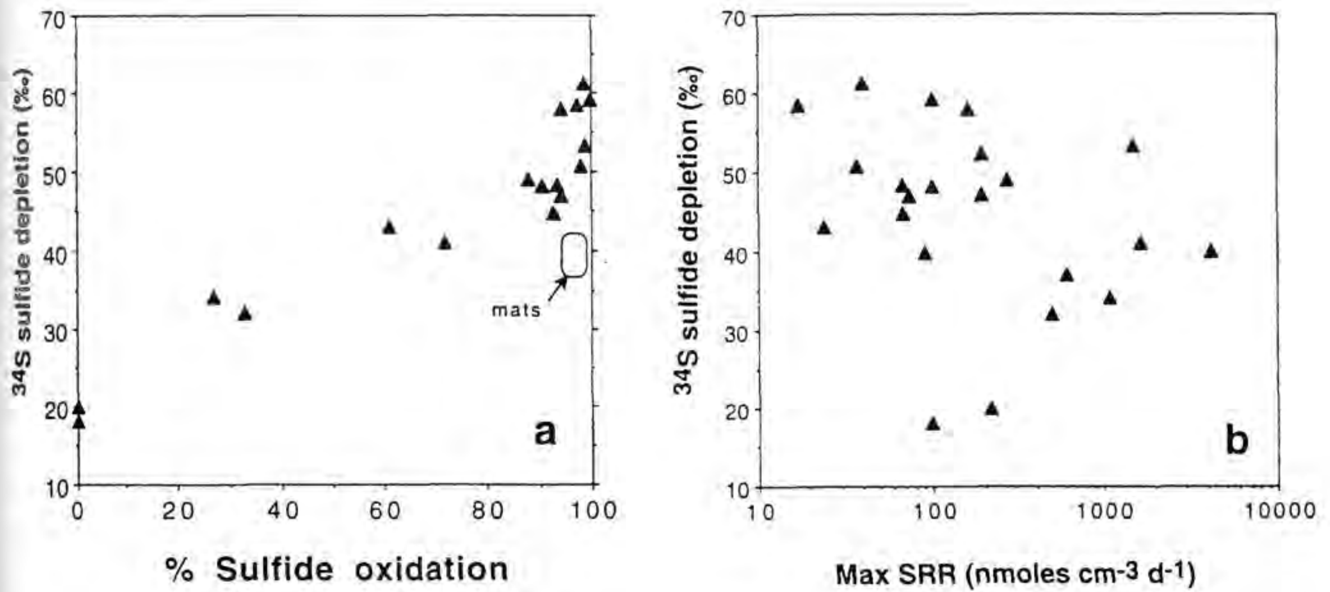


Fig. 2A. Sulfide ^{34}S depletion for sedimentary sulfides (see Fig. 1) compared to per cent of sulfide produced by sulfate reduction that is lost from the sediment (R^2=0.888). Sulfate reduction rates have in all cases been directly measured with radiotracer. Sulfide loss is calculated as the per cent difference between the depth integrated rate of sulfate reduction and the rate of sulfur burial (as pyrite and acid volatile sulfide phases) in the sediment. Fractionation values at no sulfide oxidation are from published experimental sediment incubations (NAKAI & JENSEN 1964), and from the present study, for sediment from the mouth of the Weser estuary. Data from CANFIELD & THAMDRUP 1994, and from the present study, from sediment from the Belt Seaway, Northern Denmark, Chilean upwelling zone, and Golfo Dulce, Costa Rica. Included is also a region representing the isotopic composition and per cent sulfide oxidation for cyanobacterial microbial mats from Baja, Mexica, Solar Lake, Sinai, and the photosynthetic marine sapropel at Mangrove Lake, Bermuda. In modern photosynthetic sediments sulfide is oxidized by both phototrophic pathways (not requiring oxygen), and nonphototrophic pathways (requiring oxygen, see below). In nonphotosynthetic sediments sulfide is oxidized either directly with oxygen, or, indirectly, by reaction with Fe and Mn oxides, and nitrate. these oxidants are generated, and regenerated by oxidation of their reduced forms (Fe^{2+}, Mn^{2+}, and NH_4^+) with oxygen.

Fig. 2B. ^{34}S sulfate depletion compared to maximum rate of sulfate reduction measured by radiotracer in marine sediments. An R^2=0.023 demonstrates a lack of significant correlation. Data from same sediments as in A), with a few additional sediments for which % sulfide oxidation could not be calculated.

An extensive compilation of sedimentary sulfide isotopic compositions, dating from the present through the Mesoproterozoic, is presented in Fig. 3. The data include 1840 individual analysis, of which 1213 are from the Phanerozoic (<0.544 Ga) and 627 are from the Neo and Mesoproterozoic (>0.544 Ga). Through the Phanaerozoic, the most ^{34}S-depleted sulfides follow the seawater sulfate curve with an approximate displacement of 55‰ (Fig. 3). Overall, 16% of the data show maximum discriminations of 55‰ or more, with 34% of all data having discriminations of equal or greater than 45‰ compared to seawater sulfate. In only one instance has a discrimination as great as 45‰ been reprted for sulfate reducing bacteria (Fig. 1), which cannot, therefore, be taken as a typical fractionation. Hence, marine sulfides are commonly formed in the Phanerozoic with isotopic discriminations greater than can be generated by sulfate reducing bacteria, strongly indicating the operation of the oxidative sulfur cycle as described above.

A similar pattern persists into the Neoproterozoic with the deposition of the Kaza group and Issac formation of the Windermere Supergroup at between 0.64 and 0.67 Ga (ROSS ET AL. 1995). Single isolated analysis of the Little Dal group (0.80 Ga, HAYES ET AL. 1992) and the Awatubi member of the Chuar group (0.862 Ga; HAYES ET AL. 1992) suggest that Phanerozoic patterns of fractionation may persist to 0.86 Ga. All of these predate the evolution of bioturbating organisms (KNOLL 1992), which importantly, decouples large isotopic fractionations from the possible influence of benthic infauna on sulfur cycling. Before 0.86 Ga no isotope analysis (n=383) yields discriminations of ≥45‰ compared to seawater sulfate, and only 7 analysis yield discriminations greater than 35‰.

The isotope record thus demonstrates a fundamental change in the processes controlling the isotopic composition of sedimentary sulfides between 0.67 and 1.05 Ga. This is consistent with the widescale initiation of the oxidative sulfur cycle, as driven by the accumulation of oxygen in the atmosphere and marine realm. This time frame is contemporaneous with the evolution of sulfide-oxidizing bacteria, and the liberation of oxidants during the enhanced burial of organic carbon during the late Proterozoic (KNOLL ET AL. 1986). While such a timing predates the appearance of the Ediacaran fauna, it is consistent with the evolution of the first Metazoans as indicated by amino acid comparisons (RUNNEGAR 1991).

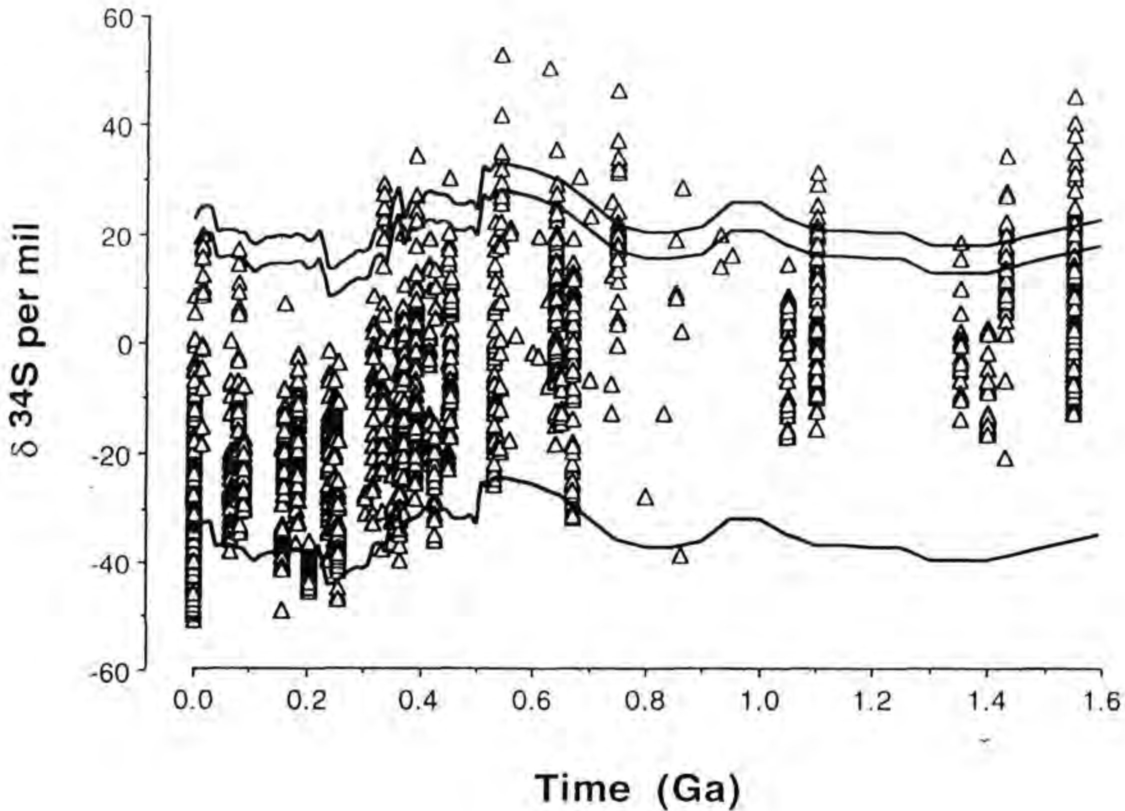


Fig. 3. Isotopic composition of sedimentary sulfides from the present through the Mesoproterozoic (1.6 Ga). Isotopic compositions from earlier times, particularly before 2 Ga, may have been influenced by low seawater sulfate levels (CAMERON 1982), and are not included here. The data only include sulfides whose association with bacterial processes is probable. Hence, sulfides from highly metamorphosed (beyond Greenschist facies) sediments, sulfides hosted in nonsedimentary rocks, and those of hydrothermal origin have not been used. Except for some copper sulfide analysis from the 1.1 Ga Nonesuch shale, Michigan (BURNIE ET AL. 1972), only pyrite analysis have been included. Also included is a band (with a width of 5‰) representing the isotopic composition of seawater sulfate (LINDH 1983, STRAUSS 1993), and a curve showing seawater sulfate displaced by 55‰. Seawater sulfate values are fairly certain in the Phanaerozoic, becoming less certain in the Proterozoic. However, to affect the interpretations offered here, the isotopic composition of seawater sulfate would need be consistently underestimated by 20‰ in sediments older than 1 Ga, for which there is no evidence. Complete list of references is available on request.

Transition from low to higher oxygen levels. Before the proposed oxygenation of the marine realm and the evolution of oxygen and nitrate respiring nonphotosynthetic sulfur bacteria, sulfide oxidation would likely have occurred by anoxygenic phototrophic bacteria (representing, in part, very ancient phylogenetic lineages, WOESE 1987), in the water column, and, on the seafloor, in photosynthetic oxygen-producing, microbial mats and stromatolites. Benthic photosynthetic communities are well known in the middle and late Proterozoic (WALTER 1976), while low oxygen conditions would favor the widespread occurrence of ocean anoxia and associated sulfidic conditions (LOGAN ET AL. 1995). The isotope record suggests that sulfur processing under such circumstances should not have led to large depletions in ^{34}S (Fig. 3). Using the physiology of anoxygenic phototrophs as a guide, we review the circumstance that may have led to reduced fractionations.

Hence, in photosynthetic systems with low sulfide availability, anoxygenic photosynthetic bacteria generally oxidize sulfide directly to sulfate (PFENNIG 1975), with only small associated fractionations (CHAMBERS & TRUDINGER 1979). Thus, the formation of elemental sulfur or other sulfur intermediates, and the associated fractionations during their disproportionation, are not favored. When sulfide availability is high, elemental sulfur is a preferred oxidation intermediate, which, with all *Clorobium* sp., *Ectothiorodospira*, some *Rhodospirillaceae*, and *Chloroflexus* sp. is excreted from the cell to the environment (PFENNIG 1975, TRÜPER & FISCHER 1982). This elemental sulfur may be further oxidized by anoxygenic phototrophs to sulfate. Metabolism by disproportioning bacteria, however, is inhibited at even relatively moderate sulfide levels of >1 mM (THAMDRUP ET AL. 1993). Thus, the reduced ^{34}S sulfide depletion in pre 0.86 sedimentary sulfides could be explained by more direct oxidation of sulfide to sulfate by anoxygenic phototrophs, or an inhibition of elemental sulfur disproportionation by high sulfide levels.

The reduced ^{34}S-sulfide depletions of modern microbial mats and photosynthetic sapropels (Fig. 2A) can be explained by a similar combination of factors. Key aspects of the mat environment include rapid rates of sulfate reduction, populations of anoxygenic phototrophs, and diel oxygen fluctuations (CANFIELD & DES MARAIS 1993). These factors have likely changed little over the course of time considered here, except for the probable introduction of nonphotosynthetic sulfide oxidizers after their late Proterozoic evolution. Hemce, low ^{34}S sulfide depletions in modern mats may be taken to represent and justify a lack of large depletions over time in similar benthic communities.

By contrast, the large ^{34}S sulfide depletions in modern euxinic basins (FRY ET AL. 1991) are not represented in the pre 0.86 sulfide record, and in particular, in

reputed ancient euxinic environments, such as for example, the 1.1 Ga Nonesuch Shale (max. 36‰) (HIESHIMA & PRATT 1991, BURNIE ET AL. 1972).
Unlike microbial mats, the invasion of oxygen would have profoundly affected euxinic water bodies. Possible changes include deepening of the oxic/anoxic interface, decreased sulfide levels due to increased aerobic carbon oxidation, the direct reaction of sulfide with oxygen, producing more complex sulfide intermediates, and, after their evolution, the colonization of, and increasing dominance of nonphotosynthetic sulfide oxidizers. In other words, a similar situation to modern euxinic environments would have developed.
A further consequence of increased oxygen is its diffusion into nonphotosynthetic marine sediments. With insufficient oxygen, the sulfide produced by sulfate reduction would diffuse from the sediment, for probable water-column oxidation by phototrophs as described above. The penetration of oxygen into the sediment allows multiple respiratory pathways of carbon oxidation (other than just sulfate reduction), and the complex recycling of modern sediments (CANFIELD ET AL. 1993). These same circumstances are envisioned to initiate the oxidative sulfur cycle, encouraging the formation of highly ^{34}S-depleted sulfides.

Constraints on oxygen levels. There is a general requirement of *Beggiatoa* to live sediment-bound in steep opposing gradients of sulfide and oxygen (JØRGENSEN 1982a). Some representatives of other nonphotosynthetic sulfide oxidizing genera are also sediment-bound requiring oxygen (see above). Hence, the evolution of these sulfide oxidizers, as with large ^{34}S sulfide depletions in nonphotosynthetic marine sediments, should be contemporaneous with oxygenation of the sediment surface. We offer an estimate of the oxygen levels required to oxygenate nonphotosynthetic marine sediments.
Oxygen diffuses to the sediment surface along a diffusional gradient through a static benthic boundary layer (Fig. 4). To penetrate to and through the sediment surface, the flux of oxygen must meet or exceed the demands for oxygen in the sediment. Sediment oxygen demand is determined by the carbon oxidation rate of the sediment regardless of whether oxygen is used to oxidize carbon directly, or the reduced products of anaerobic carbon oxidation including sulfide, Fe^{2+}, Mn^{2+}, and ammonia (CANFIELD ET AL 1993). A likely range of oxygen demand is provided from an extensive compilation of carbon oxidation rates for modern coastal sediments of < 200 meters water depth; a depth likely to maintain approximate exchange equilibrium with atmospheric oxygen. In a relatively low oxygen atmosphere, as will satisfy the minimum oxygen requirements of this calculation, greater water depths may have been completely anaerobic (LOGAN

ET AL. 1995), or have experienced significant oxygen depletions, and are thus not of interest here. Carbon oxidation rates range between 3.9 to 82 mmoles m^{-2} d^{-1}, with a median of 13.7 (n=60). We assume that late Proterozoic coastal sediments experienced a similar range of rates. Thus, the average organic carbon contents of late Proterozoic sediments (0.4 wt%, SCHIDLOWSKI 1993), are broadly similar to modern sediments (0.65 wt%, SCHIDLOWSKI 1993), accounting for some carbon loss through deep burial, and thermal maturation. The above carbon oxidation rates correspond to a similar range of oxygen demand, assuming that one mole of oxygen oxidizes one mole of organic carbon. Using Fick's first law, this range of oxygen demand is balanced by diffusion from overlying water containing oxygen at between 13 to 552 µM, with a median value of 46 µM (Fig. 4).

One might assume that bacterial evolution would be induced when somewhere between the minimum (a small number) and the mean (half) of the sediments became exposed to oxygen. Random sampling might uncover ^{34}S-depleted sulfides for a similar number of sediments experiencing oxygenation. This yields a range in oxygen concentrations of between 13 to 46 µM, or between 5 to 18% PAL, with an air saturated PAL of 254 µM at 15°C. This is our best estimate for the minimum level of atmospheric oxygen attained sometime between 670 and 1050 Ga.

This rise in oxygen levels considerably post-dates an earlier Earth surface oxidation event at around 2.0 Ga (DES MARAIS ET AL. 1992, HOLLAND ET AL. 1989, HOLLAND & BEUKES 1990), where the modelling of soils developed on basalts (HOLLAND ET AL. 1989) yields estimates of atmospheric oxygen of greater than or equal to 1 % PAL. The retention of iron during the weathering of the Kuruman Iron Formation from South Africa at between 2.2 to 1.9 Ga suggests higher levels of up to 15% or greater PAL (HOLLAND & BEUKES 1990). Our results suggest that the oxygen generated at around 2.0 Ga did not apparently maintain, into the middle and late Proterozoic, high enough levels to substantially affect sulfur fractionation, or to induce the evolution of colorless sulfur bacteria.

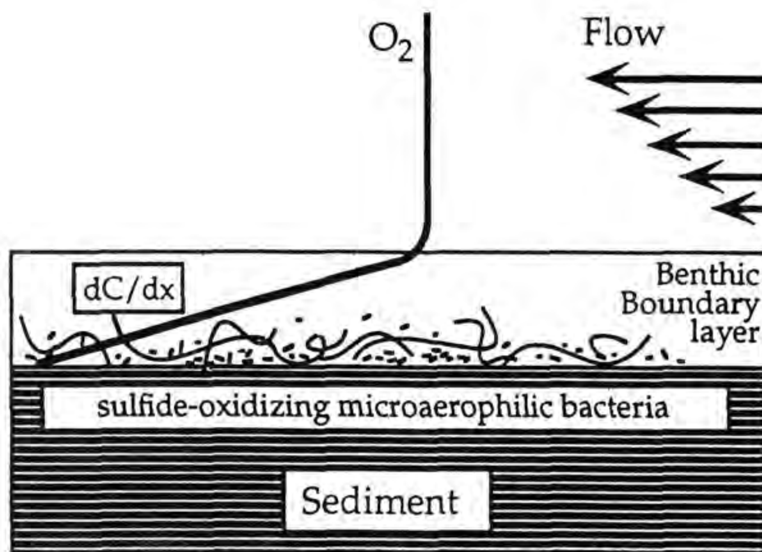
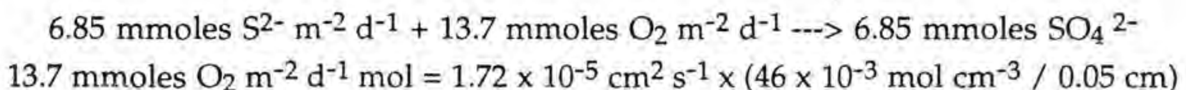


Fig. 4. Cartoon representing the model used to constrain the water-column oxygen levels needed to oxygenate surface sediments. The supply of oxygen to the sediment surface is limited by the concentration of oxygen in the overlaying water, and the thickness of the benthic boundary layer, by Fick's first law:

$$O_2 \text{ flux} = D_{O2} (dC/dx)$$

where O_2 flux is the flux of oxygen to the sediment surface, D_{O2} is the diffusion coefficient of oxygen, x is the thickness of the benthic boundary layer, with C as the concentration of oxygen in the overlying water. Hence, dC/dx is the oxygen gradient to the sediment surface, assuming that oxygen goes to zero concentration at the sediment surfac. The parameters D_{O2} , and x are fixed by the demand for oxygen as determined by the sediment metabolic rate (see text). The value of C is uniquely provided for given values of D_{O2}, x, and O_2 flux. This calculation assumes diffusion through a benthic boundary layer 500 µm thick (GUNDERSEN 1990), and a diffusion coefficient for oxygen at 15°C of 1.72×10^{-5} cm^2 s^{-1} (LERMAN 1979).



6.85 mmoles S^{2-} m^{-2} d^{-1} + 13.7 mmoles O_2 m^{-2} d^{-1} ---> 6.85 mmoles $SO_4{}^{2-}$

13.7 mmoles O_2 m^{-2} d^{-1} mol = 1.72×10^{-5} cm^2 s^{-1} x (46×10^{-3} mol cm^{-3} / 0.05 cm)

List of references are available on request.

5. ZUSAMMENFASSUNG, DISKUSSION UND AUSBLICK

Die Ergebnisse und Publikationen, die aus dieser Doktorarbeit hervorgegangen und entweder zur Veröffentlichung vorgereitet, eingereicht oder angenommen worden sind, werden zusammengefaßt und z.T mit Ausblick auf geplante oder bereits weitergeführte Arbeiten diskutiert. Hierbei werden auch bisher unveröffentlichte Ergebnisse miteinbezogen, die besonders für die Arbeiten zum Solar Lake wichtig sind. Die Ergebnisse aus Solar Lake und Mariagerfjord werden miteinander verglichen und gegenübergestellt.

5.1. SAUERSTOFFTOLERANTE SULFATREDUZIERENDE BAKTERIEN

Molecular identification of bacteria from a co-culture by Denaturing Gradient Gel Electrophoresis of 16S ribosomal DNA fragments as a tool for isolation in pure cultures. Andreas Teske, Pavel Sigalevich, Yehuda Cohen, Gerard Muyzer.

In dieser Arbeit wird eine Mischkultur aus einem *Desulfovibrio*-Stamm, nahe verwandt zu *Desulfovibrio oxyclini* (siehe folgende Arbeit), und einem fakulativ aeroben *Arcobacter*-Stamm analysiert, die aus der tagesperiodisch oxischen Oberflächenschicht der Cyanobakterienmatte des hypersalinen Experimentalteichs am Heinz-Steinitz-Laboratorium für Meeresbiologie, Eilat, Israel, in Sulfid-Sauerstoff-Agargradienten isoliert wurde. Einwöchige Chemostatenkultur unter Sauerstoff-Limitierung sowie darauf folgende wiederholte aerobe Transfers auf Agarplatten konnten *Desulfovibrio* nicht ausselektieren, der nach Transfer auf anaerobes Medium stets neu aufwuchs. Mittels DGGE und nachfolgender Sequenzierung der DGGE-Amplifikate war es möglich, die beiden Stämme der Cokultur zu identifizieren, und auf der Basis dieser Information selektive Anreicherungsbedingungen für *Desulfovibrio* und *Arcobacter* zu wählen, unter denen schließlich beide Komponenten unabhängig voneinander als Reinkulturen isoliert werden konnten. Der *Desulfovibrio*-Stamm zeigte als Reinkultur unter Sauerstoff (2%, 5%, 20%) kein Wachstum, während das *Arcobacter*-Isolat auch in Gegenwart von 20% O_2 in der Gasphase wuchs, sofern CO_2 in erhöhten Konzentrationen (5-10%) vorhanden war.
Unter Sauerstofflimitierung sind Chemostaten-Cokulturen anaerober, sulfatreduzierender Bakterien oder methanogener Archaeen mit fakultativen Aerobiern stabil (GOTTSCHAL & SZEWZYK 1985, GERRITSE ET AL. 1990). Die im Tages-Nacht-Rythmus schwankende Sauerstoffkonzentration in Cyanobakterien-matten kann durch das Umschalten eines Chemostaten von anaeroben auf oxische Bedingungen simuliert werden. Im Chemostaten bleibt

eine anaerob gestartete Cokultur eines fakultativ aeroben *Marinobacter*-Stamms mit *Desulfovibrio oxyclini* (siehe unten) in der oxischen Phase des Zyklus, unter verändertem Zellzahlenverhältnis (*Desulfovibrio:Marinobacter* 1:20) stabil (BAEV, TESKE ET AL., unveröffentlichte Ergebnisse). Auch bei Andauern der oxischen Phase über zehn Tage bleibt die hohe Zelldichte von *Desulfovibrio oxyclini*, zwischen 10^8 - 10^9 Zellen pro ml, erhalten. Diese Ergebnisse demonstrieren die relative Unempfindlichkeit einer Sulfatreduziererpopulation, vergesellschaftet mit einem aeroben Bakterium, gegenüber Sauerstoffstreß, und lassen sich auf sulfatreduzierende Bakterien in oxischen Oberflächenschichten von Cyanobakterienmatten übertragen, in denen Sulfatreduzierer mit verschiedenen aeroben Bakterienpopulationen vergesellschaftet vorliegen.

***Desulfovibrio oxyclini* sp. nov., a new sulfate reducing bacterium from the oxic layer of a hypersaline cyanobacterial mat from Solar Lake, Sinai.** Daniel Krekeler, Pavel Sigalevich, Andreas Teske, Heribert Cypionka, Yehuda Cohen.

Der *Desulfovibrio*-Stamm PIB wurde charakterisiert und als neue *Desulfovibrio*-Spezies *Desulfovibrio oxyclini* beschrieben. Stamm PIB wurde erstmals aus 10^7-fach verdünnten Anreicherungen der tagesperiodisch oxischen Oberflächenschicht der Cyanobakterienmatte des hypersalinen Experimentalteichs am Heinz-Steinitz-Laboratorium für Meeresbiologie, Eilat, Israel, isoliert. Stamm PIB konnte weiterhin aus hohen Verdünnungsstufen (10^4 - 10^6 Zellen per ml) von Most-Probable-Number-Zählungen der Sulfatreduzierer der tagesperiodisch oxischen Oberflächenschicht der Solar-Lake-Cyanobakterienmatte isoliert werden.

Desulfovibrio oxyclini ist durch physiologische Besonderheiten an die Lebensweise in tagesperiodisch oxischen Habitaten angepaßt. Neben Sulfat, Sulfit und Thiosulfat kann Sauerstoff als Elektronenakzeptor genutzt werden, der mit Wasserstoff als Elektronendonor die höchsten Respirationsraten ergibt; $\geq$ 260 nmol O_2 min^{-1} $\geq$ mg $protein^{-1}$, oder $\geq$ 1040 nmol e^- min^{-1} mg $protein^{-1}$, etwa in der Größenordnung von *E.coli* (DILLING & CYPIONKA 1990). Aerobe Atmung mit Wasserstoff ist bisher bei *Desulfovibrio desulfuricans*, *Desulfovibrio vulgaris* und *Desulfobacterium autotrophicum* festgestellt worden, nicht dagegen bei *Desulfobulbus*, *Desulfobacter*, *Desulfococcus*, *Desulfotomaculum*, und marinen *Desulfovibrio*-Stämmen (DANNENBERG ET AL. 1992), letzteres mit der hier beschriebenen Ausnahme von *Desulfovibrio oxyclini*. Aerobe Atmung ist bei *Desulfovibrio oxyclini* nicht mit Wachstum gekoppelt. Die Frage bleibt offen, wie unter oxischen Bedingungen in den Oberflächenschichten von Cyanobakterienmatten Sulfatreduktion stattfinden kann, da Sauerstoff als

bevorzugter Elektronenakzeptor die Sulfatreduktion, bei *Desulfovibrio oxyclini* wie auch bei allen anderen bisher getesteten Sulfatreduzierern, in Reinkulturen supprimiert (MARSCHALL ET AL. 1993).
Wasserstoff wird als Nebenprodukt der Nitrogenase der Cyanobakterien produziert, und kommt damit als Atmungssubstrat für Sulfatreduzierer in der Cyanobakterienmatte in Frage (HOUCHINS 1984, STEWARD 1980). Es ist geplant, weitere Desulfovibrio-Isolate aus den oxischen Schichten der Solar-Lake-Matte in Zusammenarbeit mit Prof. Heribert Cypionka auf ihre Kapazitäten für aerobe Atmung mit Wasserstoff zu testen, und parallel zu dieser physiologischen Untersuchung die für das Genus *Desulfovibrio* spezifischen [NiFe]-Hydrogenasen der Solar-Lake-Isolate molekular zu charakterisieren. Mit einem spezifischen PCR-Assay für *Desulfovibrio*-Hydrogenasen werden Sequenzabschnitte von *Desulfovibrio*-[NiFe]-Hydrogenasen amplifiziert, über DGGE analysiert und mit Referenzspezies verglichen; neue Hydrogenasenfragmente werden zur näheren Identifizierung sequenziert (WAWER & MUYZER 1995).

Molecular and microbiological analysis of sulfate-reducing bacterial populations in the surface layer of a hypersaline cyanobacterial mat of Solar Lake. Andreas Teske, Niels B. Ramsing, Manabu Fukui, Yehuda Cohen.

Die Zelldichte sulfatreduzierender Bakterien in der Oberflächenschicht des Solar Lake (Sinai) wurde mit Most-Probable-Number-Verdünnungsreihen analysiert. Die Zelldichte lactat- und acetatoxidierender Sulfatreduzierer in der tagsüber oxischen und größtenteils sauerstoffübersättigten obersten Mattenschicht (0-2 mm) liegt bei ca. 10^6 Zellen pro ml, mit Schwankungsbreiten von 10^5 bis zu 5×10^6 Zellen pro ml. In der Chemokline (2-4 mm) finden sich z.T. maximale Zelldichten von $0.4 - 4 \times 10^7$ kultivierbaren Sulfatreduzierern pro ml. In der Oberflächenschicht und in der Chemokline wurde in maximalen Zelldichten von 10^5, 10^6 und 10^7 Zellen pro ml, sowohl in den Acetat- als auch in den Lactat-Verdünnungsreihen, der actetat-und andere kurzkettige Fettsäuren oxidierende, filamentöse Sulfatreduzierer *Desulfonema* gefunden. *Desulfonema* wurde bis auf die Erstbeschreibung (WIDDEL ET AL. 1983) bisher wenig untersucht, obwohl er in mikrobiellen Matten und organischen Sedimenten sehr weit verbreitet ist. In Schlicksedimenten und Cyanobakterienmatten von Nord- und Ostseeküste, in Süßwasserquellen, und im Sediment der chilenischen Pazifikküste wurde *Desulfonema* gefunden. *Desulfonema* wurde als Begleitorganismus des mattenbildenden denitrifizierenden Sulfidoxidierers *Thioploca* vor der chilenischen Küste mit *Desulfonema*-spezifischen fluorophor-markierten 16S rRNA-Sonden nachgewiesen (FUKUI ET AL. 1995, KÜVER, unveröffentlicht).

Zur molekularen Untersuchung und Überprüfung dieser Ergebnisse wurden Nukleinsäuren aus millimeterdicken Schichten der Solar-Lake-Matte isoliert, 16S rRNA Teilsequenzen wurden PCR-amplifiziert sowie über DGGE analysiert. Die DGGE-Muster wurden mit einer 16S rRNA Sonde für die phylogenetische Gruppe *Desulfonema* und die nahe verwandten Genera *Desulfosarcina* und *Desulfococcus*, sowie mit einer für *Desulfovibrio oxyclini* und nahe verwandte Stämme spezifischen 16S rRNA Sonde analysiert. Die *Desulfonema*-Sonde hybridisierte mit einem PCR-amplifizierten 16S rRNA Fragment aus DNA der obersten 6 - 8 Millimeter der Solar-Lake Matte. Dieses dominante molekulare Isolat gehört zur Gruppe der untereinander verwandten Sulfatreduzierer *Desulfonema*, *Desulfococcus*, *Desulfosarcina*, *Desulfobotulus*, die, letzteren ausgenommen, neben einer Vielzahl anderer Kohlenstoffsubstrate auch Acetat vollständig oxidieren können. Mit einer rRNA-Sonde, die ursprünglich für *Desulfococcus*, *Desulfosarcina*, *Desulfobotulus* entworfen wurde (DEVEREUX ET AL. 1992), aber auch zu den nahe verwandten *Desulfonema* 16S rRNA Sequenzen komplementär ist, wurden sulfatreduzierende Bakterien dieser Gruppe als dominante Sulfatreduzierer-Population in der oxischen Oberflächenschicht der hypersalinen Cyanobakterienmatte von Guerrero Negro nachgewiesen, während *Desulfovibrio* die zweitstärkste Gruppe darstellte (RISATTI ET AL. 1994). Die stimulierende Wirkung von Acetat auf die Sulfatreduktion in der obersten Mattenschicht (FRÜND & COHEN 1992) ist mit diesen molekularen Befunden konsistent. Acetat, Formiat, Glykolat, Oxalat, Lactat und Ethanol werden unter anderem als Zerfalls- und Gärungsprodukte von Cyanobakterien gebildet (HEYER & KRUMBEIN 1991).

Unsere Anfangshypothese, daß vorwiegend *Desulfovibrio*, und besonders sauerstofftolerante Spezies wie *Desulfovibrio oxyclini* , die wiederholt aus hohen Verdünnungsstufen (10^4, 10^5 und 10^6 Zellen pro ml) von Most-Probable-Number Verdünnungsreihen der obersten Mattenschicht resoliert worden, die alleinigen dominanten Sulfatreduzierer der tagesperiodisch oxischen Mattenoberfläche darstellten, muß revidiert werden. Im DGGE-Bandenmuster der Solar Lake Isolate ist das 16S rRNA-Fragment von *Desulfovibrio oxyclini*, allerdings nur in vereinzelten Proben, zu erkennen. Neben den von *Desulfovibrio oxyclini* entwickelten physiologischen Anpassungen, wie Sauerstoffrespiration mit Wasserstoff, und Disproportionierung von $S_2O_3^{2-}$, ist die Strategie von *Desulfonema*, des filamentöser Sulfatreduzierers mit der Möglichkeit zur Aggregation und Bewegung in der dichten Matrix der Cyanobakterienfilamente, eine weitere und mindestens ebenso erfolgreiche Anpassung an die spezifischen Verhältnisse der oxischen Cyanobakterienmatte. Das aus der Solar Lake Matte

gewonnene dominante molekulare Isolat weist auf weitere, noch nicht bekannte Sulfatreduzierer der *Desulfonema-Desulfococcus-Desulfosarcina-Desulfobotulus*-Gruppe hin, die in der oxischen Oberflächenschicht und in der Chemocline der Cyanobakterienmatte von großer Bedeutung sind.

Die spezifischen Sulfatreduktionsraten der sulfatreduzierenden Bakterien in der oxischen Oberflächenschicht (0-2 mm) der Solar Lake-Cyanobakterienmatte liegen, bei konservativer Auswertung der MPN-Zählungen, zwischen 4 - 6 x 10^{-4} nmol SO_4^{2-}/Zelle d^{-1}. Durch Vergleich der Zelldichten mit Sulfatreduktionsraten, die zur gleichen Zeit und mit den gleichen Proben, in derselben Millimeterzonierung, gemessen wurden (HABICHT, unpublished), ergaben sich folgende spezifische Sulfatreduktionsraten in der Oberflächenzone der Solar Lake-Matten:

Tageszeit	Schicht	Kultivierbare Sulfatreduzierer cm^{-3} Lactat + Acetat	Sulfatreduktionsraten nmol cm^{-3} d^{-1} HABICHT unpubl.	Spezifische Sulfatreduktionsraten mol SO_4^{2-}/Zelle d^{-1}
12°°	0-2 mm	4.740.000	2207	4.656 x 10^{-13}
12°°	2-4 m m	2.730.000	3127	11.454 x 10^{-13}
12°°	4-7 mm	1.860.000	2779	14.941 x 10^{-13}
12°°	7-10 mm	3.030.000	1119	3.693 x 10^{-13}
12°°	10-13 mm	2.430.000	547	2.251 x 10^{-13}
24°°	0-2 mm	1.800.000	1002	5.567 x 10^{-13}
24°°	2-4 mm	11.600.000[1)]	1842	1.589 x 10^{-13}
24°°	4-7 mm	1.860.000	1831	9.844 x 10^{-13}
24°°	7-10 mm	465.000[2)]	761	16.366 x 10^{-13}
24°°	10-13 mm	1.740.000	472	2.713 x 10^{-13}
Vergleichswerte JØRGENSEN& COHEN 1977 0-5 mm		2.000.000	5400	27.000 x 10^{-13}

[1)] Sehr hohe Zelldichten auf Acetat, 9.300.000 Zellen cm^{-3}

[2)] Auffallend niedrige Zellzahlen auf Acetat und Lactat

Die MPN-Zahlen in dieser Tabelle sind immer konservativ gewählt, d.h. bei mehrdeutigen Proben und Unsicherheiten in der Zählung wurden solche Proben als negativ angesehen und die dementsprechenden kleineren Werte aus den MPN-Tabellen verwendet.

Die spezifischen Aktivitäten von H_2-, lactat- und pyruvatoxidierenden sulfatreduzierenden Bakterien in Reinkultur, 0.2 - 50 x 10^{-15} mol SO_4^{2-}/Zelle d^{-1} (JØRGENSEN 1978b), multipliziert mit den MPN-Zelldichten, liegen mindestens um Faktor 10 unter den tatsächlich gemessenen Sulfatreduktionsraten der

Cyanobakterienmatte. Die tatsächlichen Zelldichten in der Matte sind mindestens um Faktor 10 größer als die hier erhaltenen Werte. Hierbei wird vorausgesetzt, daß die spezifischen Sulfatreduktionsraten der Sulfatreduzierer in der Cyanobakterienmatte den Laborraten für Reinkulturen zumindest in der Größenordnung entsprechen. Die Variation bestimmter Parameter bei den Kulturbedingungen kann dazu führen, neue Gruppen sulfatreduzierender Bakterien mitzuzählen, die bisher nicht erfaßt wurden: Kühle Inkubationstemperaturen sind für vakuolierte Sulfatreduzierer essentiell, ähnlich *Desulforhopalus vacuolatus* (ISAKSEN & TESKE 1995), und die Verwendung von künstlichem Meerwasser mit erhöhtem Gehalt an Ca^{2+} und Mg^{2+} hat das Wachstum von marinen *Desulfonema* in den Solar Lake-MPNs ermöglicht. Auch das bisher nicht in Kultur gebrachte, dominante molekulare Isolat aus den obersten Millimetern der Mattenschicht des Solar Lake erinnert daran, daß Populationen von sulfatreduzierenden Bakterien in den Cyanobakterienmatten des Solar Lake existieren, die mangels geeigneter Kulturbedingungen bisher nicht isoliert und in MPN-Zählungen nicht erfaßt wurden.

Anhang: Einige MPN-Zählungen in den obersten Mattenschichten liefern in bestimmten Verdünnungen keine eindeutigen Ergebnisse, wenn entweder eine Probe mitten aus einer positiven Verdünnungsreihe kein Wachstum zeigt, oder wenn die oberste Stufe einer Verdünnungsreihe nicht eindeutig positiv ist. Werden diese mehrdeutigen Proben als positiv gezählt, ändern sich die Zellzahlen und dementsprechend die spezifischen Sulfatreduktionsraten (bei 0-2 mm, 12°°; 2-4 mm, 12°°; und bei 0-2 mm, 24°°). Die spezifischen Sulfatreduktionsraten in diesen Proben vermindern sich entsprechend den erhöhten MPN-Schätzungen, bleiben aber innerhalb derselben Größenordnung:

Tages zeit	Schicht	Kultivierbare Sulfatreduzierer cm^{-3} Lactat + Acetat	Sulfatreduktionsraten nmol cm^{-3} d^{-1} (HABICHT unpubl.)	Spezifische Sulfatreduktionsraten mol SO_4^{2-}/Zelle d^{-1}
12°°	0-2 mm	11.600.000[1]	2207	1.623×10^{-13}
12°°	2-4 mm	5.050.000[2]	3127	6.192×10^{-13}
12°°	4-7 mm	1.860.000	2779	14.941×10^{-13}
12°°	7-10 mm	3.030.000	1119	3.693×10^{-13}
12°°	10-13 mm	2.430.000	547	2.251×10^{-13}
24°°	0-2 mm	3.700.000[3]	1002	2.708×10^{-13}
24°°	2-4 mm	11.600.000	1842	1.589×10^{-13}
24°°	4-7 mm	1.860.000	1831	9.844×10^{-13}
24°°	7-10 mm	465.000	761	16.366×10^{-13}
24°°	10-13 mm	1.740.000	472	2.713×10^{-13}
JØRGENSEN& COHEN 1977 approx. 0-5 mm		2.000.000	5400	27.000×10^{-13}

[1] Eine negative 10^5 Acetat-Probe, vor einer positiven 10^6-Probe, wird positiv gezählt. Dadurch reichen die positiven MPN-Verdunnungen dieser Acetat-Zählung ununterbrochen bis 10^6, 10^7, 10^7.

[2] Eine schwach sulfidische 10^{7}-Probe an der Spitze einer Lactat-Reihe wird positiv gezählt, und eine 10^5 Acetat-Probe , die diese bis 10^7reichende Acetat-Reihe unterbricht, wird positiv gezählt.

[3] Eine negative 10^5 Acetat-Probe, die eine bis 10^7reichende Acetat-Reihe unterbricht, wird positiv gezählt. Eine Reihe, die bei 10^4 endet, wird als 10^5 gezählt, da der Verdacht eines vorzeitigen Abbruchs dieser Reihe besteht. Die beiden anderen Reihen dieser Acetat-MPN gehen bis 10^7.

Distribution of sulfate-reducing bacteria in a stratified fjord (Mariager Fjord, Denmark) as evaluated by Most-Probable-Number counts and Denaturing Gradient Gel Electrophoresis of PCR-amplified ribosomal DNA fragments.
Andreas Teske, Cathrin Wawer, Gerard Muyzer, Niels B. Ramsing.

Analog zum Solar Lake wurden die Populationen sulfatreduzierender Bakterien in der stratifizierten Wassersäule des Mariagerfjord, Dänemark, untersucht. Aus der permanent stratifizierten, in einen anoxischen und einen oxischen Teil gegliederten Wassersäule des Mariagerfjords wurden die zahlenmäßig dominanten Sulfatreduzierer oberhalb und unterhalb der Oxykline mittels Verdünnungsreihen (MPN) isoliert, mittels DGGE-Analyse von PCR-amplifizierten 16S rDNA-Sequenzabschnitten miteinander verglichen, und phylogenetisch eingeordnet. Innerhalb und unterhalb der Chemokline liegt die Anzahl der kultivierbaren Sulfatreduzierer (ca. 300 Zellen pro ml) etwa um Faktor 10 höher als in der oxischen Oberflächen-Wasserschicht (ca. 30 Zellen pro ml). In der Chemokline des Mariagerfjords, die durch das gleichzeitige Vorkommen verschiedener Elektronendonatoren und Akzeptoren charakterisiert ist (RAMSING ET AL. 1995), könnten sulfatreduzierende Bakterien, die dazu befähigt sind, vielfältige alternative Elektronenakzeptoren nutzen, neben Sulfat auch Fe^{3+}, MnO_2, NO_3^- und O_2. Die Zellzahlen der Lactat- und Wasserstoff-verwertenden Sulfatreduzierer liegen insgesamt um mindestens eine Größenordnung über denjenigen für acetatoxidierende Sulfatreduzierer.
Die molekulare Analyse zeigte, daß die Bakterienpopulationen in der Wassersäule des Mariagerfjord entsprechend den chemischen Gradienten der Wassersäule fein strukturiert und abgestuft sind. Jede Population zeigt wiederum unterschiedliche Profile in Präsenz (rDNA) und Aktivität (rRNA). Eine hochaktive Spezies in der Chemokline des Mariagerfjord konnte anhand der Sequenz der DGGE-Bande als neue phylogenetische Linie der Delta-Proteobakterien identifiziert werden, zu denen auch die Mehrzahl der sulfatreduzierenden Bakterien gehört.
Die Sulfatreduzierer aus der oxischen Wasserschicht des Mariagerfjord gehören zum Genus *Desulfovibrio* und zu dem neubeschriebenen, *Desulfobulbus*-verwandten Genus *Desulforhopalus* (ISAKSEN & TESKE 1995), siehe Abschnitt 4.5. Die *Desulfovibrio* Spezies der oxischen Zone des Mariagerfjord sind mit *Desulfovibrio salexigens* und mit der sauerstofftoleranten marinen Spezies *Desulfovibrio oxyclini* aus dem Solar Lake verwandt. Die erste Spezies des Genus *Desulforhopalus*, *Desulforhopalus vacuolatus*, ein psychrotropher, unvollständig Propionat oxidierender, vakuolierter Sulfatreduzierer, wurde aus dem Sediment

dem Sediment des Kysingfjord (Dänemark) isoliert, der im Gegensatz zu mesophilen Sulfatreduzierern auch bei kühlen Umgebungstemperaturen (0 - 5 °C) aktiv ist. Vakuolierte Sulfatreduzierer sind im Zusammenhang mit sauerstofftoleranter Sulfatreduktion interessant, da vakuolierte Sulfatreduzierer erstmals in der oxischen Oberflächenschicht von Sedimenten des Kattegat gefunden worden sind, wo sie den dominante Zelltyp darstellten (JØRGENSEN & BAK 1991). Während die vakuolierten Sulfatreduzierer des Kattegat-Sediments in den höchsten MPN-Verdünnungen von 10^5 und 10^6 Zellen/ml vorkamen, wurde ein *Desulforhopalus*-verwandtes molekulares Isolat aus der 10^0-MPN der oxischen Wasserschicht, in 8 m Tiefe, erhalten. Ein weiteres, *Desulforhopalus - Desulfobulbus* verwandtes molekulares Isolat wurde aus der 10^1 MPN-Verdünnung aus 13 m Tiefe, dem oberen Rand der Chemokline, erhalten.
In einer begleitenden biogeochemischen Studie des Mariagerfjord (RAMSING ET AL. 1995) wurde versucht, Sulfatreduktionsraten in der Wassersäule zu messen; allerdings blieben die Raten unterhalb der mit $^{35}SO_4^{2-}$-Tracer in der hier verwendeten spezifischen Aktivität erreichbaren Meßgrenze (ca. 1 nmol S cm^{-3} d^{-1}). Werden als als spezifische Respirationsraten sulfatreduzierender Bakterien im Mariagerfjord Werte vorausgesetzt, die mit Reinkulturen gemessen wurden, 0.2 bis 50 x 10^{-15} mol SO_4^{2-}/Zelle d^{-1} (JØRGENSEN 1978b), so erhält man für ca. 200 kultivierbare SRBs cm^{-3} mögliche Sulfatreduktionsraten von 0.00004 - 0.01 nmol S cm^{-3} d^{-1} . Mit 10^5 via in-situ-Hybridisierung nachgewiesenen SRBs cm^{-3} erhält man 0.02 - 0.5 nmol S cm^{-3} d^{-1}. Beide Raten liegen unterhalb der Meßgrenze von ca. 1 nmol S cm^{-3} d^{-1}.

Die einzigen direkten Vergleichswerte für Sulfatreduktion in einer Wassersäule stammen aus dem Schwarzen Meer; hier wurden, durch Einsatz von $^{35}SO_4^{2-}$ - Tracer mit hoher spezifischer Aktivität, Maximalwerte von 0.0035 - 0.036 nmol cm^{-3} d^{-1} in 210 und 100 m Tiefe, unterhalb der Chemokline, gemessen (ALBERT ET AL. 1995, JØRGENSEN ET AL. 1991). Übertragt man diese Werte vom Schwarzen Meer auf den Mariagerfjord, der in der Chemokline eine Dichte von ca. 200 - 300 kultivierbaren Sulfatreduzierern cm^{-3} aufweist, so erhält man einen Schätzwert der spezifischen Aktivität der Sulfatreduzierer im Mariagerfjord von 0.116 - 1.80 x 10^{-4} nmol SO_4^{2-} SRB^{-1} d^{-1}. Diese geschätzten spezifischen Aktivitäten überlappen teilweise mit den Meßwerten für Reinkulturen, 0.002 - 0.5 x 10^{-4} nmol SO_4^{2-} SRB^{-1} d^{-1} (JØRGENSEN 1978b). Damit könnte die Zelldichte der Sulfatreduzierer, die mit MPN im Mariagerfjord ermittelt wurde, für diese analoge Sulfatreduktionsrate ausreichen.

***Desulforhopalus vacuolatus* gen. nov., sp. nov., a new psychrotrophic sulfate-reducing bacterium with gas vacuoles isolated from a temperate estuarium, Kysing Fjord, Denmark.** Mai F. Isaksen, Andreas Teske.

Die erste Spezies eines neuen Genus unvollständig Propionat oxidierender sulfatreduzierender Bakterien, *Desulforhopalus vacuolatus*, wird beschrieben. *Desulforhopalus vacuolatus*,oxidiert wie das verwandte Genus *Desulfobulbus* Laktat und Propionat unvollständig zu Acetat, wächst nicht autotroph auf H_2. Im Gegensatz zum mesophilen *Desulfobulbus* ist *Desulforhopalus* psychrotroph, und wächst im Temperaturbereich von 0° - 24°C, mit Wachstumsoptimum um 18 - 19°C. Unterhalb von 10°C konnte *Desulforhopalus vacuolatus* selektiv angereichert werden. Zellen von *D. vacuolatus* besitzen Gasvakuolen, solange die Kultur wächst, und ihre Vakuolen in der stationären Phase. Die Funktion dieser Vakuolen ist noch unklar, obwohl es zwei Möglichkeiten gibt: Die Vakuolen können als Schwimmkörper dienen, vor allem bei aquatischen, anoxygenen und oxygenen phototrophen Bakterien, die Ihre Position in der Wassersäule einstellen und verändern (WALSBY 1994). Ein sauerstofftolerantes sulfatreduzierendes Bakterium könnte Vakuolen zur Positionierung nahe an der oxisch-anoxischen Phasengrenze benutzen: Vakuolierte Sulfatreduzierer sind in der oxischen Oberflächenschicht von Sedimenten des Kattegat gefunden worden, wo sie in den obersten Verdünnungsstufen der MPNs den dominanten Zelltyp darstellten (JØRGENSEN & BAK 1991). Andererseits könnten Vakuolen möglicherweise dazu dienen, bei gleichbleibendem Cytoplasmavolumen die Oberfläche der Zellmembran vergrößern, um durch Kälte verlangsamte Stoffwechsel- und Membrantransportprozesse zu beschleunigen. Vakuolierte Sulfatreduzierer sind stets bei kühleren Inkubationstemperaturen erhalten wurden: MPN-Zählungen aus Kattegat-Sedimenten (ca. 5°C in situ), die in den höchsten Verdünnungen der oxischen und suboxischen Sedimentschicht vakuolierte Bakterien ergaben, wurden bei 19°C inkubiert (JØRGENSEN & BAK 1991), das Temperaturoptimum für *Desulforhopalus vacuolatus*. MPN-Zählungen aus kalten Sedimenten des Polarmeeres um Spitzbergen, die seit Anfang Oktober 1995 bei 4°C in kubiert worden waren, ergaben in der ersten (und bisher einzigen) positiven Verdünnung vakuolierte Bakterien, die in Morphologie und Zellgröße *Desulforhopalus* sehr ähneln (KNOBLAUCH, unpublished). *Desulforhopalus vacuolatus* ist vielleicht die Spitze eines Eisbergs kälteadaptierter sulfatreduzierender Bakterien, auf die die hohe sulfatreduzierende Aktivität polarer mariner Sedimente zurückzuführen ist (NEDWELL ET AL. 1993).

5.2. VERGLEICH MARIAGER FJORD - SOLAR LAKE: DOMINANTE SULFATREDUZIERER IN OXISCHEN HABITATEN

Betrachtet man die unterschiedlichen Sauerstoffkonzentrationen, Zelldichten von sulfatreduzierenden Bakterien und Sulfatreduktionsraten von oxischen Cyanobakterienmatten und der Chemokline teilweise anoxischer Meeresbecken, so zeigt sich, daß sauerstofftolerante Sulfatreduktion in Cyanobakterienmatten das größere mikrobiologisches Rätsel darstellt. In den Chemokline des Cariaco Trench, des Schwarzen Meeres, und des Mariagerfjord liegen die Sauerstoffkonzentrationen bei 0 - 5 µmol (SOROKIN 1983, HASTINGS & EMERSON 1988, JØRGENSEN ET AL. 1991, RAMSING ET AL. 1995). Sulfatreduzierer und sulfatreduzierende Aktivität nehmen zum unteren Rand der Chemokline hin zu (SOROKIN 1964, JØRGENSEN ET AL. 1991, ALBERT ET AL. 1995). Die Sulfatreduktionsaktivität bekannter, klassich-anaerober sulfatreduzierender Bakterien wird in Reinkulturexperimenten erst ab 15 µmol O_2 vollständig inhibiert (MARSCHALL ET AL. 1993). Damit können die bekannten Sulfatreduzierer und ihre Aktivität diese "weiche" Variante sauerstofftoleranter Sulfatreduktion erklären.
In photosynthetischen Algenmatten werden jedoch tagsüber Sauerstoffkonzentrationen von mehreren 100 bis zu 1000 µmol erreicht (CANFIELD & DES MARAIS 1991, FRÜND & COHEN 1992, VISSCHER ET AL. 1992, CAUMETTE ET AL. 1994). Als Folge der sehr schmalen Gradienten in Cyanobakterienmatten sind Sulfatreduzierer und sulfatreduzierende Aktivität in der oxischen Oberflächenschicht oberhalb der Chemokline, innerhalb sowie direkt unterhalb der Chemokline konzentriert, mit häufig zu beobachtetender annähernd symmetrischer Verteilung um das Maximum der Chemokline (JØRGENSEN 1993, HABICHT unveröffentlicht). Hohe Zelldichten sulfatreduzierender Bakterien werden im tagesperiodischen Rythmus Sauerstoff in hohen Konzentrationen, bis zur Übersättigung (> ca. 250 µM), ausgesetzt.
Die Sulfatreduktionsraten, die unter derartig unterschiedlichen Bedingungen erhalten werden, unterscheiden sich extrem: Maximale Sulfatreduktionsraten von 1500 - 3000 nmol SO_4^{2-} cm^{-3} d^{-1} in der Cyanobakterienmatte des Solar Lake unterscheiden sich mindestens um drei Größenordnungen von Raten unterhalb der Nachweisgrenze von 0.5 - 1 nmol cm^{-3} d^{-1} in der Wassersäule des Mariagerfjord. Die spezifischen Sulfatreduktionsraten, die für Reinkulturen sulfatreduzierender Bakterien ermittelt worden sind (0.2 - 50 x 10^{-6} nmol SO_4^{2-} $Zelle^{-1}$ d^{-1}, JØRGENSEN 1978b), können in Verbindung mit den MPN-Zelldichten in der oxischen Oberflächenschicht des Solar Lake, die beobachteten Sulfatreduktionsraten nicht vollständig erklären. Es bleibt eine Diskrepanz von

mindestens einer Zehnerpotenz: Entweder sind in der Cyanobakterienmatte des Solar Lake mindestens zehnmal mehr sulfatreduzierende Bakterien enthalten, als in diesen MPN-Zählungen ermittelt wurde (1.8 - 4.7 x 10^6 SRB cm^{-3}), oder die spezifische Aktivität der gezählten sulfatreduzierenden Bakterien in der Solar Lake-Cyanobakterienmatte ist mindenstens zehnmal so hoch (4 - 6 x 10^{-4} nmol SO_4^{2-} SRB^{-1} d^{-1}) wie diejenige der getesteten Reinkulturen. Die Möglichkeit bleibt offen, daß in der oxischen und ebenso in der anoxischen Schicht der Matte, in der diese Diskrepanz gleichermaßen anzutreffen ist, zahlenmäßig dominante Populationen unbekannter Sulfatreduzierer vorhanden sind, die sich unter den hier verwendeten Kulturbedingungen nicht isolieren ließen.
Im Mariagerfjord besteht die Möglichkeit, daß die mit MPN ermittelte Zelldichte (200 - 300 SRB cm^{-3}) die - als Analogie mit der Chemokline des Schwarzen Meeres - geschätzten Sulfatreduktionsraten (0.0035 - 0.036 nmol SO_4^{2-} cm^{-3} d^{-1}, ALBERTS ET AL. 1995) vollständig erklären kann. Die geschätzte spezifische Aktivität der Sulfatreduzierer im Mariagerfjord von 0.116 - 1.80 x 10^{-4} nmol SO_4^{2-} SRB^{-1} d^{-1} überlappt mit den spezifischen Aktivitäten für sulfatreduzierende Reinkulturen (0.2 - 50 x 10^{-6} nmol SO_4^{2-} SRB^{-1} d^{-1}, JØRGENSEN 1978b).

Die Frage nach dominanten Typen sulfatreduzierender Bakterien in beiden Typen aerober Habitate läßt sich, nach diesen Einschränkungen, teilweise beantworten.
In der Wassersäule des Mariagerfjord überwogen lactat- und wasserstoff-verwertende Sulfatreduzierer die acetat-oxidierenden Sulfatreduzierer etwa um Faktor zehn. Die meisten identifizierten MPN-Isolate, aus den 10^1 -Stufen der MPN, gehörten zum Genus *Desulfovibrio* , doch ließen sich auch je ein MPN-Isolat als ein naher Verwandter von *Desulforhopalus vacuolatus* (Stufe 10^0, 8 m, aerobe Zone) und als ein etwas entfernterer Verwandter von *Desulfobulbus-Desulforhopalus* (Stufe 10^1, 13 m, oberer Rand der Chemokline) identifizieren. Da die 16S rRNA-Analyse durchgeführt wurde, bevor die MPNs wirklich ausgewachsen waren, wurden Isolate aus den 10^2 - Stufen nicht identifiziert. Da die MPN bei Raumtemperatur inkubiert wurde, ist anzunehmen, daß gegen *Desulforhopalus* und ähnliche psychrophile, vakuolierte Isolate selektiert wurde, und *Desulfovibrio* angereichert wurde. Es bleibt aber festzuhalten, daß *Desulfovibrio* und *Desulforhopalus* die bisher identifizierten sulfatreduzierenden Bakterien in der Chemokline und der oxischen Zone des Mariagerfjord darstellen.

In der oxischen Schicht der Cyanobakterienmatte des Solar Lake sind andere Populationen vorherrschend. *Desulfovibrio oxyclini* , ursprünglich aus einer 10^7-Verdünnung isoliert, und andere, z.T. nahe verwandte *Desulfovibrio* -Stämme, sind in Zelldichten von 10^4 - 10^6 cm^{-3} gefunden worden. *Desulfovibrio oxyclini* ist durch seinen vielseitigen Schwefelmetabolismus, die Fähigkeit zur Thiosulfat- und Sulfit-Disproportionierung, die Respiration mit verschiedenen Schwefelkomponenten und die Befähigung zur Sauerstoffatmung, an den täglichen Wechsel oxischer und anoxischer Bedingungen angepaßt. Obwohl *Desulfovibrio*-Isolate aus dem Solar Lake als Reinkulturen letztlich nicht unter einer Sauerstoffatmosphäre überleben können, bilden sie in Cokultur mit fakultativ aeroben Bakterien sehr stabile Assoziationen, die aerobe Kulturbedingungen, z.B. in Chemostatenexperimenten, und wiederholtes aerobes Ausstreichen über mehrere Tage hinweg überstehen.

Acetatoxidierende Sulfatreduzierer, unter ihnen der filamentöse Sulfatreduzierer *Desulfonema*, sind in vergleichbaren Zelldichten gefunden worden, obwohl die Zählungen gerade in der obersten Schicht (0-2 mm) mit Acetat auffällig unregelmäßig ausfielen: Abgebrochene Verdünnungsreihen und übersprungene Verdünnungsstufen, in denen nichts wächst, obwohl die Verdünnungsreihe sich danach fortsetzt, waren zu beobachten (siehe die zweite MPN-Tabelle im Anhang zur Solar-Lake Diskussion), und vermindern die Zähleffektivität der MPN für acetatoxidierende Sulfatreduzierer. Diese Unregelmäßigkeiten sind wahrscheinlich auf das Zusammenklumpen, die Koloniebildung und die Morphologie vieler acetatoxidierender sulfatreduzierender Bakterien zurückzuführen, die sich schwierig in homogene Suspension bringen lassen und in der MPN-Injektionsspritze hängenbleiben. Acetatoxidierer-spezifische rRNA-Sonden, die mit rRNA aus der Oberflächenschicht einer vergleichbaren Cyanobakterienmatte (Baja California) hybridisieren, liefern stark schwankende Hybridisierungs-intensitäten, was auf dieselben Probleme, inhomogene Verteilung und Zusammenklumpung dieser Bakterienpopulationen, zurückgeführt wird (RISATTI ET AL. 1994).

Trotz dieser Schwierigkeiten konnte ein Genus acetatoxidierender Sulfatreduzierer, das filamentöse *Desulfonema*, als ein dominantes Mitglied der Sulfatreduziererpopulation der Solar Lake Matte identifiziert werden. *Desulfonema* erscheint in Verdünnungsstufen 10^5, 10^6 und 10^7 Zellen cm^{-3} in der obersten Mattenschicht und in der Chemokline. *Desulfonema* ist durch seine filamentöse Morphologie zur Fortbewegung speziell in der dichten Matrix der Cyanobakterienmatte befähigt; die Filamente könnten sich aneinander oder an ein festes Substrat anlagern, um durch Partikelassoziation Sauerstoffstreß zu reduzieren (FUKUI & TAKII 1990). Neben *Desulfonema* ist mindestens ein

weiterer, zu *Desulfonema* verwandter, aber in seinem Metabolismus unbekannter Sulfatreduzierer in der obersten Schicht der Solar Lake Matten dominant, konnte hier aber nur als molekulares Isolat mit PCR und DGGE gezeigt werden.
Desulforhopalus wurde im Solar Lake nicht gefunden. Das relativ kühle Oberflächenwasser des Solar Lake ist im Winter 15 - 20°C, im Sommer 25°C warm, und damit für *Desulforhopalus* und ähnliche psychrophile Sulfatreduzierer weniger geeignet (COHEN ET AL. 1977). Solar Lake hat unterhalb der Thermo-und Halokline Temperaturen von 50 - 60°C, und stellt ein vielversprechendes Habitat für *Desulfurella* und andere thermophile Schwefelreduzierer dar (COHEN ET AL. 1977). Die Temperatur in den Cyanobakterienmatten der Uferzone schwankt im Jahresrythmus zwischen 25 und 40°C (KRUMBEIN ET AL. 1977), Temperaturen, bei denen *Desulforhopalus* nicht mehr wachsen kann (ISAKSEN & TESKE 1995).

Die spezifischen Bedingungen von Cyanobakterienmatten sind für die Sauerstofftoleranz sulfatreduzierender Bakterien in diesen Habitaten essentiell. Hierzu gehören die Millimeterdimensionen der Mattenschichtung und die einander entgegengesetzten, im Tagesrythmus wechselnden Redoxgradienten, die ständige Tages-Nacht-Periodizität von hoher Sauerstoffsättigung und Anoxia, das an der Mattenoberfläche auf engstem Raum konzentrierte Nährstoffangebot durch Photo- und Chemosynthese, sowie die dichte Matrix der Cyanobakterienmatte. Sauerstofftolerante Sulfatreduktion in mikrobiellen Matten ist durch die Wechselwirkung dieser Faktoren mit diversifizierten Populationen sulfatreduzierender Bakterien zu verstehen, die sich auf unterschiedliche Weise an den Sauerstoffstreß und die spezifischen Bedingungen der Matte angepaßt haben, und kann nicht durch ad-hoc-Postulate, wie die Annahme einer einzigen spezifischen Klasse völlig neuartiger sauerstofftoleranter sulfatreduzierender Bakterien, erklärt werden.

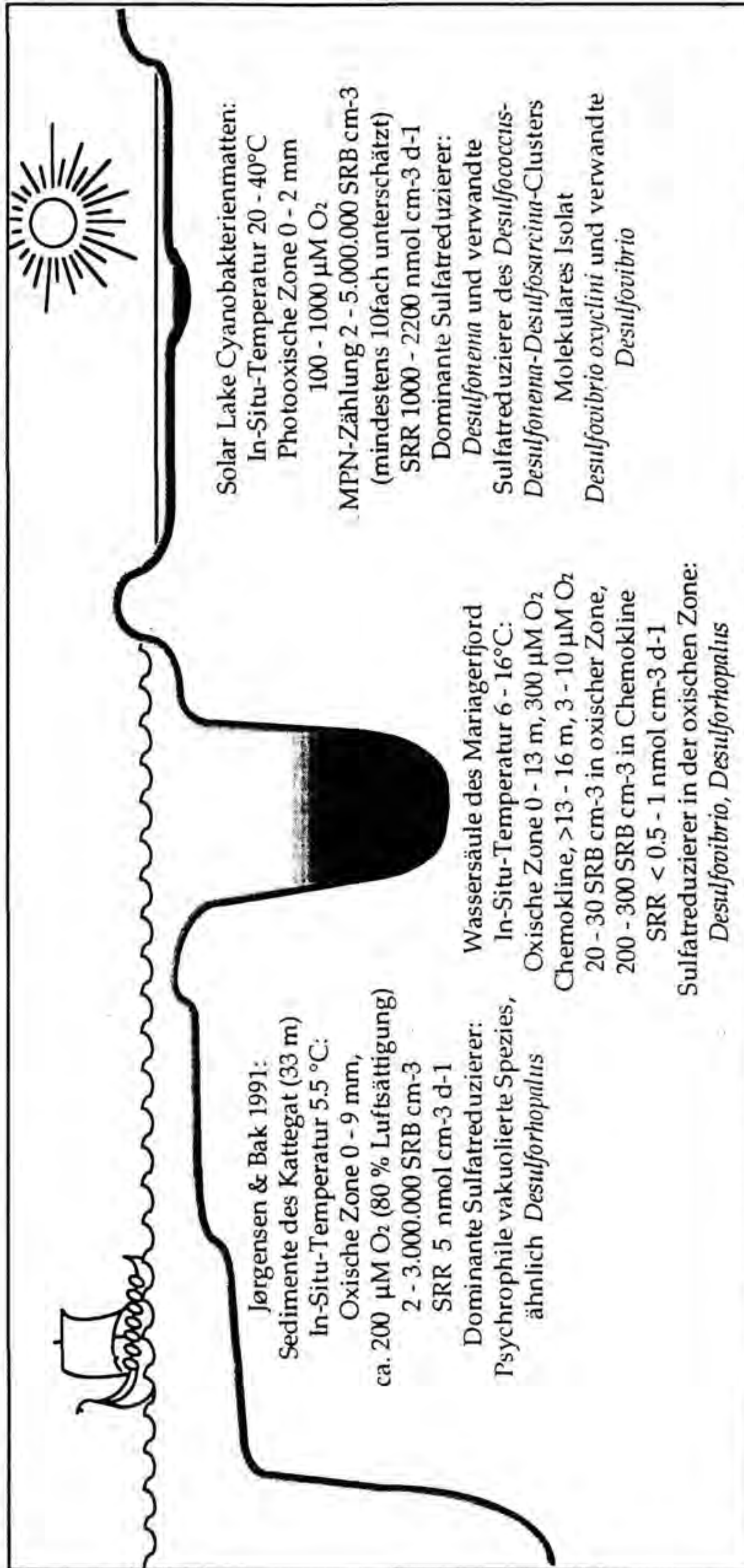


Abbildung 1. Vergleich oxischer mariner Habitate mit sauerstofftolerenter Sulfatreduktion, der jeweils gemessenen Sulfatreduktionsraten, und der bisher identifizierten Populationen sauerstofftoleranter Sulfatreduzierer.

5.3. SULFIDOXIDIERENDE BAKTERIEN

Phylogeny of *Thioploca* and Related Filamentous Sulfide-Oxidizing Bacteria. Andreas Teske, Niels B. Ramsing, Jan Küver, und Henrik Fossing.

Basierend auf 16S rDNA wurde die Phylogenie der filamentösen sulfidoxidierenden Bakterien, *Thioploca*, *Beggiatoa*, und *Thiothrix*, erstellt. Drei marine *Thioploca* Spezies, *Thioploca araucae*, *Thioploca chileae* vom chilenischen Kontinentalschelf bei Concepcion, sowie *Thioploca ingrica* aus der Uferzone des Randersfjord, Dänemark, wurden analysiert. *Thioploca* und *Beggiatoa* bilden eine monophyletische Gruppe, mit jeweils einem Zweig für *Beggiatoa* und für *Thioploca*. Diese phylogenetische Abgrenzung beider Genera ist nicht als endgültig anzusehen: Verschiedene vakuolierte marine *Beggiatoa* mit großen Filamentdurchmessern (20 - 120 µM) stehen in ihrer 16S rRNA Sequenz (unveröffentlichte Ergebnisse) und morphologisch den chilenischen marinen *Thioploca* Spezies näher als anderen *Beggiatoa* Spezies. *Thiothrix* ist nicht nahe mit *Beggiatoa* und *Thioploca* verwandt, sondern bildet eine eigene Entwicklungslinie, die mit dem freilebenden Schwefeloxidierer *Thiomicrospira* und schwefeloxidierenden intrazellulären Symbionten von marinen Invertebraten verwandt ist.

Die Entwicklungslinien *Beggiatoa/Thioploca* einerseits und *Thiothrix* andererseits gehören zum Phylum der Proteobakterien, und innerhalb dieser sehr großen Gruppe zum Zweig der Gamma-Proteobakterien. Das Phylum Proteobacteria hat sich wahrscheinlich aus anoxygenen photosynthetischen Bakterien entwickelt (WOESE 1987), und umschließt eine große Vielzahl von schwefeloxidierenden photosynthetischen und nichtphotosynthetischen Bakterien. Im Laufe dieser Untersuchung zeigte sich, daß die Entwicklungslinien von *Beggiatoa/Thioploca*, *Thiothrix*, *Thiomicrospira*, schwefeloxidierenden Symbionten, bestimmten *Thiobacillus* Spezies, und vielen phototrophen Schwefelbakterien, direkt auf der Basis der Gamma-Unterabteilung und des Beta-Gamma-Übergangsbereiches der Proteobakterien zurückgehen, als sogenannte "deep-branching lineages" innerhalb der Gamma-Proteobakterien. Hier bildete sich erstmals der Verdacht, daß diese schwefeloxidierenden Bakterien aus einer evolutionären Radiation hervorgegangen sind, die mit der Evolution des marinen Schwefelkreislaufs in Verbindung gebracht werden kann.

Phylogenetic relationships of *Thiomicrospira* species and their identification in deep-sea hydrothermal vent samples by Denaturing Gradient Gel Electrophoresis of 16S rDNA fragments. Gerard Muyzer, Andreas Teske, Carl O. Wirsen, und Holger W. Jannasch.

Basierend auf 16S rRNA-Sequenzen wurde die Phylogenie von *Thiomicrospira,* eines Genus chemolithoautotropher Schwefeloxidierer, erstellt, mit der weiterführenden Absicht, diese Sequenzinformation zur molekularen Identifizierung von *Thiomicrospira* mittels DGGE, Sequenzierung und Hybridisierung zu verwenden. Alle *Thiomicrospira* Spezies, bis auf den falsch klassifizierten *Thiomicrospira denitrificans,* bilden eine monophyletische Gruppe innerhalb der Gamma-Proteobakterien. Die nächsten Verwandten von *Thiomicrospira* sind obligat symbiontische Schwefeloxidierer, die mit marinen Bivalvia und anderen Invertebraten spezifisch assoziiert sind und ihre Wirtstiere in sulfidischen Habitaten mit autotroph fixiertem Kohlenstoff versorgen (CAVANAUGH 1994). Diese symbiontischen Schwefeloxidierer und *Thiomicrospira* sind lediglich an der Wurzel beider Linien miteinander verwandt. Das Fehlen unmittelbarer, freilebender Verwandter der obligaten Symbionten läßt auf eine lange gemeinsame Evolution der Symbionten mit ihren spezifischen Wirtstieren schließen (DISTEL ET AL. 1994). *Thiomicrospira* ist demnach ein moderner Abkömmling der letzten freilebenden Vorfahren der Bivalvia-Symbionten.
Die 16S rDNA Sequenzen von *Thiomicrospira* ermöglichten die Identifizierung von *Thiomicrospira*-DNA aus Hydrothermal Vent-Proben, und führten zu einer gegenwärtig am MPI durchgeführten Doktorarbeit über die Verbreitung von *Thiomicrospira,* seine molekulare Identifizierung und Isolierung in Reinkultur. *Thiomicrospira* ist in den verschiedensten marinen und auch Süßwasser-Standorten weit verbreitet, in Wattenmeersedimenten, Hydrothermal vents, chilenischen *Thioploca*-Matten und Sedimenten, Solequellen, der Chemokline des Schwarzen Meeres, und Bremer Kläranlagen. Die 16S rRNA Sequenzen von *Thiomicrospira* erhärteten den Verdacht, der sich bei der *Thioploca-Beggiatoa*-Studie gebildet hatte, daß die mikroaerophilen, nichtphotosynthetischen Schwefeloxidierer auf eine gemeinsame evolutionäre Radiation, bei der Entstehung der Gamma- und Beta-Proteobakterien, der beiden jüngsten großen Bakterien-Phyla (VAN DE PEER ET AL. 1994), zurückgehen.

5.4. EVOLUTION DES OXIDATIVEN SCHWEFELZYKLUS

Late Proterozoic rise in atmospheric oxygen from phylogenetic and stable isotopic studies. Donald E. Canfield, Andreas Teske

Die Entwicklung mikroaerophiler, schwefeloxidierender Bakterien, sowie das Einsetzen des aeroben oxidativen marinen Schwefelzyklus sind an den Anstieg des Sauerstoffgehalts der Erdatmosphäre gekoppelt. Beide Ereignisse sind - mit Unschärfen - datierbar, doch fallen sie in einen konsistenten Zeitrahmen, das späte Proterozoikum, etwa zwischen 650 und 1050 ma.
Durch anaerobe Sulfatreduktion produziertes Sulfid ist um durchschnittlich 20‰, im Extremfall 30 - 40‰, am leichteren Schwefelisotop ^{34}S verarmt. Durch direkte Oxidation mit Sauerstoff, oder über indirekte Sauerstoffoxidation via Fe^{3+} oder Mn^{4+}, wird mikrobiell produziertes Sulfid zu Elementarschwefel reoxidiert, der von schwefeldisproportionierenden Bakterien zu Sulfid und Sulfat dispropotioniert wird. Durch mehrere Zyklen von Reoxidation und Disproportionierung verstärkt sich die ^{34}S-Depletion des Sulfids auf Werte von 40 - 60 ‰ gegenuber dem Ausgangssulfat. Diese Isotopensignatur, die in Freiland- und Laborversuchen eine 80 - 100%ige In-situ-Reoxidation des mikrobiell produzierten Sulfids erfordert, ist nur marinen Sedimenten, die jünger sind als ca. 670 ma, konsistent nachzuweisen, sie erscheint jedoch nicht, mit einer einzigen Ausnahme im unvollständig dokumentierten Intervall 670 - 1060 ma, in proterozoischen Sedimenten älteren Datums. Im Zeitraum vor 1060 ma liegt die maximale ^{34}S-Depletion bei 30 - 40‰.
Die Evolution des oxidativen marine Schwefelzyklus ist mit der Entwicklung sulfatreduzierender und sulfidoxidierender Prokaryonten gekoppelt. Sulfatreduzierende Bakterien sind einerseits der Delta-Gruppe der Proteobakterien und den grampositiven Bakterien zugehörig, sind aber auch mit der sehr alten Entwicklunglinie *Thermodesulfobacterium*, nahe an der gemeinsamen Wurzel von Bakterien und Archaeen, und innerhalb der Archaeen im Genus *Archaeoglobus*, vertreten. Dem frühen Ursprung bakterieller Sulfatreduktion entsprechend, zeigt sich die Isotopensignatur sulfatreduzierender Bakterien, ^{34}S-Depletion von ca. 20 - 40‰ gegenüber dem unfraktionierten marinen Sulfat, bereits in archaealen und früh- sowie mittelproterozoischen marinen Sedimenten (HAYES ET AL. 1992). Die Reoxidation zum Sulfat fand wahrscheinlich durch anoxygene Photosynthese statt: Anaerobe, sulfidoxidierende photosynthetische Bakterien erscheinen in zahlreichen Genera innerhalb der Proteobakterien, und bilden darüber hinaus einige sehr alte Phyla, wie die *Chlorobiaceae* und *Chloroflexaceae*, nahe an der

gemeinsamen Wurzel der Bakterien und der Archaeen (WOESE 1987, PIERSON & OLSON 1989). Die aeroben oder mikroaerophilen, nichtphotosynthetischen schwefeloxidierenden Bakterien sind als Angehörige der Beta- und Gamma-Proteobakterien, der beiden jüngsten großen Bakterienphyla (VAN DE PEER 1994), jüngeren Ursprungs: *Beggiatoa, Thioploca, Thiothrix, Thiomicrospira,* schwefeloxidierende Symbionten, und bestimmte *Thiobacillus* Species entstammen dem späten Proterozoicum, und das Alter ihrer evolutionären Radiation wurde, durch Kalibrierung der Mutationsraten ihrer 16S rRNA Gene, auf ca. 600 - 900 ma geschätzt.
Die Evolution dieser mikroaerophilen Schwefeloxidierer fällt zeitlich mit dem Auftreten der ^{34}S-Isotopensignatur zusammen, die auf Sauerstoff-Oxidation mikrobiell produzierten Sulfids in Verbindung mit mehrfachen Disproportionierungszyklen zurückzuführen ist. Diese Indikatoren können als Sauerstoffbarometer dienen, um den Sauerstoffgehalt der Erdatmosphare zwischen 600 und 1000 ma anzugeben und frühere Schätzungen zu präzisieren: Wenn Minimal- bis zu Medianwerte der Kohlenstoffmineralisierungs- und Sulfatreduktionsraten der heutigen Schelfmeer-Sedimente in der Modellrechnung vorausgesetzt werden, kann das entstehende Sulfid in der Mehrzahl der Sedimente vollständig zu Schwefel und schließlich zu Sulfat reoxidiert werden, wenn am oberen Ende der Diffusionsgrenzschicht (500 µm) und in der Wassersäule mindestens 13 - 46 µM O_2 im Meerwasser gelöst enthalten sind. Sulfidoxidation und Disproportionierung, ausgelöst durch diese Sauerstoff-Mindestkonzentration, hinterlassen ihre charakteristische - 40 bis 60‰ $\partial^{34}S$-Isotopensignatur. Aerobe Schwefelbakterien bevorzugen Konzentrationen von O_2 im µM-Bereich, die innerhalb der Diffusionsgrenzschicht gegeben sind. 13 - 46 µM O_2 in der Wassersäule entsprechen 5 - 18% der heutigen Sauerstoff-Sättigungs-Konzentration. Setzt man Sauerstoff-Equilibrium von Wassersäule der Schelfmeere und Atmosphäre voraus, wird der Sauerstoffgehalt der Atmosphäre zwischen 670 und 1060 ma auf 5-18% des heutigen Gehalts geschätzt. Die Erdatmosphäre erreichte Sauerstoffkonzentrationen, die zur Atmung geeignet waren, in den letzten 400 ma des Proterozoikums. In diese Zeit fallen bedeutende evolutionäre Radiationen sowohl einzelliger eukaryontischer Organismen, wie der Acritarchs, als auch die Entfaltung der Metazoen, verbunden mit einer Zunahme des evolutionären Tempos (KNOLL 1992, 1994). Datierbare evolutionäre Radiationen der Prokaryonten und die Isotopensignaturen mikrobiell katalysierter geochemischer Prozesse fügen sich zu einem integrierten und stetig weiter verfeinerten Bild der biologischen und geochemischen Evolution der Erde zusammen.

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