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1. Gutachter: Prof. Dr. Friedrich Widdel
2. Gutachter: Dr. Gerard Muyzer

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The Power of the Modal Bacter

„Life therefore began with a bacterial mode. Life still maintains a bacterial mode in the same position. So it was in the beginning, is now, and ever shall be - at least until the sun explodes and dooms the planet."

Stephen Jay Gould, Life's Grandeur - The spread of excellence from Plato to Darwin.

für Christel und Lukas

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Bremen, im September 1999

Summary

In the present study the microbial communites at a shallow submarine hydrothermal vent were investigated. In particular, the relation between the composition and the structure of the microbial communities and the changing environmental conditions along a transect from the center of the vent to the surrounding area was studied. Synecological studies were carried out by 16S rRNA based techniques in parallel to autecological studies with bacteria isolated from the habitat.

1. A trend from a diverse range of bacterial populations which were present in approximately equal abundance in the transition zone from the strongly hydrothermally-influenced sediments to normal sedimentary conditions, to a community dominated by few populations close to the center of the vent was revealed. Temperature was found to be an important parameter in determining this trend. This supported the hypothesis that extreme environmental conditions will lead to a community structure with few dominating populations.

2. In total 91 unique bacterial populations could be detected. The sequencing of excised and reamplified bands from denaturing gradient gel electrophoresis gels revealed the presence of a wide spectrum of bacterial lineages. However, only a low percentage of the sequences was closely related to the sequences of cultivated bacteria. Because of the input of allochtonous organic matter the microbial community seemed to be net heterotrophic. However, autotrophic populations were also detected and they contributed to the new production of organic matter.

3. The concentration of rRNA in general decreased sharply with sediment depth and the concentration was higher in regions closer to the vent center. This indicates that the microbial activity was stimulated by the hydrothermalism. *Bacteria* made up at least 78% (average 95%) of the prokaryotic rRNA. Along the steepest temperature gradient the proportion of archaeal rRNA was increasing. Nevertheless, even in the hottest sediment layer where a quantification was possible (in situ temperature 82 °C) archaeal rRNA made up only 11.9% of the prokaryotic rRNA. This suggests that *Archaea* were generally a minor component of the microbial community at this vent site

4. Viable cell counts of specific physiological groups of bacteria (autotrophic sulfur oxidizers, sulfate reducers, and iron reducers), which were determined by using the most-probable number (MPN) technique, constituted at most about 4 % of the total cell counts as determined by the acridin-orange-direct-count (AODC) method. Both counting procedures revealed highest cell numbers in a transition zone from the strongly hydrothermally-influenced sediments to normal sedimentary conditions.

5. Like at deep-sea hydrothermal vents, members of the sulfur-oxidizing genus *Thiomicrospira* constituted an important component of the microbial communities. One type (Milos-T2) that was isolated from high dilutions was shown to be dominant. This also demonstrated the rare occurence of a congruence between results of molecular based analysis and cultivation based analysis.

6. The diversity among the sulfur-oxdizing bacterial community was high. New species that belong to the genera *Thiomicrospira* and *Halothiobacillus*, both of which have been described at other vent sites before, could be isolated. In addition, five sulfur-oxidizing bacteria that form new lineages among the γ-subclass of the Proteobacteria and are most closely related to symbionts, could be isolated. Some of these are able to oxidize reduced sulfur compounds anaerobically using nitrate as electron acceptor. Important parameters that appear to govern the distribution of the sulfur-oxidizing bacteria are the *in situ*

temperature, the substrate concentration, i.e. hydrogen sulfide, and the availability of oxygen.

7. A thermophilic sulfate-reducing bacterium could be isolated (strain MT-96), which represents a new species of the genus *Desulfacinum*. In contrast to the most closely related thermophilic species *Desulfacinum infernum* and *Thermodesulforhabdus norvegicus*, which were isolated from oil field waters, the completely oxidizing strain MT-96 was isolated from a geothermally influenced habitat containing no oil related substrates.

8. Experiments to investigate the fractionation of the stable sulfur isotopes by strain MT-96 were carried out. The hydrogen sulfide produced in the experiments was enriched in ^{32}S by approximately 19‰ compared to sulfate, which indicates that stable isotope discrimination by this thermophile is within the range found previously for mesophilic sulfate-reducing bacteria and only slightly higher than observed for the thermophilic Gram-positive *Desulfotomaculum nigrificans*. Field data on the sulfur isotope partitioning between sulfate and reduced sulfur species at hydrothermal vents from Milos are close to the results observed in the experiments of the present study.

Zusammenfassung

Im Rahmen der vorliegenden Dissertation wurden mikrobielle Lebensgemeinschaften an einer submarinen Flachwasser-Hydrothermalquelle untersucht. Insbesondere wurde die Beziehung zwischen der Zusammensetzung und der Struktur der mikrobiellen Gemeinschaften und den sich ändernden Umweltparametern entlang eines Transektes vom Zentrum der Quelle in das umgebende Sediment untersucht. Mittels molekularbiologischer Methoden basierend auf der 16S rRNA wurden synökologische Untersuchungen durchgeführt, die durch autökologische Untersuchungen an aus dem Habitat isolierten Bakterien ergänzt wurden.

1. Es gab einen Trend von einer diversen Bakteriengemeinschaft in der Übergangszone zwischen den von der Hydrothermalflüssigkeit stark beeinflußten Sedimenten und den von der Hydrothermalflüssigkeit unbeeinflußten Sedimenten, in denen die Populationen in annähernd gleicher Zahl vertreten waren, zu einer Gemeinschaft im Zentrum der Quelle, die von wenigen Populationen dominiert wurde. Ein wichtiger Faktor, der diesen Trend bestimmte, war die Temperatur. Dies bestätigte die Hypothese, daß extreme Umweltbedingungen zu einer Gemeinschaftsstruktur mit einer geringen Anzahl an dominanten Populationen führen.

2. Insgesamt konnten 91 unterschiedliche bakterielle Populationen identifiziert werden. Die Sequenzierung von ausgeschnittenen und reamplifizierten Banden aus Gelen einer denaturierenden Gradienten-Gel-Elektrophorese zeigte das Vorhandensein eines weiten Spektrums verschiedener evolutiver Linien der Bakterien. Allerdings war nur ein kleiner Teil der 16S rRNA Sequenzen nahe mit den Sequenzen von kultivierten Bakterien verwandt. Die mikrobielle Gemeinschaft kann aufgrund des Eintrages von allochtonem organischen Material insgesamt als nettoheterotroph charakterisiert werden. Autotrophe Populationen konnten jedoch auch nachgeweisen werden und tragen zur Produktion neuen organischen Materials bei.

3. Die Konzentration an rRNA nahm generell mit der Sedimenttiefe drastisch ab und war in Regionen näher zum Zentrum der Quelle höher. Dies deutet darauf hin, daß die hydrothermale Aktivität die mikrobielle Aktivität stimulierte. Bakterien machten mindestens 78% (durchschnittlich 95%) der prokaryotischen rRNA aus; somit waren hauptsächlich Bakterien für diese Aktivität verantwortlich. Entlang des steilsten Temperaturgradienten konnte allerdings ein Anstieg des Anteils archaeeller rRNA auf bis zu 11.9% bei einer *in situ* Temperatur von 82 °C beobachtet werden. *Archaea* scheinen insgesamt an der untersuchten Hydrothermalquelle eher eine untergeordnete Komponente der mikrobiellen Gemeinschaft zu sein.

4. Die Lebendzellzahlen verschiedener physiologisch definierter Gruppen von Bakterien (autotrophe Schwefeloxidierer, Sulfatreduzierer, Eisenreduzierer), die mittels der most-probable number (MPN) Methode ermittelt wurden, machten maximal knapp 4 % der unter Anwendung der Acridine-Orange Färbetechnik bestimmten Gesamtzellzahlen aus. Sowohl die Gesamtzellzahlen als auch die MPN-Zahlen zeigten die höchsten Werte in der Übergangszone.

5. Vertreter des schwefeloxidierenden Genus *Thiomicrospira* stellten wie an Tiefsee-Hydrothermalquellen einen wichtigen Bestandteil der vorgefundenen mikrobiellen Gemeinschaft. Ein aus dem Habitat isolierter Typ (Stamm Milos-T2) konnte dabei als vorherrschend identifiziert werden. Dies demonstriert den seltenen Fall einer Kongruenz zwischen den Ergebnissen der auf molekularen Methoden basierenden bzw. auf Kultivierungsmethoden basierenden Analysen.

6. Die Diversität innerhalb der physiologische Gruppe der schwefeloxidierenden Bakterien war hoch. Es konnten neue Arten der Genera *Thiomicrospira* und *Halothiobacillus* iso-

liert werden, die schon von anderen Hydrothermalquellen bekannt waren. Zusätzlich konnten fünf weitere schwefeloxidierende Bakterien, die neue Linien innerhalb der γ-Untergruppe der Proteobakterien darstellen und die nahe mit Symbionten verwandt sind, isoliert werden. Einige dieser Isolate können reduzierte Schwefelverbindungen anaerob mit Nitrat als Elektronenakzeptor oxidieren. Wichtige Parameter, welche für die Verteilung der schwefeloxidierenden Bakterien von Bedeutung waren, sind die *in situ* Temperatur, die Substratkonzentration, wie z.B. Schwefelwasserstoff, und die Verfügbarkeit von Sauerstoff.

7. Es wurde ein thermophiles sulfatreduzierendes Bakterium isoliert und charakterisiert (Stamm MT-96), das eine neue Art der Gattung *Desulfacinum* darstellt. Im Gegensatz zu den am nächsten verwandten, thermophilen Arten *Desulfacinum infernum* und *Thermodesulforhabdus norvegicus*, die aus Nordsee-Ölfeldern isoliert wurden, wurde die hier beschriebene Art aus einem geothermal beeinflußten marinen Sediment isoliert, in dem keine ölähnlichen Verbindungen vorkommen.

8. Mit dem Stamm MT-96 wurden Versuche zur Fraktionierung der stabilen Schwefelisotope durchgeführt. Der in den Experimenten produzierte Schwefelwasserstoff war im ^{32}S-Gehalt um 19‰ angereichert. Somit weist dieses thermophile Gram-negative Bakterium eine ähnliche Diskriminierung des stabilen Schwefelisotops ^{32}S auf wie bereits zuvor untersuchte, mesophile sulfatreduzierende Bakterien und nur eine leicht erhöhte Fraktionierung gegenüber dem thermophilen, Gram-positiven Sulfatreduzierer *Desulfotomaculum nigrificans*. Die Ergebnisse deuten außerdem darauf hin, daß die Sulfatreduktion an der untersuchten Quelle einen wichtigen Beitrag zur Schwefelisotopenzusammensetzung liefert.

Inhaltsverzeichnis

Teil 1: Darstellung der Ergebnisse im Gesamtzusammenhang

A Einleitung

1. Geologie von Hydrothermalquellen

Im Jahre 1977 kam es zu der spektakulären Entdeckung von Tiefsee-Hydrothermalquellen und der mit diesen assoziierten, bis dahin unbekannten Lebensgemeinschaften an einem Dehnungsrücken im Pazifischen Ozean vor Galapagos (4, 23). Seit dieser Zeit wurden weitere submarine Hydrothermalsysteme sowohl in der Tiefsee (132, 150) als auch im Küstenbereich beschrieben (27, 44, 63, 83, 143, 144, 150). Allen diesen Systemen ist gemeinsam, daß sie an tektonisch aktiven Zonen der Erdkruste lokalisiert sind. Die Dehnungszonen der tektonischen Platten sowie deren aktive Subduktionszonen, die unter anderem zur Bildung von vulkanischen Bögen führen, sind die hauptsächlichen Orte für submarine hydrothermale Aktivität (132). In diesen Regionen dringt Meerwasser durch Risse im Meeresboden mehrere Kilometer tief ein. Auf diesem Weg wird das Wasser durch die Erdwärme bis auf annähernd 400°C erhitzt, wobei Minerale aus dem magmatischen Basalt gelaugt werden (71, 132). Dies führt dazu, daß die hydrothermalen Flüssigkeiten meist sauer und reduziert und unter anderem mit Schwermetallen und reduzierten Schwefelverbindungen, vor allem Schwefelwasserstoff, angereichert sind (71, 132).

Das erhitzte Wasser steigt wieder auf und wird mit variierender Strömungsgeschwindigkeit in das umgebende Meerwasser ausgestoßen. Dabei unterscheidet man Quellen mit geringer Strömungsgeschwindigkeit (0,5 bis 2 cm s^{-1}), bei denen sich die Hydrothermalflüssigkeit vor dem Austritt mit dem kühleren Seewasser mischt, von solchen, in denen die Hydrothermalflüssigkeit mit hoher Strömungsgeschwindigkeit (1 bis 2 m s^{-1}) ohne vorherige Vermischung austritt (71). In der Tiefsee kann dieser zweite Prozeß zu den Strukturen der sogenannten 'black smokers' (schwarze Raucher) führen, aus denen die Hydrothermalflüssigkeit aufgrund des hohen hydrostatischen Druckes mit Temperaturen von bis zu 380 °C ausströmt. Beim Kontakt dieser Flüssigkeit mit dem 2-4 °C kalten, oxischen Tiefseewasser, kommt es zu einer Präzipitation der in der Hydrothermalflüssigkeit enthaltenen Metallsulfide und von Calciumsulfat. Dies führt zum Aufbau der bis zu mehrere Dezimeter hohen Schlote. Die schwarze Farbe der als Wolke feiner Partikel austretenden Metallsulfide erinnert dabei an den aus Schornsteinen aufsteigenden Rauch.

An flacheren Hydrothermalquellen, wie dem in dieser Arbeit untersuchten System, kommt es dagegen aufgrund des geringeren hydrostatischen Druckes zum Kochen des Wassers und damit zu einer Phasentrennung zwischen der Flüssigkeit und den Gasen. Diese Arten von Hydrothermalquellen werden deshalb auch als Gaseohydrothermalquellen bezeichnet (26, 27, 144). Die Phasentrennung kann unter anderem zur Folge haben, daß sich die Salinität der Hydrothermalflüssigkeit erhöht, da ein Teil des Wassers als Wasserdampf entweicht. Neben den oben beschriebenen Hydrothermalquellen, gibt es auch submarine Hydrothermalsysteme, die direkt mit vulkanischer Aktivität verbunden sind. Diese sind sowohl für das Flachwasser (83, 143, 144) als auch für die Tiefsee an sogenannten 'hot-spot' Vulkanen beschrieben worden (86).

Das in dieser Arbeit untersuchte Hydrothermalsystem liegt in der Bucht von Palaeochori im Südosten der Insel Milos im Ägäischen Meer (28, 134, 165). Die Insel Milos ist Teil des südägäischen vulkanischen Bogens, der durch die Subduktion der afrikanischen Platte unter die

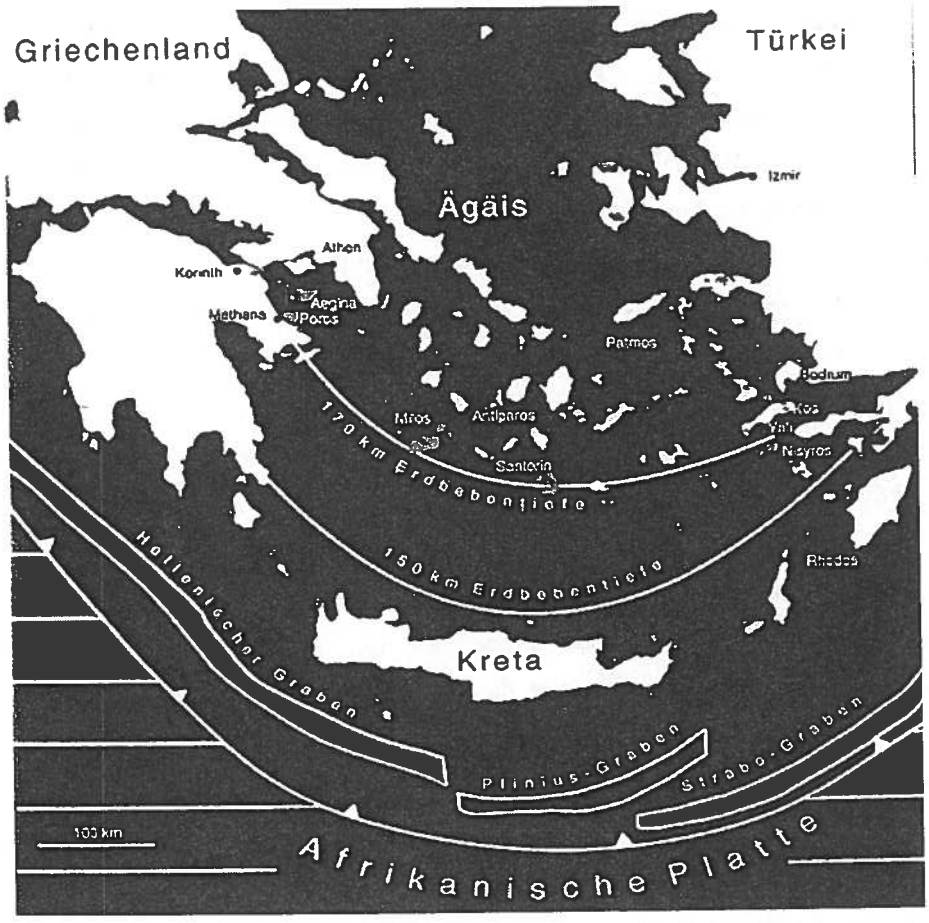

Abbildung 1. Darstellung der tektonischen Verhältnisse in der Ägäis und Lokalisation des südägäischen vulkanischen Bogens (entlang der Linie, welche die 170 km Erdbebentiefe markiert), sowie der Inseln Milos, Santorin und Nysiros.

eurasische Platte am Hellenischen Graben gebildet wurde und sich vom Pelepones bis zur türkischen Küste erstreckt (Abb. 1; 47). Auf diesem Bogen sind zwei noch rezent vulkanisch aktive Inseln lokalisiert, Santorin und Nysiros. Auf der Insel Milos, die ebenfalls vulkanischen Ursprungs ist, findet sich rezent nur noch solfaratische Aktivität. Die letzte vulkanische Aktivität liegt 80.000 Tausend Jahre zurück (46). Die Insel Milos, die eine Fläche von ca. 35 km² geothermisch aktivem Meeresboden aufweist, ist eine der am besten untersuchten hydrothermalen Gegenden der Ägäis (24, 27). Hydrothermale Aktivität wurde an vielen Orten um die Insel Milos nachgewiesen, wobei die Bucht von Palaeochori als eine der aktivsten Zonen erkannt wurde. In dieser Bucht gibt es eine große Anzahl von unterschiedlichen Quellen, die von der sublittoralen Zone bis in Tiefen von bis zu 120 m nachgewiesen wurden (27).

In der Abbildung 2 ist ein hypothetischer Querschnitt der Bucht von Palaeochori dargestellt, der die geologischen Voraussetzungen für die hydrothermale Aktivität in dieser Bucht veranschaulichen soll (24).

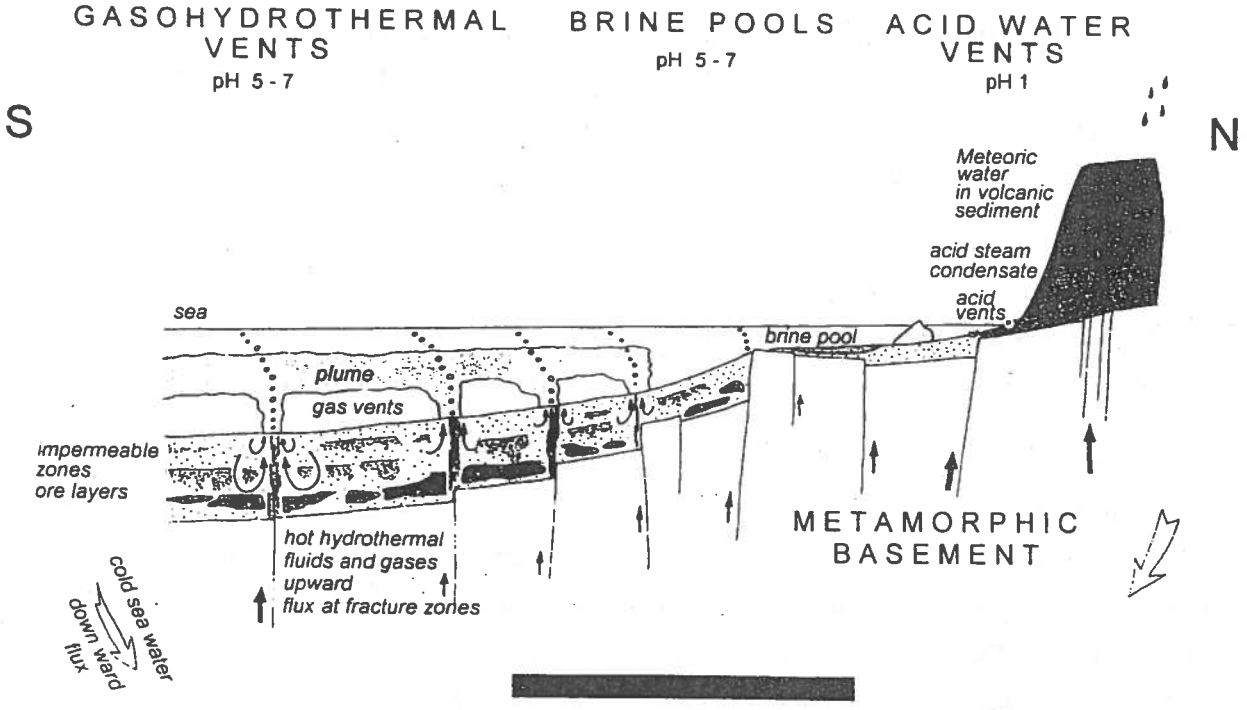

Abbildung 2. Hypothetischer Querschnitt der Bucht von Palaeochori. Siehe Text für weitere Erklärungen (aus Ref. 24).

Der Untergrund besteht aus brüchigem, metamorphem Gestein, welches in Blöcken entlang der Verwerfungsspalten angeordnet ist. Dieser Untergrund ist von marinen und vulkanoklastischen Sedimenten in unterschiedlicher Dicke überzogen. Die Verwerfungsspalten dienen als Durchlaß für die heißen, sauren und reduzierten Flüssigkeiten und Gase aus dem geothermalen Reservoir, das in 1,1 bis 1,3 km Tiefe liegt und eine Temperatur von 300 °C bis

323 °C aufweist (152). Die hydrothermalen Flüssigkeiten, für die ein magmatischer Ursprung nachgewiesen wurde (11, 110), können entweder diffus durch das Sediment oder an definierten Öffnungen austreten. Sie variieren in ihrer Zusammensetzung aufgrund von Interaktionen mit dem Gestein in der Erdkruste und dem Sediment, von Vermischungen mit dem umgebenden Seewasser und von mikrobieller Aktivität (24). In der Bucht von Palaeochori können prinzipiell drei verschiedene Typen von Hydrothermalquellen unterschieden werden: (A) Saure Quellen mit pH-Werten um eins, die an Land bzw. in sehr flachem Wasser (< 2 m) auftreten. (B) Salzlaken-Pools, die dort entstehen, wo hochsaline Hydrothermalflüssigkeiten diffus austreten und sich in Senken im Sediment ansammeln. (C) Die schon oben erwähnten Gaseohydrothermalquellen, an denen es zu einem gleichzeitigen Austritt von Gasen und Flüssigkeiten kommt. Die pH-Werte der beiden letzten Quelltypen liegen bei ca. 5-7.

Der in dieser Arbeit untersuchte Quellentyp gehörte der dritten Gruppe an. Es wurde eine fast konzentrische, solitäre Quelle mit einem Durchmesser von ca. vier Metern in einer Wassertiefe von acht Metern untersucht (134, 165). Neben einem definierten Austritt von Flüssigkeiten und Gasen im Zentrum der Quelle fand hier auch ein diffuser Ausstrom von Flüssigkeiten über eine größere Fläche statt. Dieser Typ eignete sich für die in dieser Arbeit durchgeführten Untersuchungen besonders gut, da gezielte Untersuchungen entlang eines Transektes vom Zentrum der Quelle in die vom Hydrothermalismus unbeeinflußten Bereiche durchgeführt werden konnten (134, 165).

2. Biologie der Hydrothermalquellen

Die Ökosysteme der Tiefsee-Hydrothermalquellen beruhen größtenteils auf der Produktion von organischem Material durch chemolithoautotrophe Prokaryoten (71, 85, 150). Diese Mikroorganismen oxidieren in der Hydrothermalflüssigkeit enthaltene reduzierte Verbindungen, in den meisten Fällen mit dem im umgebenden Seewasser enthaltenen Sauerstoff, und konservieren daraus die für den Aufbau von Biomasse aus Kohlendioxid (CO_2) benötigte Energie. Die wichtigsten Verbindungen in diesem Zusammenhang sind reduzierte Schwefelverbindungen, wie z.B. Schwefelwasserstoff (71). Wie zahlreiche Untersuchungen gezeigt haben, dient das so produzierte organische Material als Basis für die üppigen Lebensgemeinschaften an den Tiefsee-Hydrothermalquellen, an denen keine direkte Primärproduktion über Photosysnthese erfolgen kann (41, 73, 87, 101, 113, 124, 125, 161, 162). Diese Lebensgemeinschaften sind jedoch nicht völlig unabhängig vom Sonnenlicht, da die Oxidation der reduzierten Schwefelverbindungen mit Sauerstoff erfolgt, der letztendlich aus der oxygenen Photosynthese stammt (70, 85). Daher könnten diese Lebensgemeinschaften aufgrund des großen Reservoirs von im Meerwasser gelösten Sauerstoff nur für eine gewisse Zeit autark existieren.

In Hydrothermalsystemen, die in der euphotischen Zone lokalisiert sind, kommt es dagegen aufgrund des eindringenden Lichtes zu einem direkten Beitrag von photosynthetisch produzierten organischem Material zur Primärproduktion (27, 144). Es ist deshalb zu erwarten, daß sich die trophische Struktur der Lebensgemeinschaften an diesen flachen Hydrothermalquellen von jener an den Tiefsee-Hydrothermalquellen unterscheidet (144). In der Tiefsee steht nur ein Bruchteil der in der euphotischen Schicht der Ozeane produzierten Biomasse als Nahrung zur Verfügung (siehe z.B. Ref. 65). Die küstennahen Tiefsee-Hydrothermalquellen im Guaymas Becken stellen eine Ausnahme dar, da hier aufgrund der relativ hohen Sedimentationsrate von photosynthetisch produziertem (vor allem Diatomeendetritus) und terrigenem Material eine bis zu 400 m dicke, organisch angereicherte Sedimentschicht die Quellen überlagert (135). Inwieweit an den Tiefsee-Hydrothermalquellen oder auch an den Systemen in flacheren Meerestiefen anoxygene, lithoautotrophe Prokaryoten zur Primärproduktion beitragen, ist noch weitgehend ungeklärt (85). Diese Produktion vollzieht sich unabhängig vom Sonnenlicht, da die Biomasse aus geothermisch erzeugten Elektronendonatoren und -akzeptoren, wie z.B. Wasserstoff, Kohlendioxid und Schwefel, aufgebaut wird. Diese Produktion erklärt jedoch nicht die hohen Dichten der an den Hydrothermalquellen vorgefundenen Fauna (siehe unten).

2.1 Fauna an Hydrothermalquellen

Die an den Hydrothermalquellen vorkommende Fauna war seit der Entdeckung der ersten Tiefsee-Hydrothermalquellen vor etwa 20 Jahren Gegenstand zahlreicher Untersuchungen. Zu Beginn standen vorwiegend physiologische und morphologische Untersuchungen sowie Bestandsaufnahmen im Vordergrund (20, 35, 41, 45, 76, 125, 150). Eine Besonderheit dieser Fauna ist es, daß eine Vielzahl der Organismen in einer mehr oder weniger engen Symbiose mit schwefeloxidierenden Bakterien lebt (112). In einigen Fällen ist diese Symbiose soweit fortgeschritten, daß der Wirt keine Möglichkeit mehr besitzt, feste Nahrung von außen aufzunehmen. So besitzt der Röhrenwurm *Riftia pachyptila*, einer der häufigsten Tiere an Tiefsee-Hydrothermalquellen, keine Mundöffnung und keine Verdauungsorgane (76). Durch ein ausgeklügeltes System versorgt der Wurm die in einem bestimmten Organ, dem Trophosom, sitzenden Bakterien mit den von diesen zur Energiekonservierung benötigten Substanzen Sauerstoff und Schwefelwasserstoff (112). Andere Tiergruppen, wie z.B. Muscheln und Schnecken, haben ebenfalls enge Symbiosen mit schwefeloxidierenden Bakterien entwickelt (112). Weiterhin wurde deutlich, daß sich die vorwiegend endemische Fauna der Tiefsee-Hydrothermalquellen durch sehr hohe Individuendichten und eine hohe biologische Produktivität auszeichnet (150). Allerdings ist die Artenvielfalt dieser Lebensgemeinschaften niedriger ist als in der vom Hydrothermalismus unbeeinflußten Umgebung (150). Dies wurde auf die besonderen Umweltbedingungen an den Hydrothermalquellen

zurückgeführt, die es nur spezialisierten Lebensformen erlauben, diesen Lebensraum zu besiedeln. An den Hydrothermalquellen existieren extreme Gradienten von Umweltparametern, wie z.B. der Temperatur, des pH-Wertes und der H_2S-Konzentration (8, 71, 85). Außerdem wurde deutlich, daß die Hydrothermalquellen relativ kurzlebige Habitate darstellen und es immer wieder zu mehr oder weniger starken Störungen, wie z.B. durch vulkanische Aktivität, kommen kann (29). Aus diesem Grund wurde in den letzten Jahren auch vermehrt die räumliche und zeitliche Variabilität der Fauna sowie die sukzessive Besiedlung nach Störungen untersucht (45, 105, 131, 133, 151). Da die Tiefsee-Hydrothermalsysteme keinen anthropogenen Einflüssen unterworfen sind, dienten sie teilweise auch als Testfeld, um an anderen Systemen aufgestellte ökologischen Hypothesen zu überprüfen (151).

An den Hydrothermalquellen in flacheren Meeresgebieten fehlt in der Regel die für die Tiefseequellen so charakteristische Fauna (27, 44, 63, 83, 143, 144). Vor allem fehlen dort die engen Symbiosen zwischen Tieren und Bakterien. Eine Ausnahme stellt das Vorkommen eines Röhrenwurms der Ordnung *Vestimentifera* an einer Hydrothermalquelle in 82 m Wassertiefe vor Japan dar (55). Außerdem wurden an manchen flachen submarinen Hydrothermalquellen Nematoden der Unterfamilie der *Stilbonematinae* gefunden (83, 149). Diese Nematoden gehen enge Ektosymbiosen mit vermutlich schwefeloxidierenden Bakterien ein; ihr Vorkommen ist jedoch nicht auf hydrothermale Gebiete beschränkt, sondern sie finden sich generell in sulfidischen Habitaten (116). Wie in der Tiefsee zeigte sich auch an den flachen Hydrothermalquellen, daß die Diversität der Fauna zur Quelle hin abnahm und sich die Gemeinschaft in den vom Hydrothermalismus beeinflußten Bereichen durch wenige Arten, die jedoch hohe Individuendichten erzielen, auszeichnete (44, 83, 144, 149).

2.2 Mikrobielle Gemeinschaften an Hydrothermalquellen

Für das genauere Verständnis von Hydrothermalsystemen und deren Auswirkungen auf die Umwelt ist es notwendig, Informationen über die in diesen Habitaten vorkommenden mikrobiellen Gemeinschaften zu erhalten. Die mit den Hydrothermalquellen assoziierten Mikroorganismen verändern die in der Hydrothermalflüssigkeit enthaltenen Bestandteile, wie zum Beispiel H_2S, H_2, CH_4, CO_2, Metalle und organische Verbindungen, zum einen durch deren Aufnahme und Umwandlung in mikrobielle Biomasse (69, 71) und zum anderen, indem sie an deren Präzipitation mitwirken (34, 69, 82). In vielen Fällen bilden sie die Grundlage für alles an den Hydrothermalquellen vorgefundene höhere Leben (71, 85, 124).

Obwohl seit 20 Jahren vornehmlich die mikrobiellen Gemeinschaften der Tiefsee-Hydrothermalquellen untersucht werden, ist über diese Gemeinschaften weniger bekannt als über die oben beschriebene Fauna (7, 70, 85, 111). So gibt es bisher erst wenige Arbeiten, in denen die räumliche Heterogenität und die Struktur der mikrobiellen Gemeinschaften beschrieben wurde. Die Frage der räumlichen Heterogenität scheint jedoch gerade für

Mikroorganismen von entscheidender Bedeutung zu sein, da sich die Hydrothermalsysteme durch sehr kleinskalige Gradienten von sowohl chemischen als auch physikalischen Umweltparametern auszeichnen, die einer Vielzahl von Mikroorganismen potentielle Nischen eröffnen (7, 8, 70, 71, 85). Im besten Fall erfolgt die Erfassung der Verteilung von mikrobiellen Populationen in direkter Relation zu den physikochemischen Parametern in den für Mikroorganismen relevanten Größenordnungen (2). Dadurch lassen sich Aussagen über die Gründe einer beobachteten Verteilung von Mikroorganismen bzw. einer Veränderung der mikrobiellen Gemeinschaftsstruktur treffen. Allerdings wurden in den bisherigen mikrobiologischen Untersuchungen von marinen Hydrothermalquellen kaum simultane Bestimmungen der Umweltparameter durchgeführt.

Dies steht im Gegensatz zu terrestrischen geothermalen Systemen, an denen eine Vielzahl sehr detaillierter Untersuchungen der mikrobiellen Gemeinschaften und der diese strukturierenden Faktoren stattfanden. In diesem Zusammenhang verdienen die Untersuchungen von David Ward und Mitarbeitern besondere Erwähnung, da sie modellhaft am Beispiel der Octopus Quelle, einer heiße Quelle im Yellowstone National Park in den USA, den Kenntnisstand über die mikrobielle Diversität und die diese steuernden Faktoren enorm vergrößert haben (154). Als mögliche Ursachen für den unterschiedlichen Kenntnisstand mariner und terrestrischer Hydrothermalquellen werden Schwierigkeiten der Probennahme angeführt, die sich an submarinen Standorten und im speziellen in der Tiefsee sehr viel aufwendiger und kostspieliger gestaltet (85). Der Vorteil des in dieser Arbeit untersuchten Systems lag in seiner relativ guten Zugänglichkeit und der Möglichkeit, durch den Einsatz von Gerätetauchern sehr detaillierte und wiederholte *in situ* Messungen verschiedener Umweltparameter vornehmen zu können.

2.2.1 Untersuchungen an einzelnen Organismen

In vielen Fällen wurden die mikrobiellen Gemeinschaften der Hydrothermalquellen untersucht, indem Vertreter von spezifischen physiologischen Gruppen von Mikroorganismen, die als wichtig für das Ökosystem erachtet wurden, über Kultivierungsverfahren isoliert wurden (7, 70, 85). In Einzelfällen gelang es auch, ohne Kultivierung, physiologische und ökologische Untersuchungen an einzelnen Populationen durchzuführen. Aufgrund der potentiellen Bedeutung von Schwefelverbindungen für diese Ökosysteme gab es eine Vielzahl von Untersuchungen, die sich mit Mikroorganismen des Schwefelkreislaufs beschäftigten (7, 70, 85).

Mikroorganismen des oxidativen Teils des Schwefelkreislaufs

Hier wären die schon erwähnten chemolitho(auto)trophen Prokaryoten zu nennen, die reduzierte Schwefelverbindungen als Elektronendonatoren für eine Atmung mit O_2 oder NO_3^{2-}

und zur CO_2-Fixierung nutzen. Diese Organismen leben entweder an der Grenzfläche zwischen der anoxischen Hydrothermalflüssigkeit und dem oxischen Meerwasser oder in Symbiose mit Tieren. Interessanterweise bedurfte es des Umweges über die Tiefseehydrothermalquellen, um zu erkennen, daß diese Symbiosen in einer Vielzahl von Evertebraten in sulfidischen Habitaten vorkommen (19, 20, 41, 42). Obwohl diese Symbionten bisher nicht kultiviert werden können, gibt es mittlerweile einige Arbeiten, in denen die Phylogenie und Physiologie untersucht wurden (112). Anhand von Sequenzvergleichen der 16S rRNA wurde festgestellt, daß alle schwefeloxidierenden Endosymbionten zwei monophyletische Gruppen innerhalb der γ-Untergruppe der Proteobakterien bilden (31, 94). Bisher war es allerdings nicht mit Sicherheit möglich, den evolutiven Ursprung der Symbionten zu ermitteln. Als die nächsten freilebenden Verwandten wurden vielfach Vertreter der schwefeloxidierenden Gattung *Thiomicrospira* identifiziert (31, 33, 94, 120). Einige Wirte geben ihre Symbionten vertikal, d.h. über die Keimbahn, weiter, andere sind jedoch darauf angewiesen, im Laufe ihrer Ontogenese die Symbionten aus der Umgebung aufzunehmen (18, 43, 49, 50, 95). Dies läßt den Schluß zu, daß es Populationen freilebender Schwefeloxidierer geben müßte, die unter bestimmten Bedingungen eine Symbiose eingehen können. In den Fällen, in denen die Symbionten vertikal weitergegeben werden, enthalten die Wirte jeweils einen für sie spezifischen Symbionten, und zumindest bei den vesicomyden Muscheln gibt es Hinweise auf eine kongruente Evolution und damit eine Co-Spezialisierung von Wirt und Symbiont (17, 31, 90, 119). In den Fällen, in denen die Symbionten in jeder Generation extern aufgenommen werden, wurde beobachtet, daß verschiedene Wirte die gleichen Symbionten enthalten können (43, 95). Die physiologischen Studien ergaben, daß die Endosymbionten die Fähigkeit besitzen, CO_2 zu fixieren und damit autotroph zu wachsen (112). Weiterhin konnte gezeigt werden, daß die in dieser Hinsicht untersuchten Symbionten die reduzierten Schwefelverbindungen über den Adenylylphosposulfat-(APS)-Weg oxidieren, einen Weg, der zum Beispiel in *Thiomicrospira* bisher nicht nachgewiesen werden konnte (112). In einigen Fällen gibt es Hinweise darauf, daß die Symbionten in der Lage sind, eine anaerobe Sulfidoxidation mit Nitrat als Elektronakzeptor an Stelle von Sauerstoff durchzuführen (60, 61, 99).

Bei den freilebenden Formen an submarinen Hydrothermalquellen können prinzipiell drei verschiedene Typen von Schwefeloxidierern unterschieden werden. Eine erste Gruppe bilden filamentöse Formen wie *Beggiatoa* und *Thiotrix* (44, 63, 68, 74, 83, 98, 113, 143, 144, 161) und, wie erst kürzlich vor Milos entdeckt, auch *Thioploca* (25). Von diesen gibt es bis heute keine Reinkulturen. Einige physiologische Studien und *in situ* Untersuchungen an *Beggiatoa* konnten zum Verständnis von deren Ökologie beitragen (51, 101, 113). Besonders große *Beggiatoa* Arten bilden an den Hydrothermalquellen im Guyamas Becken auf dem Sediment in der Nähe von Austritten hydrothermaler Flüssigkeit dichte Matten (bis zu mehreren Zentimetern Dicke) (113). *In situ* Untersuchungen mit Mikroelektroden für Temperatur und

Sauerstoff zeigten, daß die Dicke der Matten durch einen durch den Ausstoß von Hydrothermalwasser induzierten konvektiven Porenwassertransport bestimmt wird. Mit dessen Hilfe werden die Bakterien effizient mit Sauerstoff und Schwefelwasserstoff versorgt. Dadurch wird eine Diffusionslimitierung durch den im umgebenden Meerwasser nur in geringen Konzentrationen vorhandenen Sauerstoff überwunden (51). Außerdem besitzen *Beggiatoa* die Fähigkeit zu gleiten und damit die Möglichkeit, sich aktiv an der Grenzfläche zu positionieren. Spätere Untersuchungen zeigten dann, daß diese Arten von *Beggiatoa* intrazellulär Nitrat konzentrieren und als alternativen Elektronenakzeptor für die Oxidation von Schwefelwasserstoff verwenden können (101). Damit wäre auch eine Möglichkeit geschaffen, die oben beschriebene Diffusionslimitierung durch Sauerstoff zu umgehen. Während *Thioploca* und *Beggiatoa* zusammen eine phylogenetische Linie innerhalb der γ-Untergruppe der Proteobakterien darstellen (1, 147, 148), bildet *Thiotrix* eine eigenständige Gruppe innerhalb der γ-Untergruppe der Proteobakterien (123, 137, 147). Diese filamentösen Schwefelbakterien besitzen nicht die Fähigkeit zu gleiten. Sie heften sich vielmehr an einem Untergrund an und sind somit auf einen stationären Gradienten angewiesen. Bei allen an submarinen Hydrothermalquellen vorkommenden filamentösen Schwefelbakterien konnte bis heute nicht nachgewiesen werden, ob diese obligat chemolithoautotroph wachsen, oder ob sie auch organische Verbindungen als Elektronendonator und Kohlenstoffquelle verwenden können. Für die in Guyamas Becken vorkommenden *Beggiatoa* wurde gezeigt, daß sie die Enzyme des Calvinzyklus besitzen und somit über die Fähigkeit verfügen, Kohlendioxid zu fixieren (113).

Eine zweite Gruppe freilebender schwefeloxidierender Bakterien umfaßt zum einen säureproduzierende chemolithoheterotrophe Bakterien und zum anderen Tetrathionat produzierende, heterotrophe Bakterien (37, 81, 129). Während die ersten nicht in der Lage sind, autotroph zu wachsen, jedoch reduzierte Schwefelverbindungen als Elektronendonatoren für eine Atmung mit O_2 oder NO_3^{2-} nutzen können, oxidieren Vertreter der zweiten Gruppe, Thiosulfat zu Tetrathionat, welches ins Medium abgegeben wird. Die Fähigkeit, Thiosulfat zu Tetrathionat zu oxidieren, ist bei vielen Mikroorganismen vorhanden. Nach phänotypischen Merkmalen wurden diese heterotrophen schwefeloxidierenden Bakterien den Gattungen *Pseudomonas*, *Vibrio*, *Acinetobacter* und *Alcaligenes* zugeordnet (37, 81, 129). Es wurden allerdings keine Stammbeschreibungen vorgenommen.

Eine dritte Gruppe freilebender schwefeloxidierender Bakterien stellen die obligat chemolithoautotrophen Bakterien dar. Von diesen wurden bisher mehrmals Vertreter der Gattung *Thiomicrospira* aus verschiedenen Tiefseehydrothermalsystemen (15, 37, 75, 109, 128, 129, 160) und *Thiobacillus hydrothermalis* von einem Tiefseehydrothermalsystem im Fji Becken (38) isoliert. Diese Bakterien sind obligat auf reduzierte Schwefelverbindungen als Elektronendonatoren und auf Kohlendioxid als Kohlenstoffquelle angewiesen. Sowohl die verschiedenen *Thiomicrospira*-Arten als auch *Thiobacillus hydrothermalis* sind mesophil,

neutrophil und wachsen optimal bei einer NaCl-Konzentration, die der des Meerwasser entspricht (13, 38). Während die Gattung *Thiomicrospira* eine monophyletische Gruppe innerhalb der γ-Untergruppe der Proteobakterien darstellt, deren kultivierte Vertreter alle marin und obligat chemolithoautotroph sind (13), befinden sich Vertreter der Gattung *Thiobacillus* in der α- β- und γ-Untergruppe der Proteobakterien (89). Thiobacilli wurden bisher vorwiegend aus Süßwasser-Standorten isoliert. Die phylogenetische Heterogenität ist historisch bedingt, da früher allein die Fähigkeit, reduzierte Schwefelverbindungen zu Sulfat zu oxidieren, zusammen mit der stäbchenförmigen Morphologie als taxonomisches Merkmal zur Einordnung in die Gattung *Thiobacillus* verwendet wurde (89). Phylogenetische Analysen haben jedoch gezeigt, daß dieser Metabolismus innerhalb der Gram-negativen Bakterien weit verbreitet ist. Neben einer genetischen Heterogenität weisen Thiobacilli auch eine physiologische Heterogenität auf. So gibt es neben den obligat chemolithoautotrophen Formen auch fakultativ chemolithoautotrophe und chemolithoheterotrophe Formen. Die beobachtete Heterogenität innerhalb der Thiobacilli hat zu taxonomischen Neugliederungen der Gattung, vorwiegend basierend auf 16S rRNA-Sequenzen, geführt (62, 88, 89, 102).

Neben den bisher beschriebenen Mikroorganismen gibt es aber auch andere, neue Organismen, deren Stoffwechsel noch nicht eindeutig aufgeklärt wurde. Ein erst kürzlich beschriebenes schwefeloxidierendes Bakterium könnte an Tiefsee-Hydrothermalquellen wesentlich zur Primärproduktion beitragen. Mehrfach wurde an verschiedenen Tiefsee-Hydrothermalquellen nach vorherigen sogenannten 'diking events' der Ausstoß von weißen, flockigen, unterhalb des Meeresbodens entstandenem Material beobachtet (Ref. 29 und darin enthaltene Literatur). Es ist wahrscheinlich, daß die Produktion dieses Materials hauptsächlich auf ein mesophiles, aerobes chemolithoautotrophes Bakterium zurückzuführen ist (145, 146). Dieses vibrioförmige Bakterium, das bisher nicht in Reinkultur vorliegt, bildet filamentösen Schwefel, der morphologisch und auch chemisch dem flockigen Material der Tiefseequellen ähnelt. Phylogenetisch ist es der *Arcobacter*-Gruppe innerhalb der ε-Unterguppe der Proteobakterien zuzuordnen (146).

Bisher ist nicht bekannt, ob die obligat chemolithoautotrophen oder die chemolithoheterotrophen schwefeloxidierenden Bakterien an den Hydrothermalquellen dominieren. Aus Anreicherungskulturen wurden vorwiegend chemolithoheterotrophe Organsimen isoliert (37, 129). Dies sagt jedoch wenig über die numerische Dominanz *in situ* aus, da Anreicherungskulturen die am schnellsten wachsenden und nicht unbedingt die numerisch häufigsten Organismen favorisieren (36, 67, 155). In Kompetionsversuchen in Chemostaten wurde nachgewiesen, daß die Dominanz des jeweiligen Stoffwechseltyps von der Konzentration organischer Kohlenstoffquellen abhing. Bei niedrigen Konzentrationen dominierte der obligat chemolithotrophe Typ, während bei höheren Konzentrationen chemolithoheterotrophe Formen besser wuchsen (91). Aufgrund der räumlichen Heterogenität der Hydrothermalquellen ist die

Existenz von Mikrohabitaten vorstellbar, die sowohl den einen, als auch den anderen Typen favorisieren (37). Außerdem ist eine Koexistenz beider Stoffwechseltypen denkbar (72).

Mikroorganismen des reduktiven Schwefelkreislaufs

Im reduktiven Teil des Schwefelkreislaufs werden oxidierte Schwefelverbindungen, wie z.B. Sulfat oder Schwefel, von heterotrophen Mitgliedern der Domänen *Archaea* und *Bacteria* als terminale Elektronenakzeptoren für den anaeroben Abbau von organischen Material verwendet und letztlich zu Sulfid reduziert. Einige dieser Organismen können jedoch auch autotroph wachsen und es wurden auch obligat autotrophe Formen isoliert (7, 9, 70, 85). Diese Organismen kommen meist im anaeroben Bereich der Hydrothermalquellen vor. Infolgedessen sind viele thermophil (optimale Wachstumstemperatur zwischen 50 und 80°C) bzw. hyperthermophil (optimale Wachstumstemperatur >80°C). Das Konzept einer natürlichen Lebensgemeinschaft unterhalb des Meeresbodens (subseafloor community) bzw. allgemein innerhalb der Erdkruste (deep subsurface community) wurde von Gold (48) als Hypothese basierend auf nur wenigen Daten formuliert. Mittlerweile gibt es allerdings deutliche Hinweise, die dieses Konzept unterstützen (29, 30, 64, 93, 114, 139, 141). Während eine Vielzahl (hyper)thermophiler *Archaea* bekannt sind, die an marinen Hydrothermalquellen am reduktiven Schwefelkreislauf beteiligt sind (Ref. 9 und darin enthaltene Literatur), gibt es erst wenige thermophile Vertreter der *Bacteria*. Hier wären Vertreter der *Thermotogales* (7), sowie *Desulfurobacterium thermolithotrophum* (92) und der alkanverwertende, sulfatreduzierende Stamm TD3 (130) zu nennen. Mesophile sulfatreduzierende Bakterien wurden an drei verschiedenen Tiefseehydrothermalsystemen nachgewiesen und isoliert (40).

Über eine Vielzahl von intermediären Schwefelverbindungen stehen der oxidative und der reduktive Teil des Schwefelkreislaufs miteinander in Verbindung. Als wichtiges Intermediat wurde dabei Thiosulfat erkannt, das sowohl von Organismen des oxidativen als auch von Organismen des reduktiven Schwefelkreislaufs verwertet werden kann (78). Allerdings ist über den Schwefelkreislauf an Hydrothermalquellen nur wenig bekannt. Es gibt aber Hinweise, daß Thiosulfat auch dort von Bedeutung sein kann (126, 164).

Mikroorganismen mit anderen metabolischen Fähigkeiten

Neben den am Schwefelkreislauf beteiligten Organismen, wurden auch Organismen mit anderen physiologischen Fähigkeiten isoliert. Hierzu zählen heterotrophe Vertreter der Gattungen *Bacillus* und *Thermus* (96, 97), *Hyphomonas* (157), und Vertreter der *Spirochaeten* (54), aber auch autotrophe Organismen, wie methanogene *Archaea* (77) und mangan-oxidierende Bakterien (39). Es wäre hier noch zu erwähnen, daß z.B. die Vertreter der *Thermococcales* und *Thermotogales* zwar fermentativ ohne elementaren Schwefel wachsen können, jedoch in Anwesenheit von Schwefel deutlich besser wachsen (7, 9, 70, 138). Der

Schwefel dient in diesem Fall nicht als Elektronenakzeptor zur Energiekonservierung, sondern vielmehr als "electron sink" für Reduktionsäquivalente, die während der Fermentation entstehen. Sowohl von Jannasch (70) als auch Karl (85) wird darauf hingewiesen, daß es keine Untersuchungen gibt, in der die potentielle Bedeutung von Eisen (Fe^{3+}) als Elektronenakzeptor an Hydrothermalquellen untersucht worden ist. Dies ist überraschend, da relativ große Mengen an Eisen mit der hydrothermalen Flüssigkeit ausgestoßen werden (70, 85, 132).

2.2.2 Untersuchungen der mikrobiellen Gemeinschaften an Hydrothermalquellen mittels molekularbiologischer Methoden

Obwohl anhand der isolierten Organismen wichtige Informationen über deren potentielle Fähigkeiten und ihre Anpassungen an das jeweilige Habitat gewonnen wurden, blieb deren ökologische Bedeutung für das System in den meisten Fällen unklar. Dies liegt zum einen daran, daß in den meisten Fällen Organismen aus Anreicherungen isoliert wurden, deren Inokulum, das heißt die Umweltprobe, nicht verdünnt wurde. Wie bereits erwähnt, kann dies dazu führen, daß nicht unbedingt die häufigsten, sondern die unter den Kultivierungsbedingungen am schnellsten wachsenden Organismen isoliert werden (36, 67, 155). Zum anderen wurde durch die Anwendung von molekularbiologischen Methoden in den letzten Jahren immer deutlicher, daß nur ein Bruchteil der in einem Habitat vorkommenden Prokaryoten mit herkömmlichen Methoden kultivierbar ist und daß diese in den wenigsten Fällen die ökologisch relevanten Arten darstellen (3, 117, 153, 154, 156). Daraus wird deutlich, daß eine umfassende Untersuchung der mikrobiellen Gemeinschaft und der trophischen Struktur eines Habitats nur mit Hilfe von Kultivierungsmethoden unzureichend ist.

Mit Hilfe der molekularbiologischen Methoden wurde es möglich, Populationen *in situ* nachzuweisen, ohne daß diese vorher kultiviert werden müssen (3, 153). Der Nachweis erfolgt hierbei über spezifische Markermoleküle, wobei sich die 16S rRNA und das diese codierende Gen (16S rDNA) als sehr geeignet herausgestellt haben. Für eine detaillierte Beschreibung der in dieser Arbeit hauptsächlich verwendeten denaturierenden Gradienten-Gel-Elektrophorese (DGGE) sowie anderer in der molekularen Ökologie angewandter molekularbiologischer Methoden, sei auf entprechende Übersichtsartikel und die darin enthaltene Literatur verwiesen (3, 106-108, 117, 136, 153). Die 16S rRNA erfüllt bei allen Prokaryoten die gleiche Funktion und enthält sowohl konservierte als auch variable Sequenzbereiche. Dies macht sie als phylogenetisches Markermolekül geeignet. Da mittlerweile Datenbanken zur Verfügung stehen, in denen rund 16.000 16S rRNA Sequenzen hinterlegt sind, ist es möglich, neue Sequenzen mit vorhandenen zu vergleichen und phylogenetisch zuzuordnen. Dies ermöglichte in der Geschichte der Mikrobiologie außerdem zum ersten Mal eine Systematik, die eine evolutionäre Abstammung der Mikroorganismen berücksichtigte (163). Dadurch wurde es auch möglich, die Vielfalt der Mikroorganismen als Produkt ihrer Anpassung an das Habitat und Evolution zu betrachten

(154). Die Anpassung von Organismen an bestimmte ökologische Nischen ist die treibende Kraft der evolutiven Diversifikation und der Bildung von eigenständigen Populationen (10, 118, 154).

Bisher gibt es erst wenige Arbeiten, in denen mikrobielle Gemeinschaften submariner Hydrothermalquellen mit molekularbiologischen Methoden untersucht wurden. Diese Untersuchungen zeigten, daß die bakteriellen Gemeinschaften nur von wenigen Populationen dominiert wurden (103, 104, 109, 121). Dies stand im Gegensatz zu den Ergebnissen anderer Untersuchungen an weniger extremen Standorten und wurde mit den besonderen Umweltbedingungen erklärt, die, ähnlich wie bei der Fauna, nur spezialisierten Populationen ein Wachstum ermöglichen.

Unter Verwendung der denaturierenden Gradienten-Gel-Elektrophorese (DGGE) (108) konnten Muyzer und Mitarbeiter (109) vier Phylotypen in zwei Proben (Oberflächen von Sulfidstrukturen) von je zwei Tiefsee-Hydrothermalsystemen am Mittelozeanischen Rücken (Snake-Pit bzw. TAG Hydrothermalfeld) identifizieren. Unter diesen konnten zwei Populationen nachgewiesen werden, die phylogenetisch in die schwefeloxidierende Gattung *Thiomicrospira* fielen. Eine dieser beiden Populationen war anhand der Sequenz eines 550 bp langen Fragments der 16S rRNA sogar fast identisch mit *Thiomicrospira crunogena*, einem Isolat aus dem gleichen Habitat. Dies zeigt den seltenen Fall einer Kongruenz zwischen Kultivierungsmethoden und molekularbiologischen Methoden und ist ein deutlicher Hinweis darauf, daß *Thiomicrospira* spp. tatsächlich wichtige Vertreter der mikrobiellen Gemeinschaft darstellen. Eine dritte Population zeigte Ähnlichkeit mit Vertretern der sulfatreduzierenden Gattung *Desulfovibrio* und ist somit am reduktiven Teil des Schwefelkreislaufs beteiligt.
In einer Untersuchung von Polz und Mitarbeitern (121) am Snake-Pit Hydrothermalfeld wurde festgestellt, daß der bakterielle Epibiont einer die Fauna dominierenden Garnele (*Rimicaris exoculata*) ebenfalls die mikrobielle Gemeinschaft an dieser Quelle beherrscht. Interessanterweise stellte dieser Phylotyp, welcher der ε-Untergruppe der Proteobakterien zuzuordnen ist, auch auf der Oberfläche von Sulfidstrukturen 50% der aus diesem Habitat extrahierten rRNA. *Archaea* und *Eukarya* konnten in diesen Proben nicht nachgewiesen werden. Der genaue Metabolismus des Epibionten von *Rimicaris exoculata* ist nicht bekannt, es sprechen allerdings einige Hinweise dafür, daß es sich auch hierbei um ein schwefeloxidierendes Bakterium handelt (121, 124, 161).

Eine weitere Untersuchung beschäftigte sich mit der mikrobiellen Gemeinschaft einer mikrobiellen Matte, die sich an einem Hydrothermalsystem auf der Sedimentoberfläche des submarinen Vulkans Loihi Seamount vor Hawaii bildete. Die Erstellung einer Klonbibliothek zeigte, daß die mikrobielle Gemeinschaft von zwei sogenannten operationalen taxonomischen Einheiten (operational taxonomic units; OTU) bestimmt wurde. Dabei war die häufigste OTU (60,5% der Klone) mit dem schwefeloxidierenden Bakterium *Thiovolum* sp. assoziiert (104).

Kürzlich konnte gezeigt werden, daß die zweithäufigste OTU eine hohe Verwandtschaft mit einem aus Süßwasser isolierten neutrophilen chemolithoautotrophen Fe^{2+}-Oxidierer zeigt (111). Dies unterstützt im nachhinein die frühe Vermutung, daß die Eisenoxidation an dem Loihi Hydrothermalsystem einen wichtigen mikrobiellen Prozeß darstellt (86). Insgesamt zeigen die Ergebnisse der vorgenannten Untersuchungen, daß die mikrobiellen Gemeinschaften an Tiefseehydrothermalquellen von chemolitho(auto)trophen Populationen dominiert werden und die Produktion neuer Biomasse über Chemosynthese, vorwiegend basierend auf reduzierten Schwefelverbindungen, vermutlich maßgeblich zur Erhaltung dieser Ökosysteme beiträgt.

Die Zusammensetzung der mikrobiellen Gemeinschaften der Hochtemperaturbereiche von Hydrothermalquellen ist bisher vorwiegend über Kultivierungsmethoden analysiert worden. Aufgrund der häufigen Isolierung von *Archaea* aus diesen Habitaten wurde davon ausgegangen, daß *Archaea* die mikrobielle Gemeinschaft dominieren (138). Ferner zeigten Analysen der aus Sulfidstrukturen eines 'black-smokers' extrahierten Lipide, daß in den Regionen, die den höchsten *in situ* Temperaturen ausgesetzt waren, die Konzentration der für *Archaea* spezifischen Etherlipide deutlich höher lag als die Konzentration der für Bakterien charakteristischen Phospholipide (56). Analysen der mikrobiellen Gemeinschaften mittels molekularbiologischer Methoden zeigten dann jedoch überraschenderweise ein anderes Bild. So konnte zwar nachgewiesen werden, daß die archaeelle Vielfalt in marinen und terrestrischen Hydrothermalsystemen sehr groß ist (5, 6, 28, 142); die Dominanz der *Archaea* war in diesen Habitaten jedoch eher die Ausnahme und es waren die Bakterien, die in vielen Fällen dominierten (52, 66, 127, 142). Mit Hilfe der fluoreszierenden *in situ* Hybridisierung (FISH) konnte gezeigt werden, daß sich in den Sulfidstrukturen eines black-smokers des Snake-Pit Hydrothermalfeldes auf dem Mittelozeanischen Rücken eine hohe Zahl von Prokaryoten befand (52). Die mikrobielle Gemeinschaft bestand zu etwa gleichen Teilen aus Bakterien und Archaea, wobei auch hier die Bakterien leicht dominierten. Der Unterschied zu der Arbeit von Hedrick et al. (56) könnte darin begründet sein, daß die untersuchten Proben unterschiedlichen *in situ* Temperaturen ausgesetzt waren. So könnte es sein, daß in Bereichen in denen die Temperatur über 100 °C liegt *Archaea* dominieren. Es ist bis heute kein Bakterium bekannt, das bei diesen Temperaturen wächst. Harmsen et al. (52) konnten darüber hinaus zeigen, daß die morphologische Vielfalt *in situ* höher war als die gleichzeitig über Kultivierung bestimmte Vielfalt. Es konnten keine Zellen identifiziert werden, die mit den Sonden für *Bacillus* und *Thermus* hybridisierten (53), obwohl Vertreter dieser Genera aus diesem Habitat isoliert werden konnten. Dies deutet darauf hin, daß diese Isolate wohl nicht zu den *in situ* dominanten Populationen zählen. Auch in dieser Arbeit zeigte sich, daß eine gleichzeitige Verwendung molekularbiologischer und auf Kultivierung basierender Methoden deutliche Vorteile bringt. So konnte über FISH nachgewiesen werden, daß 40% der Bakterienzellzahlen einer bis dahin unbekannten Gruppe von Bakterien zugeordnet

werden konnten (53). Über Kultivierung konnte aus den gleichen Proben ein Vertreter dieser Gruppe isoliert und charakterisiert werden (92).

3. Zielsetzung der Arbeit

Die hier vorliegende Arbeit war Teil des von der Europäischen Union im Rahmen des Programms 'Marine Science and Technology (MAST)' geförderten Projekts 'Hydrothermal Fluxes and Biological Production in the Aegean'. Im Rahmen dieses Projekts war es das Ziel der Arbeitsgruppen am Max-Planck-Institut für Marine Mikrobiologie, eine Hydrothermalquelle vor der Küste der Insel Milos sowohl biogeochemisch als auch mikrobiologisch genauer zu untersuchen, um so zu deren Gesamtverständnis beizutragen. Die Quelle sollte gewissermaßen als Modellsystem für ähnliche Systeme an anderen Orten dienen.

Aufgrund der an der ausgewählten Hydrothermalquelle auftretenden Gradienten von physikochemischen Parametern stellte diese Quelle ein ideales System dar, um den Einfluß sich verändernder Umweltbedingungen auf die mikrobielle Gemeinschaft zu untersuchen. Hauptziel der vorliegenden Arbeit war es somit, die räumliche Heterogenität der mikrobiellen Gemeinschaft und deren Struktur entlang eines Transektes vom Zentrum der Quelle in das angrenzende Sediment zu untersuchen. Die gleichzeitige Bestimmung von physikochemischen Parametern entlang des gleichen Transektes sollte es ermöglichen, die beobachteten Veränderungen in der mikrobiellen Gemeinschaft mit den Umweltparametern in Beziehung zu setzen. Um die mikrobielle Gemeinschaft zu untersuchen, wurden sowohl synökologische, d.h. die gesamte Gemeinschaft betreffende, als auch autökologische, d.h. einzelne Populationen und Organismen betreffende, Untersuchungen durchgeführt. Beide Ansätze sind wichtig, um ein Gesamtverständnis der an einem Habitat ablaufenden mikrobiellen Prozesse zu erhalten.
Dazu wurden zum einen molekularbiologische, kulturunabhängige Verfahren und zum anderen mikrobiologische, auf Kultivierung beruhende Verfahren verwendet. In einer Vielzahl von Untersuchungen hat sich die denaturierende Gradienten-Gel-Elektrophorese (DGGE) als geeignete Methode erwiesen, um Veränderungen in der mikrobiellen Gemeinschaft aufzuzeigen. In der vorliegenden Arbeit wurde aus diesem Grund die DGGE verwendet, um räumliche Veränderungen in der Gemeinschaftsstruktur und der Zusammensetzung der mikrobiellen Gemeinschaften zu untersuchen. Dazu wurde aus den verschiedenen Proben genomische DNA extrahiert und die bakterielle 16S rDNA mittels PCR amplifiziert. Die daraus erhaltenen vervielfältigten Fragmente wurden anschließend mittels DGGE nach ihren Sequenzunterschieden aufgetrennt. Daraus ergeben sich für jede Probe charakteristische Bandenmuster, die dann miteinander verglichen werden können. Idealerweise kann dabei jede Bande einer bestimmten Population zugeordnet werden. Es ist allerdings zu beachten, daß nur Populationen erfaßt werden, die mehr als 0,1% bzw. 1% der Gesamtpopulationen ausmachen. Um phylogenetische Informationen über die in diesem Habitat dominanten Bakterien zu erhalten, wurden weiterhin DGGE-Banden ausgeschnitten, mittels PCR reamplifiziert und anschließend sequenziert. Die phylogenetische Analyse sollte es erlauben, Aussagen über die trophische Struktur der

mikrobiellen Gemeinschaft zu treffen. Es war weiterhin von Interesse zu untersuchen, inwieweit sich die relative Häufigkeit von *Bacteria* zu *Archaea* entlang des an dieser Hydrothermalquelle auftretenden Temperaturgradienten verändert. Hierzu wurde die slot-blot Hybridisierung von aus der Umwelt extrahierter RNA verwendet. Mit Hilfe dieser Methode läßt sich der Anteil spezifischer Organismengruppen an der Gesamtgemeinschaft unter Verwendung von spezifischen rRNA-Sonden quantitativ erfassen. Darüberhinaus können über einen Vergleich der rRNA–Konzentration mit der Zellzahl Aussagen über Unterschiede in der Aktivität der mikrobiellen Gemeinschaft gemacht werden, da der Gehalt an ribosomaler RNA pro Zelle im allgemeinen mit deren Aktivität korreliert.

Bei den kultivierungsabhängigen Methoden wurde vorwiegend die most-probable number Methode verwendet. Diese Methode hat den Vorteil, daß sie eine Abschätzung spezifischer physiologischer Gruppen von Mikroorganismen erlaubt. Gleichzeitig wird es damit ermöglicht numerisch dominante und somit vermutlich ökologisch relevante Organsimen zu isolieren. Aufgrund der Bedeutung von Schwefelverbindungen für Hydrothermalsysteme wurden hier im Besonderen Organismen des Schwefelkreislaufs untersucht. Darüberhinaus interessierte jedoch auch die Verteilung von dissimilatorischen Eisenreduzierern. Diese Organismen spielen in Sedimenten eine wichtige Rolle beim anaeroben Abbau von organischem Material. Ungeachtet der teilweise hohen Konzentrationen an Eisen in Hydrothermalflüssigkeiten, gibt es bisher jedoch keine Untersuchungen darüber, inwieweit durch Eisen vermittelte mikrobielle Prozesse an Hydrothermalquellen eine Rolle spielen. Die aus den Verdünnungsreihen erhaltenen Isolate sollten weiter phänotypisch untersucht und phylogenetisch analysiert werden. Zur Untersuchung der Bedeutung des schwefeloxidierenden Genus *Thiomicrospira* wurde eine direkte Kombination aus mikrobiologischen (MPN, Isolation und Charakterisierung) und molekularbiologischen (spezifische PCR, DGGE, Hybridisierung) Methoden verwendet.

B Ergebnisse und Diskussion

Im folgenden soll aufgezeigt werden, inwieweit die Ergebnisse der einzelnen Publikationen miteinander im Zusammenhang stehen. Dieser Abschnitt kann jedoch nicht die Diskussionen der Publikationen ersetzen, und für eine ausführliche Betrachtung der Einzelergebnisse sei von daher auf diese verwiesen.

1. Untersuchungen zur Zusammensetzung und Struktur mikrobieller Lebensgemeinschaften entlang eines Umweltgradienten

Aufgrund von steilen physikalischen und chemischen Gradienten bieten Hydrothermalquellen eine Vielzahl an Habitaten und Mikronischen, die potentiell von einer großen Zahl metabolisch unterschiedlicher Mikroorganismen besiedelt werden können (7, 8, 71, 85). Die dort vorkommenden Organismen sind in vielfältiger Weise an der Veränderung der austretenden Hydrothermalflüssigkeit beteiligt (7, 69-71, 82, 85). Von daher sind Informationen über die Zusammensetzung der mikrobiellen Gemeinschaften wichtig, um die Funktionsweise dieser Systeme besser zu verstehen. Allerdings gibt es bisher kaum Untersuchungen über die räumliche Verteilung der an marinen Hydrothermalquellen vorkommenden mikrobiellen Populationen (52, 53, 63, 144), sowie über die Veränderungen in der mikrobiellen Gemeinschaften entlang der dort vorherrschenden Gradienten (56). Da Kultivierungsmethoden alleine nicht geeignet sind, um die generelle Zusammensetzung von mikrobiellen Gemeinschaften zu erfassen (3, 106, 117, 153, 154), wurden in der vorliegenden Arbeit auch kultivierungsunabhängige Verfahren angewandt.

Zunächst wurde die räumliche Verteilung der bakteriellen Populationen entlang eines Umweltgradienten an einer Flachwasser-Hydrothermalquelle in der Nähe der Insel Milos im ägäischen Meer (Griechenland) untersucht. Die Untersuchungen erfolgten zu zwei Zeitpunkten unter Anwendung der Acridine-Orange Färbetechnik zur Bestimmung der Gesamtzellzahlen, von Kultivierungsmethoden, sowie der denaturierenden Gradienten-Gel-Elektrophorese (DGGE) von mit PCR amplifizierten 16S rRNA Genfragmenten (Publikationen 1 und 2). Die Proben für diese Analysen wurden entlang eines Transektes vom Zentrum der Quelle in die Umgebung genommen. Zeitgleich wurden entlang des gleichen Gradienten geochemische Parameter bestimmt. Die über die most-probable number (MPN) Technik ermittelten Zellzahlen von metabolisch definierten Gruppen von Bakterien machten generell nur einen kleinen Teil der Gesamtzellzahlen aus (siehe unten; Punkt 2). Mittels DGGE konnten insgesamt 91 unterschiedliche Populationen identifiziert werden. Die Sequenzierung von ausgeschnittenen und reamplifizierten Banden zeigte das Vorhandensein eines weiten Spektrums verschiedener evolutiver Linien der Bakterien. Allerdings war nur ein kleiner Teil der Sequenzen nahe mit kultivierten Bakterien verwandt, was sich mit anderen Untersuchungen deckt (66, 104, 154,

156). Mittels Sequenzierung konnte auch gezeigt werden, daß in fast allen Proben eine dominante Population vorkam, die der phylogenetischen Gruppe *Cytophaga-Flavobacteria-Bacteroides* zugeordnet werden konnte. Dies weist daraufhin, daß diese Population eine hohe Toleranz gegenüber sich verändernden Umweltparametern aufweist. Allerdings ist aufgrund der Konserviertheit des 16S rRNA Gens nicht auszuschließen, daß es sich bei denen in verschieden Zonen vorkommenden Populationen um sogenannte Ökotypen handelt, die sich an bestimmte Umweltbedingungen entlang des Gradienten angepaßt haben (118, 154). Die anhand der Sequenzierung der DGGE-Banden gewonnenen Daten lassen darauf schließen, daß, obwohl autotrophe Populationen nachgewiesen werden konnten (siehe unten; Punkt 2 und 3), die mikrobielle Gemeinschaft insgesamt als nettoheterotroph charakterisiert werden kann. Die DGGE-Analyse zeigte weiterhin einen Trend von einer diversen Bakteriengemeinschaft in der Übergangszone, in denen die Populationen in annähernd gleicher Zahl vertreten waren, zu einer Gemeinschaft im Zentrum der Quelle, die von wenigen Populationen dominiert wurde. Ein wichtiger Faktor, der diesen Trend bestimmte, war die Temperatur. Dies bestätigte die Hypothese, daß extreme Umweltbedingungen zu einer Gemeinschaftsstruktur mit einer limitierten Anzahl an dominanten Populationen führen. Dieser Befund ist in Übereinstimmung mit Untersuchungen an Tiefsee-Hydrothermalquellen und terrestrischen Geothermalsystemen (66, 103, 109, 156). Allerdings wurde in diesen Untersuchungen im Gegensatz zur vorliegenden Arbeit nicht untersucht, inwieweit sich die Gemeinschaftstruktur mit dem abnehmenden Einfluß der Hydrothermalflüssigkeit auf das Sediment verändert. Der hier geschilderte Trend konnte allerdings bei einer zweiten Probennahme nicht gefunden werden. Dies lag wahrscheinlich an einer Störung der oberen Sedimentschichten durch einen der zweiten Probennahme vorangegangenen Sturm. Zusammenfassend kann festgestellt werden, daß die sich verändernden Umweltbedingungen einen starken Einfluß auf die Verteilung von bakteriellen Populationen und die Struktur der bakteriellen Gemeinschaft ausübte. Darüberhinaus konnten dominante bakterielle Populationen identifiziert werden. Dabei konnten sowohl Ähnlichkeiten mit Tiefsee-Hydrothermalquellen als auch mit terrestrischen Geothermalsystemen festgestellt werden. In zukünftigen Untersuchungen sollte es das Ziel sein, die relative Häufigkeit der als wichtig erkannten Populationen zu bestimmen, z.B. über fluoreszente *in situ* Hybridisierung (FISH), und diese zu kultivieren. Außerdem ist es von großem Interesse, die relative Bedeutung der Photosynthese und der Chemosynthese als Produzenten von organischem Material für das System zu bestimmen (Publikationen 1 und 2).

Die höchsten Konzentrationen an prokaryotischer ribosomaler RNA (rRNA) (definiert als die Summe aus bakterieller und archaeller RNA) wurden in den obersten Sedimentschichten gefunden und nahmen in tieferen Sedimentschichten stark ab. Dies zeigt an, daß die mikrobiellen Prozesse hauptsächlich an der Oberfläche stattfanden. Darüberhinaus wurde eine höhere Konzentration in näher zum Quellzentrum gelegenen Regionen nachgewiesen, was darauf hin-

deutet, daß die hydrothermale Aktivität die mikrobielle Aktivität stimulierte. Bakterien machten mindestens 78% (durchschnittlich 95%) der prokaryotischen rRNA aus, was anzeigt, daß die Bakterien hauptsächlich für diese Aktivität verantwortlich waren. Entlang des steilsten Temperaturgradienten stieg der Anteil archaeeller rRNA an. Trotzdem machten *Archaea* selbst in den heißesten Sedimentschichten, in denen eine Quantifizierung möglich war (*in situ* Temperatur 82°C), nur 11,9% der prokaryotischen rRNA aus. Dies zeigt an, daß *Archaea* an der untersuchten Hydrothermalquelle nur eine untergeordnete Komponente der mikobiellen Gemeinschaft darstellten. Dies war etwas überraschend, deckt sich aber mit Befunden aus anderen Untersuchungen (52, 66, 142). Ungeklärt bleiben allerdings die Faktoren, die es den Bakterien ermöglichen in einem Habitat zu dominieren, welches ursprünglich als Reich der *Archaea* angesehen wurde. Weitere Studien sind erforderlich, um die ökologische Rolle von *Archeaa* und *Bacteria* in Habitaten mit hohen Temperaturen im Detail zu erforschen und um die Faktoren die deren relative Häufigkeit steuern aufzuklären (Publikation 3).

2. Häufigkeit und Verteilung spezifischer physiologischer Gruppen von Bakterien und des schwefeloxidierenden Genus *Thiomicrospira*

Kultivierungsmethoden und Radiotracer-Experimente haben gezeigt, daß Schwefelverbindungen an Hydrothermalquellen wichtige Substrate für die mikrobielle Gemeinschaft darstellen (70, 71, 85). Von großer Bedeutung sind aerobe chemolithoautotrophe Prokaryoten, die aus der Oxidation reduzierter Schwefelverbindungen Energie konservieren. Diese Organismen, die allgemein als schwefeloxidierende Bakterien (SOB) bezeichnet werden, leben entweder an der Grenzfläche zwischen dem oxischen Meerwasser und der sulfidhaltigen Hydrothermalflüssigkeit oder als Symbionten von Evertebraten (70, 71, 85, 112). Sie werden als die hauptsächlichen Produzenten von organischem Material an den Tiefsee-Hydrothermalquellen angesehen (41, 71, 85, 87, 125, 161, 162). Andererseits werden oxidierte Schwefelverbindungen von einer Vielzahl von Archaeen und Bakterien als Elektronenakzeptoren zur anaeroben Oxidation von organischem Material verwendet (7, 70, 85). Ein wichtiger Prozess in diesem Zusammenhang ist die dissimilatorische Sulfatreduktion durch sulfatreduzierende Bakterien (SRB) (27, 79, 80, 109, 144). Neben der Sulfatreduktion wird die Fe(III)-Reduktion als wichtigster Prozeß des anaeroben Abbaus von organischem Material in marinen Sedimenten angesehen (16). Bisher gab es an marinen Hydrothermalquellen allerdings keine Untersuchungen über die Bedeutung von dissimilatorischen Fe(III)-reduzierenden Mikroorganismen (70, 85), obwohl die hydrothermalen Flüssigkeiten generell erhöhte Eisenkonzentrationen aufweisen (70, 85, 132). In der vorliegenden Arbeit wurde die Häufigkeit und Verteilung von SOB, SRB und dissimilatorisch Fe(III)-reduzierenden Bakterien (DIRB) entlang des Transektes bestimmt. Diese physiologisch definierten Gruppen können auch als Gilden bezeichnet werden, da sie jeweils eine Gruppe von Arten umfassen, die eine bestimmte

Ressource in ähnlicher Weise nutzen (10). Die über Kultivierung erhaltenen Zellzahlen wurden mit den gleichzeitig bestimmten Gesamtzellzahlen in Beziehung gesetzt (Publikation 1).

Die über die most-probable number (MPN) Technik ermittelten Zellzahlen der verschiedenen Gilden machten nur einen kleinen Teil der Gesamtzellzahlen aus. Sowohl die Gesamtzellzahlen als auch die MPN-Zahlen zeigten die höchsten Werte in der Übergangszone zwischen den von der Hydrothermalflüssigkeit stark beeinflußten Sedimenten und den von der Hydrothermalflüssigkeit unbeeinflußten Sedimenten. Die hohen Gesamtzellzahlen sowie relativ hohen Abundanzen von SRB und DIRB in 200 cm und 235 cm Entfernung zum Zentrum der Quelle waren wahrscheinlich zum einen durch die moderaten Umweltbedingungen, d.h. neutralen pH und Temperaturen um 20 °C, bedingt und zum anderen auf das Vorhandensein von Diatomeen und anderen Primärproduzenten, wie z.B. SOB, zurückzuführen. Aus der höchsten positiven Verdünnung der MPN für SRB (10^{-6}) konnte ein Sulfatreduzierer isoliert werden, der auf Ebene der 16S rRNA 97% Ähnlichkeit mit *Desulfobacter halotolerans* (12) aufwies. *Desulfobacter* spp. haben ein relativ enges Substratspektrum und werden als Spezialisten für den Abbau von Acetat angesehen (158). Von daher läßt das Vorhandensein von *Desulfobacter* spp. in solch hoher Zahl darauf schließen, daß Acetat, als eines der Endprodukte des anaeroben Abbaus von organischem Material, in diesem Habitat ein wichtiges Substrat darstellt. In der Zone mit den höchsten Zellzahlen von SRB konnten auch die höchsten Sulfatreduktionsraten gemessen werden (165). Der Befund, daß die DIRB in vergleichbarer Zahl wie die SRB vorhanden waren, deutet zudem darauf hin, daß der Abbau von organischem Material durch dissimilatorische Eisenreduktion einen wichtigen Prozeß an der untersuchten Hydrothermalquelle darstellt. Unterstützt wird diese Hypothese durch die Beobachtung, daß die höchste Konzentration an Fe(III) (165) mit der höchsten Zellzahl der DIRB korrelierte.

Die höchsten Zahlen an SOB konnten in 200 cm Entfernung vom Zentrum der Quelle nachgewiesen werden. Dort konnten SOB aufgrund der erhöhten Eindringtiefe von Sauerstoff auch noch in tieferen Sedimentschichten nachgewiesen werden. Neben dieser Zone konnte auch ein sich in einer bestimmter Zone auf dem Sediment bildendes Präzipitat als günstiges Habitat für Bakterien im allgemeinen und SOB im besonderen identifiziert werden. Hier scheint ein ähnliches Bakterium wie das von Taylor und Wirsen (145) beschriebene, filamentösen Schwefel bildende chemolithoautotrophe Bakterium, welches als *Arcobacter* sp. identifiziert wurde (146), eine wichtige Rolle zu spielen. Das Präzipitat hat sehr große Ähnlichkeit mit dem von Taylor und Wirsen (145) beschriebenen filamentösen Schwefel und dem an den Tiefsee-Hydrothermalquellen vorgefundenen Material. Darüberhinaus konnte ich unter Anwendung von FISH nachweisen, daß der in dem Präzipitat vorherrschende Organismus eine vibrioide *Arcobacter*-Art ist. Diese Befunde, die noch weiterer Überprüfung bedürfen, deuten darauf hin, daß dieser bisher unbekannte Organismus eine wichtige Rolle in marinen hydrothermalen Ökosystemen einnimmt (Publikation 1). Mittels FISH konnte weiterhin nachgewiesen werden,

daß die im Präzipitat vorkommenden Bakterien metabolisch aktiv waren, da über 90% der mit DAPI gefärbten Zellen mit der für Bakterien spezifischen Sonde EUB 338 hybridisiert werden konnten (24).

Da es Befunde gibt, die anzeigen, daß *Thiomicrospira* spp. an Tiefsee-Hydrothermalquellen eine wichtige ökologische Rolle spielen (75, 109, 128, 129, 160), war es von Interesse zu untersuchen, ob dies auch für im Küstenbereich lokalisierte Hydrothermalquellen zutrifft. Basierend auf den in der Publikation 1 durchgeführten Untersuchungen erfolgte eine detaillierte Untersuchung über die Verteilung und Diversität des an der Flachwasser-Hydrothermalquelle vorkommenden schwefeloxidierenden Genus *Thiomicrospira* (Publikation 4). Die Zellzahlen nahmen sowohl zum Zentrum der Quelle hin (horizontale Verteilung) als auch mit der Sedimenttiefe (vertikale Verteilung) ab. Dies korrespondierte mit einer Zunahme der Temperatur (von ca. 25 °C auf 60 °C) und einer Abnahme des pH-Wertes (von ca. pH 7 auf 5). Mit Hilfe einer spezifischen PCR (14) konnte nachgewiesen werden, daß Vertreter der Gattung *Thiomicrospira* spp. zu den abundantesten kultivierbaren Schwefeloxidierer gehörten und in einer Zone sogar die dominante Form darstellten. Eine phylogenetische Analyse zeigte, daß die aus den höchsten MPN-Verdünnungen erhaltenen Sequenzen zwei eng verwandte Cluster innerhalb der *Thiomicrospira*-Linie bildeten (Cluster 1 und 2). Aus Anreicherungen, die mit hohen Verdünnungen (10^{-5}) inokuliert wurden, konnten zwei neue Stämme, Milos-T1 und Milos-T2, isoliert werden. Eine phylogenetische Analyse zeigte, daß das Isolat Milos-T1 am nächsten mit den kürzlich beschriebenen Arten *Thiomicrospira kuenenii* und *Hydrogenovibrio marinus* verwandt ist, während das Isolat Milos-T2 am nächsten mit den aus den MPN erhaltenen Sequenzen des Cluster 2 verwandt war. Die Vorherrschaft des Stammes Milos-T2 an der untersuchten Hydrothermalquelle wurde durch dessen Identifikation in verschiedenen Umweltproben über eine Hybridisierung des DGGE-Gels und durch das Sequenzieren einer entsprechenden DGGE-Bande nachgewiesen. Insgesamt unterstützen die in dieser Arbeit dargestellten Ergebnisse die früheren Hinweise, daß *Thiomicrospira* einen wichtigen Bestandteil der an marinen Hydrothermalquellen vorgefundenen mikrobiellen Gemeinschaft darstellt (Publikation 4).

3. Isolierung und Charakterisierung neuartiger Bakterien

Aus den MPN-Reihen konnten verschiedene schwefeloxidierende und sulfatreduzierende Bakterien isoliert werden, die im folgenden beschrieben werden.

3.1 Schwefeloxidierende Bakterien

Die bisher aus marinen Hydrothermalquellen isolierten und charakterisierten obligat chemolithoautotrophen schwefeloxidierenden Bakterien gehören mit Ausnahme des von einer im

Fiji Becken lokalisierten Tiefseequelle isolierten *Thiobacillus hydrothermalis* (38) alle der Gattung *Thiomicrospira* an (75, 128, 160). In der vorliegenden Arbeit konnte neben zwei neuen Stämmen der Gattung *Thiomicrospira* (Publikation 7) und weiteren, neuen schwefeloxidierenden Bakterien, die neue Linien innerhalb der γ-Untergruppe der Proteobakterien darstellen (siehe unten; Publikation 6), auch Bakterien isoliert werden, die am nächsten zu *Thiobacillus hydrothermalis* verwandt sind (Publikation 5). Ein hier näher charakterisierter Stamm wurde aus der niedrigsten Verdünnung isoliert. Basierend auf der annähernd vollständigen Sequenz der 16S rRNA bildet der Stamm Milos-BII1T eine phylogenetische Gruppe mit *Thiobacillus hydrothermalis*, *Thiobacillus neapolitanus*, *Thiobacillus halophilus* und *Thiobacillus* sp. W5. Diese Bakterien sind alle obligat chemolithoautotroph. Aufgrund ihrer phylogenetischen Verwandtschaft und ihrer physiologischen Ähnlichkeit wurde von Kelly und Wood (89) vorgeschlagen, diese Bakterien in einem neuen Genus innerhalb der γ-Untergruppe der Proteobakterien, dem Genus *Halothiobacillus*, zusammenzufassen. Der hier beschriebene Stamm Milos-BII1T stellt somit eine neue Art dieses Genus dar, für den wir den Namen *Halothiobacillus kellyi* vorschlagen. Die Zellen waren Gram-negative, sehr bewegliche Stäbchen. Der Organismus war weiterhin obligat autotroph sowie strikt aerob. Interessanterweise konnte dieser Organismus nicht aus den höchsten MPN-Verdünnungen isoliert werden, obwohl seine Wachstumscharakteristika, wie z.B. die pH- und Temperatur-Optima, ihn gut an die Umweltbedingungen angepaßt erscheinen lassen. Dies könnte darin begründet sein, daß in dem Habitat keine Substratlimitierung vorlag und somit andere, an höhere Substratkonzentrationen angepaßte Arten, wie z.B., *Thiomicrospira*, einen Vorteil haben. Erste Untersuchungen hierzu wurden in einer Diplomarbeit durchgeführt (57). Andererseits wurde aus den niedrigsten Verdünnungen und aus Anreicherungen immer ein anhand der 16S rRNA mit dem Stamm Milos-BII1T identischer Organismus isoliert. Dieser Befund zeigt deutlich den Vorteil einer Verdünnungsreihe, um zum einen eine größere Zahl verschiedener Organismen zu isolieren und zum anderen die numerisch dominanten und somit wahrscheinlich ökologisch relevanten Arten zu isolieren (36, 67, 155, siehe unten).

Es gelang weiterhin, andere neue SOB aus den MPN zu isolieren (Publikation 6). Davon wurden fünf ausgewählt und detaillierter charakterisiert. Alle Stämme waren obligat chemolitho-autotroph, hatten pH-Optima im neutralen pH-Bereich und wuchsen am besten bei marinen NaCl-Konzentrationen. Die Stämme Milos-NDII1.1 und Milos-NDII1.2 wurden aus anaeroben MPN-Reihen mit Thiosulfat als Elektronendonor und Nitrat als Elektronenakzeptor isoliert. Darüberhinaus konnte auch für den Stamm Milos-OAII2 eine anaerobe Thiosulfatoxidation mit Nitrat als terminalem Elektronenakzeptor nachgewiesen werden. Aufgrund eines Sequenz-vergleichs der 16S rRNA konnte festgestellt werden, daß die nächsten Verwandten dieser Bakterien mit verschiedenen Evertebraten assoziierte schwefeloxidierende Symbionten sind.

Dies läßt es als wahrscheinlich erscheinen, daß der letzte gemeinsame Vorfahre der Endosymbionten ein schwefeloxidierendes Bakterium war. Die Tatsache, daß die Verwandtschaft zwischen den Endosymbionten und den hier beschriebenen Isolaten auf die Wurzel beider Linien beschränkt ist, zeigt an, daß die Symbiose sehr früh entstanden sein muß (109). Dies führte zu einer Co-Evolution zwischen den Symbionten und deren jeweiligen Wirten auf der einen, und der unabhängigen Evolution der freilebenden Formen auf der anderen Seite. Neben den phylogenetischen Daten sprechen auch physiologische Daten für eine nahe Verwandtschaft dieser Gruppen. Bei einer Vielzahl von Symbionten konnte eine Oxidation reduzierter Schwefelverbindungen unter anaeroben Bedingungen mit Nitrat als Elektronenakzeptor nachgewiesen werden (58-61, 99, 159). Darüberhinaus wurde bei allen bisher untersuchten Symbionten festgestellt, daß sie für den energiekonservierenden Schritt vom Sulfit zum Sulfat den Adenylylphosphosulfat (APS)-Weg verwenden (112). Keine von beiden metabolischen Fähigkeiten, d.h. weder die anaerobe Thiosulfatoxidation noch die Oxidation von Sulfit zu Sulfat über den APS-Weg, konnten bei *Thiomicrospira* nachgewiesen werden (112). Arten dieser Gattung wurden basierend auf der 16S rRNA jedoch vielfach als die nächsten freilebenden Verwandten der Endosymbionten angesehen (31, 32, 109, 120, 122). Im Gegensatz dazu konnten beide Fähigkeiten bei einzelnen, hier beschriebenen Isolaten nachgewiesen werden. Es war auffällig, daß die Isolate unterschiedliche Temperaturoptima aufwiesen. Dies könnte bedeuten, daß die Isolate an ihre jeweilige *in situ* Temperatur angepaßt waren, da die Isolate nahe des Zentrums der Quelle die höchsten Temperaturoptima aufwiesen. Um die jeweils am besten angepaßten Formen aus einem Habitat zu isolieren, scheint es daher wichtig zu sein, die Proben bei der *in situ* Temperatur zu inkubieren (155) (Publikation 6).

Zusammenfassend konnte gezeigt werden, daß die Diversität innerhalb der Gilde der schwefeloxidierenden Bakterien groß war. Dies lag unter anderem an der Anwendung der Verdünnungstechnik und der Inkubation bei der jeweiligen *in situ* Temperatur (36, 154, 155). Es konnten neue Arten der Genera *Thiomicrospira* und *Halothiobacillus* isoliert werden, die schon von anderen Hydrothermalquellen bekannt waren. Ob das Vorkommen der neuen SOB auf das untersuchte Hydrothermalsystem beschränkt ist, oder ob diese auch in anderen Systemen zu finden sind, muß noch überprüft werden. Offen bleibt auch die Frage, welche Faktoren das gleichzeitige Vorhandensein von physiologisch ähnlichen Bakterien in teilweise vergleichbar hohen Zahlen in einem Habitat ermöglichen (Publikationen 4, 5 und 6).

3.2 Sulfatreduzierende Bakterien

Der hier beschriebene Stamm MT-96T stellt neben dem Stamm TD3 erst das zweite thermophile, sulfatreduzierende Bakterium dar, das von einem marinen Hydrothermalsystem isoliert wurde (Publikation 7). Der Stamm MT-96T kann eine große Anzahl verschiedener Substrate von einfachen organischen Verbindungen bis zu langkettigen Fettsäuren als

Elektronendonator verwenden. Ferner war in der Gegenwart von Sulfat auch autotrophes Wachstum mit H_2 und CO_2 möglich. Die Zellen hatten eine ovale Form, eine durchschnittliche Breite von 0,8 bis 1 μm und eine Länge von 1,5 bis 2 μm. Die Zellen waren in der Regel nicht beweglich, obwohl gelegentlich bewegliche Formen beobachtet wurden. Es wurden keine Sporen gebildet und die Zellen waren Gram-negativ. Der Stamm wuchs bei Temperaturen von 37 °C bis 64 °C, mit einem Optimum bei 60 °C. Wachstum wurde bei einer NaCl-Konzentration von 15 bis 78 g pro Liter beobachtet, mit einem Optimum zwischen 32 und 36 g NaCl pro Liter. Dies könnte auf eine Adaptation an die erhöhte Salinität der hydrothermalen Flüssigkeit hinweisen. Die Sequenzanalyse der 16S rRNA ergab, daß dieser Organismus in die δ-Untergruppe der Proteobakterien fällt und daß die Sequenz eine Ähnlichkeit von <95.3% zu bereits beschriebenen Arten aufweist. Der Stamm MT-96T war phänotypisch und phylogenetisch am nächsten mit *Desulfacinum infernum* verwandt und repräsentiert eine neue Art innerhalb des Genus *Desulfacinum*, für die wir den Namen *Desulfacinum hydrothermale* vorschlagen. Im Gegensatz zu den nahe verwandten, thermophilen Arten *Desulfacinum infernum* und *Thermodesulforhabdus norvegicus*, die aus Nordsee-Ölfeldern isoliert wurden, wurde die hier beschriebene Art aus einem geothermal beeinflußten marinen Sediment isoliert, in dem keine ölähnliche Verbindungen vorkommen. Zwei weitere thermophile sulfatreduzierende Bakterien, die aufgrund einer 16S rRNA Sequenzanalyse am nächsten mit dem Stamm MT-96T bzw. *Thermodesulforhabdus norvegicus* verwandt sind, konnten aus einer Hydrothermalquelle in der Nähe von Milos in einer Wassertiefe von 120 m isoliert werden. Die hier dargestellten Ergebnisse unterstützen die Idee der Existenz einer thermophilen mikrobiellen Gemeinschaft unterhalb des Meeresbodens (114) (Publikation 7).

Ein wesentlicher Prozeß, der zur Fraktionierung der stabilen Schwefelisotope ^{34}S und ^{32}S in Sedimenten führt, ist die dissimilatorische Sulfatreduktion. Diese findet auch an geothermal beeinflußten Habitaten, wie der in dieser Arbeit untersuchten Hydrothermalquelle, statt. Eine wichtige, bisher allerdings wenig untersuchte Frage ist, ob sich die Isotopenfraktionierung durch mesophile und thermophile Sulfatreduzierer grundlegend unterscheidet. Diese Frage hat auch eine Bedeutung hinsichtlich der Interpretation des Isotopensignals in der erdgeschichtlichen Vergangenheit, in der die Temperaturen höher lagen (115). Zur Beantwortung dieser Fragestellung wurde die Fraktionierung von Schwefelisotopen ($^{34}S/^{32}S$) während der Reduktion von gelöstem Sulfat anhand einer statischen Kultur des Stammes MT-96T bei 60°C untersucht (Publikation 8; siehe Publikation 7 für eine ausführliche Beschreibung des Isolats). Der in den Experimenten produzierte Schwefelwasserstoff war gegenüber Sulfat um etwa 19‰ in ^{32}S angereichert. Somit wies das untersuchte thermophile Bakterium eine ähnliche Diskriminierung der stabilen Isotope auf wie bereits zuvor untersuchte, mesophile sulfatreduzierende Bakterien und nur eine leicht erhöhte Fraktionierung gegenüber dem thermophilen, Gram-positiven Sulfatreduzierer *Desulfotomaculum nigrificans* (21, 22, 84, 100).

Die an dem Hydrothermalsystem direkt gemessenen Werte für die Schwefelisotopenverteilung zwischen Sulfat und den reduzierten Schwefelverbindungen lagen im Bereich der mit dem Isolat erhaltenen Werte. Dies deutet darauf hin, daß die Sulfatreduktion an der untersuchten Quelle einen wichtigen Beitrag zur Schwefelisotopenzusammensetzung liefern kann (Publikation 8).

Neben den thermophilen Sulfatreduzieren konnten auch mesophile Sulfatreduzierer isoliert werden, die jedoch in dieser Arbeit nicht weiter charakterisiert wurden. Aus der höchsten postiven Verdünnung (10^{-6}) konnte ein Sulfatreduzierer isoliert werden, der auf Ebene der 16S rRNA 97% Ähnlichkeit mit *Desulfobacter halotolerans* aufwies (siehe oben). Darüberhinaus konnte aus Anreicherungen ein eisenreduzierendes Bakterium isoliert werden. Anhand der 16S rRNA und der Fettsäurenzusammensetzung konnte dieses Bakterium der Gattung *Desulfovibrio* innerhalb der δ-Untergruppe der Proteobakterien zugeordnet werden. Dieses Bakterium wurde in einer Diplomarbeit näher charakterisiert (140).

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Teil 2: Publikationen

A Publikationsliste mit Erläuterungen

Diese Dissertation beruht auf den im folgenden aufgelisteten Publikationen. Zu jeder Publikation gibt es eine kurze Erläuterung, welche meine Beiträge zu den Arbeiten aufzeigen soll.

1. Spatial Heterogeneity of bacterial populations along an environmental gradient at a shallow submarine hydrothermal vent near Milos island (Greece)

Stefan M. Sievert, Thorsten Brinkhoff, Gerard Muyzer, Wiebke Ziebis, and Jan Kuever

Entwicklung des Konzeptes unter Mitarbeit von Jan Kuever, Wiebke Ziebis, und Gerard Muyzer. Durchführung aller mikrobiologischen Versuche und der Gesamtzellzahlbestimmung. Eigenständige Durchführung der molekularbiologischen Versuche nach einer Einführung durch Thorsten Brinkhoff. Erstellung des Manuskriptes unter der redaktionellen Mitarbeit der Co-Autoren.

2. Identification of 16S rDNA-defined bacterial populations at a shallow submarine hydrothermal vent near Milos island (Greece)

Stefan M. Sievert, Jan Kuever, and Gerard Muyzer

Entwicklung des Konzeptes mit Jan Kuever und Gerard Muyzer. Durchführung aller Versuche. Phylogenetische Analyse zusammen mit Gerard Muyzer. Erstellung des Manuskriptes unter der redaktionellen Mitarbeit der Co-Autoren.

3. Relative abundance of *Archaea* and *Bacteria* along a thermal gradient of a shallow-water hydrothermal vent quantified by rRNA slot-blot hybridization

Stefan M. Sievert, Wiebke Ziebis, Jan Kuever, and Kerstin Sahm.

Entwicklung des Konzeptes. Durchführung der RNA-Extraktion, des 'blottens' und der Auswertung. Durchführung der Hybridisierung und Auswertung der Daten mit Kerstin Sahm. Erstellen des Manuskriptes unter der redaktionellen Mitarbeit von Kerstin Sahm.

4. Distribution and diversity of sulfur-oxidizing *Thiomicrospira* spp. at a shallow-water hydrothermal vent in the Aegean Sea (Milos, Greece)

Thorsten Brinkhoff, Stefan M. Sievert, Jan Kuever, and Gerard Muyzer

Entwicklung des Konzeptes mit Thorsten Brinkhoff, Jan Kuever und Gerard Muyzer. Durchführung der mikrobiologischen Versuche. Redaktionelle Mitarbeit am Manuskript.

5. *Halothiobacillus kellyi* sp. nov., a mesophilic obligately chemolithoautotrophic sulfur-oxidizing bacterium isolated from a shallow-water hydrothermal vent in the Aegean Sea and emended description of the genus *Halothiobacillus*

Stefan M. Sievert, Thorsten Heidorn and Jan Kuever

Entwicklung des Konzeptes mit Jan Kuever. Mit Ausnahme der Chemostaten-Versuche, Durchführung aller mikrobiologischen Versuche sowie der phylogenetischen Analyse. Erstellung des Manuskriptes mit Jan Kuever.

6. Novel sulfur-oxidizing bacteria from a shallow submarine hydrothermal vent, most closely related to obligate symbionts of invertebrates

Stefan M. Sievert, Gerard Muyzer, and Jan Kuever.

Entwicklung des Konzeptes mit Jan Kuever. Durchführung der Versuche mit technischer Unterstützung von Ingrid Kunze und Simone Deutschel. Phylogenetische Analyse zusammen mit Gerard Muyzer. Erstellung des Manuskriptes unter der redaktionellen Mitarbeit der Co-Autoren.

7. *Desulfacinum hydrothermale*, sp. nov., a thermophilic completely oxidizing sulfate-reducing bacterium from geothermally heated sediments near Milos Island (Greece)

Stefan M. Sievert, and Jan Kuever

Entwicklung des Konzeptes mit Jan Kuever. Durchführung aller Arbeiten. Erstellung des Manuskriptes mit Jan Kuever.

8. Sulfur isotope fractionation during dissimilatory reduction of sulfate by a thermophilic Gram-negative bacterium at 60°C

Michael E. Böttcher, Stefan M. Sievert, and Jan Kuever.

Entwicklung des Konzeptes mit Michael E. Böttcher. Durchführung der mikrobiologischen Versuche. Redaktionelle Mitarbeit am Manuskript.

B Publikationen

Spatial Heterogeneity of Bacterial Populations Along an Environmental Gradient at a Shallow Submarine Hydrothermal Vent near Milos Island (Greece)

Stefan M. Sievert, Thorsten Brinkhoff, Gerard Muyzer, Wiebke Ziebis, and Jan Kuever

Applied and Environmental Microbiology, 65:3834-3842, 1999

ABSTRACT

The spatial heterogeneity of bacterial populations at a shallow-water hydrothermal vent in the Aegean Sea close to the island of Milos (Greece) was examined at two different times by using acridine orange staining for total cell counts, cultivation-based techniques and denaturing gradient gel electrophoresis (DGGE) analysis of PCR-amplified 16S rRNA gene fragments. Concurrent with measurements of geochemical parameters, samples were taken along a transect from the center of the vent to the surrounding area. Most-probable-number (MPN) counts of metabolically defined sub-populations generally constituted a minor fraction of the total cell counts; both counting procedures revealed highest cell numbers in a transition zone from the strongly hydrothermally-influenced sediments to normal sedimentary conditions. Total cell counts ranged from 3.2×10^5 cells ml^{-1} in the water overlying the sediments to 6.4×10^8 cells g (wet weight) of $sediment^{-1}$. MPN counts of chemolithoautotrophic sulfur-oxidizing bacteria varied between undetectable and 1.4×10^6 cells g^{-1}. MPN counts for sulfate-reducing bacteria and dissimilatory iron-reducing bacteria ranged from 8 cells g^{-1} to 1.4×10^5 cells g^{-1} and from undetectable to 1.4×10^6 cells g^{-1}, respectively. DGGE revealed a trend from a diverse range of bacterial populations which were present in approximately equal abundance in the transition zone, to a community dominated by few populations close to the center of the vent. Temperature was found to be an important parameter in determining this trend. However, at one sampling time this trend was not discernible, possibly due to storm-induced disturbance of the upper sediment layers.

INTRODUCTION

Submarine hydrothermal vents are well known for extremes in geochemical conditions, as well as for the exotic life they support. Cultivation-based studies and radiotracer experiments have revealed that at most marine hydrothermal vents, sulfur compounds seem to be important substrates for microbes (23, 26). On one hand chemolithoautotrophic prokaryotes gain energy by the oxidation of reduced sulfur compounds. These organisms, which either live free at the interface between the anoxic hydrothermal fluid and the oxygenated seawater or in symbioses with animals, are thought to be the main primary producers at deep-sea vents (23, 25, 26, 53), where light is absent. However, at shallow-water vents light is present and thus primary production by photosynthetic organisms can take place (see, e.g., references 10 and 41). On the other hand, oxidized sulfur compounds are used by many heterotrophic members of the *Archaea* and *Bacteria* as electron acceptor for the anaerobic degradation of organic matter, although some can also grow autotrophically. The habitat for these organisms are the anoxic parts of the hydrothermal system, and correspondingly many of them are thermophiles or hyperthermophiles (5, 26). However, due to sharp physical and chemical gradients, hydrothermal vents offer a variety of habitats and microniches, which can potentially be inhabited by metabolically diverse microorganisms (4, 5, 23, 26). Little is known, however, about the spatial distribution of the microbial populations thriving in these ecosystems (19, 22, 41), and the changes in community structure occurring along these gradients (20).

Cultivation-based methods are not well suited to investigate the general composition of microbial communities, since only a small percentage of the microorganisms are cultivable (2) and cultivation may strongly bias our view of community structure (48). The advent of molecular tools in microbial ecology, e.g., the analysis of 16S rRNA sequences in natural samples, has made it possible to circumvent this limitation. Identification of the dominant populations *in situ* leads to a better understanding of how microbial communities are structured. However, molecular studies suffer from the drawback that physiology can only rarely be inferred from the 16S rRNA sequence data alone. Thus, cultivation and molecular methods may complement each other (19, 30, 39, 43). Nonetheless, the application of molecular tools for studying the microbial community structure at submarine hydrothermal vents to date has mainly focused on an assessment of the bacterial diversity and on an inventory of taxa that are present, without making concomitant measurements of the geochemical parameters (19, 27, 28, 30, 34).

We have chosen a submarine shallow hydrothermal vent in the Aegean Sea near the island Milos (Greece) to investigate the relation between changes in physicochemical parameters and bacterial population distributions. By using denaturing gradient gel electrophoresis (DGGE) of PCR-amplified 16S rRNA gene fragments, we examined the spatial heterogeneity of bacterial populations on a vertical as well as horizontal scale along a transect from the center of the vent to

the surrounding area. DGGE is a powerful tool to discern changes in microbial community structure in a variety of habitats (for a review see ref. 32). The DGGE analysis of the dominant phylotypes occurring at the vent system was accompanied by investigations of the vertical and horizontal distribution of specific physiological groups of bacteria, i.e., autotrophic sulfur-oxidizing bacteria (SOB), sulfate-reducing bacteria (SRB) and dissimilatory iron-reducing bacteria (DIRB), by using the most-probable-number (MPN) approach. The transect was concurrently physicochemically characterized by performing high resolution *in situ* profiling of temperature, pH, redox-potential, and O_2-concentrations.

MATERIAL AND METHODS

Study Site. The study site was a solitary gaseous hydrothermal vent located 8 m deep in Palaeochori Bay (24°31.220' E; 36°40.391' N), a sandy bay in the south-eastern part of the island Milos in the Aegean Sea (Greece). The mean composition of the discharged gases from different seeps was 80.5 % CO_2, 1.2 %, H_2S, 0.8 % CH_4 and 0.4 % H_2 (11). The reduced hydrothermal fluid has an elevated salinity of up to 58 ‰ compared to 39 ‰ of the ambient seawater (44). Macrofauna which are dependent on endosymbiotic bacteria are absent at the Milos vents (10, 16, 44).

Sampling. Sampling was carried out along a transect from the center of the almost circular vent to the surrounding area. At a specific area around the center of the vent site, a conspicuous white precipitate formed on the sediment surface, which increased in thickness under calm weather conditions (see Fig. 1 and 2). Sediment cores (polycarbonate tubes with an inner diameter of 37 mm) were taken by SCUBA divers at 10 cm, 123 cm, 165 cm and 235 cm distance from the vent center in June 1996 and at 30 cm, 117 cm and 200 cm distance from the vent center in September 1996. Physicochemical measurements were carried out along the same transect.

Slicing of sediment cores. Sediment cores were immediately subsampled by extruding the sediment from the polycarbonate tubes and slicing each core. In June 1996, the first 30 mm of every sediment core were subsampled at 10-mm intervals. In September 1996, the layers from 0 to 5 mm, 8 to 13 mm and 16 to 26 mm were subsampled. At both sampling times, the upper sample of each core consisted of water from just above the sediment surface which was sampled with a sterile syringe. In case of the cores from 123 cm distance (June) and 117 cm distance (September) the upper sample consisted of the white precipitate, which had a thickness of 2-5 mm. In September a second core was taken at 117 cm from the center of the vent 1 week later, and no precipitate was present.

Physicochemical measurements. Vertical profiles of pH, temperature, redox-potential and O_2-concentration were determined *in situ* along a transect radiating out from the

center of the vent site. The sensors were attached to a micro manipulator mounted on a tripod. The instruments were self-contained and held in watertight housings. The signals were transferred to and stored by a 12 bit data logger. The sensors for temperature and pH were combined and thus both parameters were measured at the same location and the pH measurements were temperature compensated. Vertical profiles of redox potential were measured with a platinum electrode. The dissolved-oxygen was measured by using Clark-type microelectrodes with a built-in reference and a guard cathode (35). Measurements were done at horizontal distances of 20 - 30 cm along the transect up to a distance of 3.5 m from the center and in vertical increments of 1 cm for pH, temperature, and redox-potential and 250 μm for dissolved oxygen.

Total cell counts. A sediment sample (1 cm^3) was fixed by the addition of 9 ml borate buffered formaldehyde (4% [wt/vol]) in 3.5 % (wt/vol) NaCl. Water samples were fixed by the addition of borate buffered formaldehyde (4% [wt/vol] final concentration). The borate buffered formaldehyde was prepared by adding 4 % (wt/vol) borax (Sigma, St. Louis, Mo.) to 37 % (wt/vol) formaldehyde. After 16h, the solution was filtered through Nuclepore polycarbonate filters (pore size, 0.2 μm; Costar, Cambridge, Mass.) to get a particle-free solution. Samples were stored at 4°C in the dark. For further processing samples were put on ice and sonicated (three times for 15 s at 30-s intervals) with a microtip (Sonopuls HD 200; Bandelin, Berlin, Germany), to dislodge the cells from the sediment particles. After allowing the particles to settle for 30 s, the supernatant was collected. The remaining sediment was washed 8 times with 5 ml of a filter-sterilized 3.5 % (wt/vol) NaCl-solution. The supernatants were combined, the sample was diluted, and a minimum of 2 ml was filtered through black Nuclepore polycarbonate filters (pore size, 0.2 μm). Two filters per sample were stained with acridine-orange (final concentration, 0.01 % [wt/vol]) and mounted on glass slides with low-fluorescence immersion oil (type A; Cargille, Cedar Grove, N.J.), and cells were counted with a Axiolab epifluorescence microscope (Zeiss, Oberkochen, Germany) at a magnification of 1000x. In addition to fixed samples, we examined fresh material by phase contrast with a Zeiss Standard20 microscope, to get a general impression of the appearance of the material.

MPN counts. The MPN technique was used to estimate the abundance of sulfate-reducing bacteria (SRB) and dissimilatory iron-reducing bacteria (DIRB) in June and of sulfur-oxidizing bacteria (SOB) in September. Subsamples were serially diluted (1:10) with artificial seawater medium without substrate. Between every dilution step, samples were vigorously shaken on a vortex-mixer to disaggregate cell clumps and to dislodge cells from sediment particles. Sonication was not used in this case, since we believedthat it would have adverse effects on the viability of the cells. From each dilution, three replicate tubes containing growth medium were inoculated and incubated at their approximate *in situ* temperature. For anaerobic bacteria, the first dilution step was carried out in a glove box flushed with N_2. All further

dilution steps and the inoculation were done by transferring an aliquot of fluid with syringes from one anaerobic tube to the next by injection through butyl rubber stoppers. The tubes of the anaerobic MPN series were filled with medium under anaerobic conditions (a mixture of 90 % N_2 and 10 % CO_2 in the headspace) and closed with butyl rubber stoppers (50). The numbers of cultivable bacteria were determined as described previously (3).

(i) MPN counts of SRB. Artificial seawater medium defined for SRB (51), contained 10 mM acetate as carbon source and non-chelated trace element mixture number 1. In marine sediments acetate-oxidizing SRB dominate (see, e.g., reference 24), and hence acetate is the main electron donor for sulfate reduction (see, e.g., reference 38). The presence of SRB in the MPN tubes was determined by a semiquantitative detection of sulfide (8) and microscopically verified.

(ii) MPN counts of DIRB. Artificial, sulfate-free seawater (51) was supplemented with washed (three times with distilled water) and autoclaved maghemite (γ-Fe_2O_3, 40 mM) with a surface area of 130 m^2 g^{-1} (Bayer, Krefeld, Germany) as electron donor, acetate (5 mM) as electron acceptor, and trace element solution with EDTA (51). In marine sediments maghemite yields cell numbers of DIRB comparable to those obtained with ferrihydrite (12). Tubes were counted positive if the color changed from red (maghemite) to black (magnetite). Positive tubes of DIRB were also checked microscopically.

MPN counts of SOB. Mineral medium with 20 mM thiosulfate (added by sterile filtration to the autoclaved medium) as sole electron donor was used. The composition of the mineral medium (in grams per liter) was: NaCl, 29; $(NH_4)_2SO_4$, 1; $MgSO_4$ x 7 H_2O, 1.5; $CaCl_2$ x 2 H_2O, 0.42; K_2HPO_4, 0.5; KCl, 0.7; vitamin B_{12}, 0.05; and trace element solution with EDTA, 1 ml $liter^{-1}$ (51). Bromthymolblue was added as pH indicator at a concentration of 4 mg $liter^{-1}$. K_2HPO_4 was autoclaved separately and added to the medium after autoclaving. To test for the presence of SOB able to grow under anaerobic conditions, MPN series were set up with the same medium as for aerobic SOB, but supplemented with 10 mM nitrate as electron acceptor and buffered with 15 mM $NaHCO_3$. This MPN series was performed under strict anaerobic conditions. The MPN series for SOB (aerobically and anaerobically) for the second core (117 cm from the vent center) was performed using the same medium as described above, but the salinity was increased to 55 ‰ by adding NaCl. This salinity was similar to that of the outflowing brine (44). In all cases, growth was determined by pH changes upon acid formation due to the oxidation of thiosulfate and microscopically verified. The MPN cultures were incubated in the dark to avoid growth of phototrophic organisms.

DNA extraction. Subsamples from sliced sediment cores were immediately deep-frozen in liquid N_2. For long-term storage, the samples were kept at -80°C. DNA was extracted using the method of Zhou et al. (54). We modified the method by using 2 g of sample and including five cycles of thawing at 30°C and freezing in liquid nitrogen before starting the

extraction. The lysis efficiency was monitored by epifluorescence microscopy after staining subsamples after the extraction step with acridine orange. No intact cells could be observed. The extracted DNA was stored at -20°C until further analysis.

PCR. PCR amplifications were performed by a touchdown PCR with the bacterial primer pair GM5F-GC clamp and 907R (30). The annealing temperature was lowered from 65°C to 55°C in 20 cycles, and after the final annealing temperature of 55°C was reached, 16 more cycles were performed. In all cases, bovine serum albumine (Sigma) was added to the PCR solution (final concentration, 3 mg ml^{-1}) to prevent inhibition of enzymatic amplification by humic substances. Amplification products were analyzed by electrophoresis in 2 % (wt/vol) SeaKem LE agarose (FMC Bioproducts, Rockland, Maine) gels stained with ethidium bromide (0.5 μg ml^{-1}) before being subjected to further analysis.

DGGE. DGGE analysis of PCR amplified 16S rRNA gene fragments was performed as described by Muyzer et al. (31) using the D-Gene™ system (Bio-Rad, Hercules, Calif.). In this analysis, 1 mm thick, 6 % (wt/vol) polyacrylamide gels with a 20-70% denaturant gradient were run for 20 h in 1 x TAE buffer (40 mM Tris, 20 mM acetic acid, 1 mM EDTA, [pH 8.3]) at a constant voltage of 100 V. After electrophoresis, the gels were stained with ethidium bromide (0.5 μg ml^{-1}) and photographed on a UV transillumination table (302 nm) with a Polaroid camera. The photograph of the gel was scanned with Fotolook version 2.05 (Agfa) software and edited with Photoshop 4.0 (Adobe) software. A linear regression analysis between the numbers of DGGE bands in each lane and various physicochemical parameters was performed with the statistical StatView version 4.02 (Abacus) software.

RESULTS

Physicochemical characterization of the vent site. The solitary vent site at a water depth of 8 m had a characteristic concentric zonation of colored surface deposits surrounding the gas outlet (Fig. 1 and 2). The sediment at the center was covered with a bright yellow sulfur deposit. This was surrounded by a zone covered with white flocculent material on top of black sediment. This zone was approximately 110 cm wide and extended to a distance of 150 cm from the vent center. The white zone was followed by a ring of gray sediment (ca. 30 cm wide) and a brown or green outer zone (ca. 40 cm wide). The transitions between the different zones were very distinct. In situ measurements of pH and temperature demonstrated that the shallow vent site constitutes an extreme environment (Fig. 2). At about 300 cm from the vent center, there was a distinct increase in temperature with depth. This gradient became more pronounced within the gray zone, with an increase of 10°C between the sediment surface and 5 cm sediment depth. The sediment temperature reached a maximum of 103°C at a depth of 10 cm

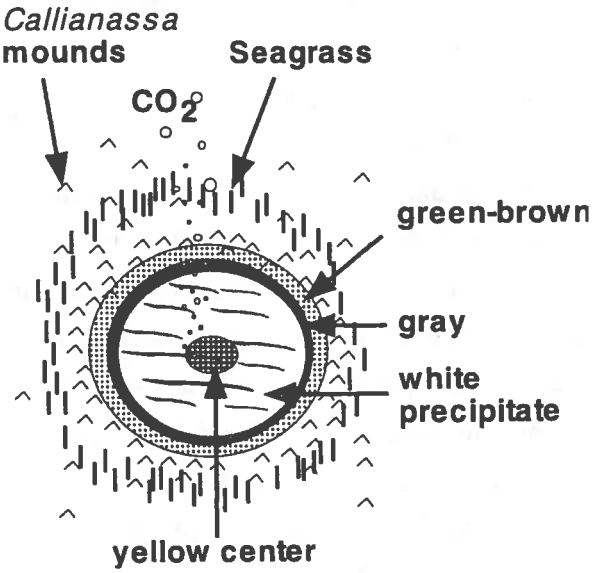

Figure 1. Schematic diagram of the solitary hydrothermal vent illustrating the zonation pattern observable on the sediment surface. See text for further explanations.

at the vent center (data not shown). At this location the temperature increased by 50°C between the sediment surface and 5 cm sediment depth (Fig. 2). The pH values decreased horizontally towards the center, but in contrast to temperature there was no change in pH with increasing sediment depth (Fig. 2). At a distance of 200 cm pH values decreased below 6. The redox potential decreased from positive values to low negative values at the same location at 200 cm from the center (data not shown). Sediments outside the venting region showed dissolved oxygen profiles with the characteristic shape of diffusive transport from the overlying water to the sediment (data not shown). The penetration depth of 3 mm was typical of coastal sediments (35, 55). In the zone between 200 cm and 230 cm from the center oxygen penetrated 2-3 times deeper into the sediment, indicating that transport processes other than diffusion are responsible for the high concentrations (data not shown). The outflow of gas and hot fluid probably induces a convective flow of pore water entrainment to the sediment surface and into the water column. This upward transport of reduced solutes is compensated by an inflow of oxic overlying water within a distinct zone. Oxygen was not detected towards the vent center or beneath the white flocculent material in the reduced black sediment (data not shown).

Total cell counts. Total cell numbers varied between 9.5 x 10^5 cells ml^{-1} (in overlying water at 10 cm from the vent center) and 5.5 x 10^8 cells g (wet weight) of $sediment^{-1}$ (uppermost sediment layer at 235 cm from the vent center) in June (Fig. 3A) and between 3.2 x 10^5 cells ml^{-1} (overlying water at 30 cm distance) and 6.4 x 10^8 cells g^{-1} (uppermost sediment layer at 200 cm from the vent center) in September (Fig. 3B). The highest numbers were observed at both sampling times in the upper sediment layers, and numbers decreased with increasing sediment depth. On a horizontal scale, two regions could be separated. The highest numbers were found at 200 cm and 235 cm distance from the vent center in a transition zone between obviously hydrotherm-

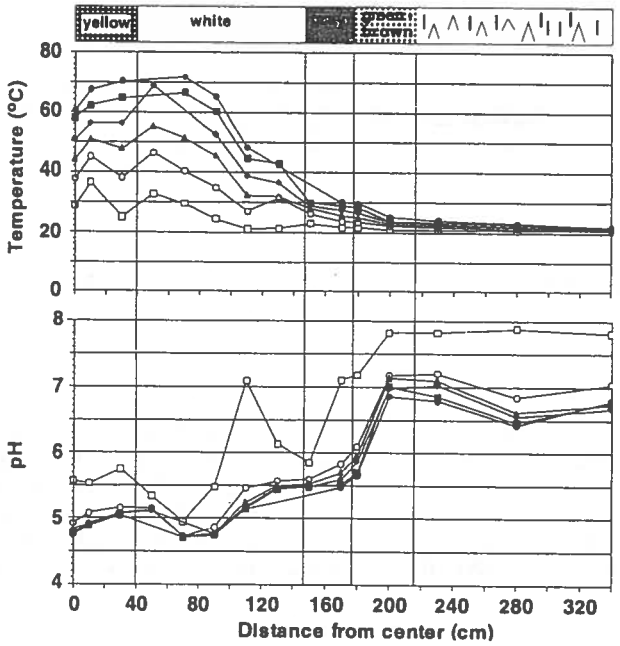

Figure 2. Vertical profiles of temperature and pH along a transect from the surrounding area towards the vent center for June 1996. The profiles from September 1996 were nearly identical (sediment depth is indicated by the following symbols: □ 0 cm; ○ 1 cm; Δ 2 cm; ◆ 3 cm; ■ 4 cm; ● 5 cm).

ally influenced sediments (higher temperature, low pH, and redox-potential) and ambient sediment conditions (mesophilic temperature, neutral pH, and positive redox-potential).

In the region which was more significantly affected by the hydrothermal fluid, i.e., between the center and 200 cm, numbers were significantly lower at a given depth compared to the outer region. Differences among the inner three cores, or between the outer two cores, were not apparent, however. In the water just above the sediment surface the situation was quite different. The highest numbers were found in the white precipitate at a distance of 123 cm in June and at 117 cm in September (Fig. 3A and B). When the precipitate was absent no enrichment of bacteria relative to the ambient seawater was observed (117 cm II; Fig. 3B). Microscopic observations of unfixed, fresh material revealed that the white precipitate had afilamentous structure. The filaments had a diameter of 0.5 to 2 μm and were not stainable with the nuclear stain acridine orange. A highly motile, vibroid prokaryote was a dominant morphotype in the white precipitate. Gliding diatoms were also entangled in the fluffy material.

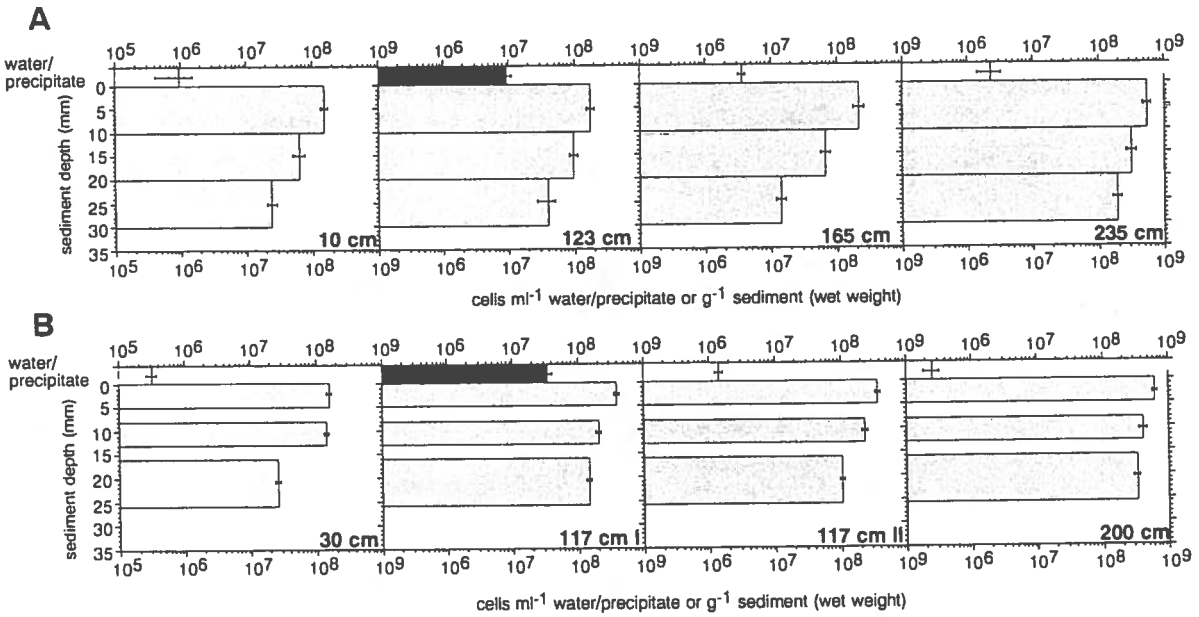

Figure 3. Total cell counts at specified locations along a transect from the vent center towards the surrounding area for June 1996 (A) and September 1996 (B). The error bars indicate the 95% confidence interval. The cores at 117 cm distance from September 1996 were obtained 1 week apart. During the first sampling (117 cm I), the white precipitate on the sediment surface was present, whereas during the second sampling (117 cm II), it was absent. The open bars indicate samples from the water above the sediment surface, the medium-shaded bars indicate sediment samples, and the dark-shaded bars indicate samples of the white precipitate.

MPN counts of SRB. The numbers of acetate oxidizing SRB varied between 8 and 1.43 x 10^5 cells g (wet weight) of sediment^{-1} (Fig. 4), with the highest numbers at 235 cm distance from the center (Fig. 4D). In general, numbers declined from the top sediment layer to the deeper layers. SRB were also detected in the oxygenated water above the sediment surface at all distances, as well as in oxidized sediment layers at 235 cm distance (Fig. 4D; 0-10 mm). At a maximum SRB comprised 0.026 % of the total counts (Fig. 4).

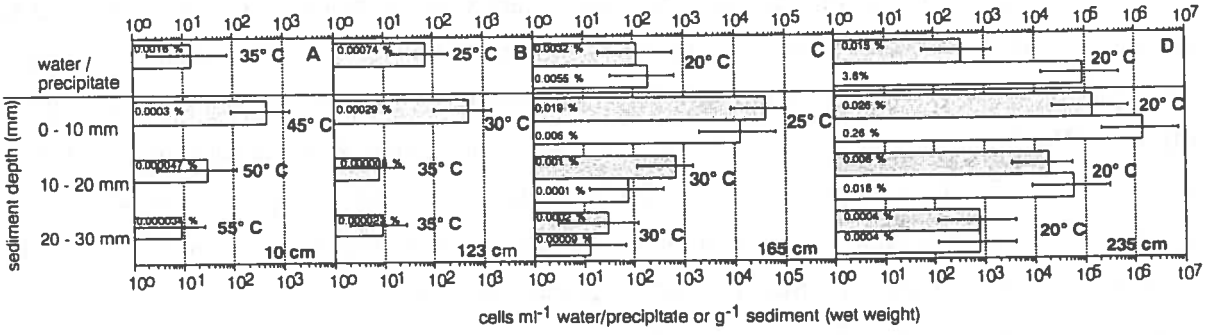

Figure 4. MPN counts of SRB (shaded bars) and DIRB (open bars) at 10 cm (A), 123 cm (B), 165 cm (C) and 235 cm (D) from the vent center in June 1996. The error bars denote the 95% confidence interval. The incubation temperature and the percentage of the total counts accounted for by the MPN counts are shown outside and inside the bars, respectively.

MPN counts of DIRB. The numbers of DIRB capable of growing on acetate varied between undetectable and 1.4 x 10^6 cells g (wet weight) of sediment^{-1} (Fig. 4). As with the SRB, highest numbers were found at 235 cm from the vent center (Fig. 4D) and they generally decreased from the surface to deeper layers. At 30 cm and 123 cm, numbers of DIRB were below the detection limit (Fig. 4A and B). At a distance of 235 cm, DIRB were present in numbers equal to or greater than SRB (Fig. 4D). DIRB constituted up to 3.8 % of the total cell numbers (Fig. 4).

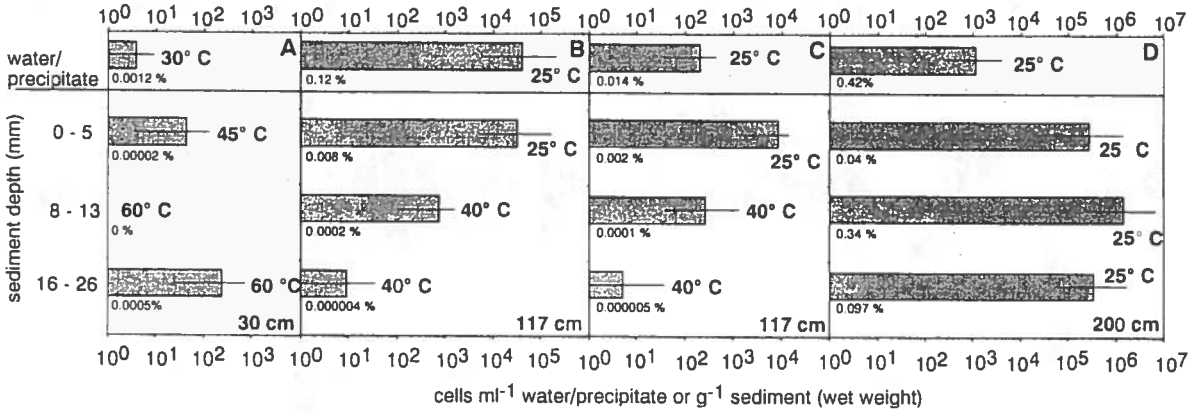

Figure 5. MPN counts of SOB at 30 cm (A), 117 cm (B and C), and 200 cm (D) from the vent center in September 1996. The bars indicate the 95% confidence interval. The incubation temperature and the percentage of the total cells accounted for by the MPN counts are given beside and below the bars, respectively. The two cores at 117 cm distance were obtained 1 week apart. During the first sampling, a white precipitate was present on the sediment surface (B), whereas during the second sampling (C), it was absent. The MPN series for the second core (C) was performed with medium with an increased salinity of 55 ‰.

MPN counts of sulfur-oxidizing bacteria (SOB). The numbers of aerobic SOB in September varied between undetectable and 1.42 x 10^6 cells g (wet weight) of sediment^{-1} and accounted at most for 0.41 % of the total cell numbers (Fig. 5). The highest numbers were recorded at 200 cm from the vent center, which corresponds to the zone with the brownish to greenish surface (Fig. 5D). In the zone with the white precipitate on the surface, the highest numbers of SOB were found in the white precipitate and not in the sediment (Fig. 5B). The MPN counts with the higher salinity medium showed numbers comparable to the MPN counts with the standard salt concentration. The single exception was for the samples collected from the water overlying the sediments (Fig. 5B and C). MPN counts of anaerobic SOB showed growth only in the first two dilutions of the samples collected 200 cm from the vent center (data not shown).

DGGE analysis. PCR-amplified 16S rRNA fragments obtained from environmental DNA were separated by DGGE to profile the bacterial communities at different locations along the transect in June and September (Fig. 6). For June, there was a trend of decreasing numbers of bands when approaching the vent center (Fig. 6A). In addition, there was a vertical decrease,

which was most obvious at 10 cm and 123 cm (Fig. 6A, lanes 2-5 and 6-9). The only physicochemical parameter that showed a significant correlation ($P < 0.05$) to the numbers of DGGE bands was temperature (Fig. 7A; $r = 0.73$; $P = 0.002$). The significance of this negative correlation increased when only sediment samples were included (Fig. 7B; $r = 0.82$; $P = 0.001$).

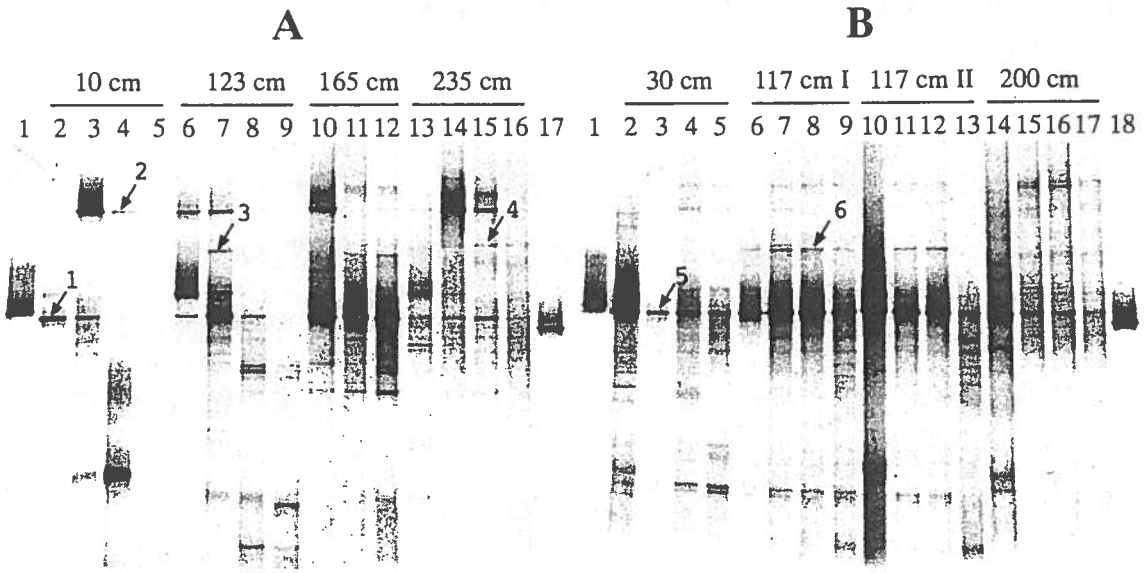

Figure 6. DGGE analysis of 16S rDNA fragments obtained after PCR amplification with the bacterial primer pair GM5F-GC-clamp and 907R of genomic DNA from environmental samples and standards with a known melting behavior for June 1996 (A) and September 1996 (B). The environmental samples were taken at specific locations along a transect from the vent center towards the surrounding area. The two cores at 117 cm distance in September were taken 1 week apart. During the first sampling (117 cm I) the white precipitate on the sediment surface was present, whereas during the second sampling (117 cm II), it was absent. No PCR-product could be obtained from the overlying water at 165 cm. The numbered bands are described in the text. (A) DGGE pattern of the samples taken in June 1996. Lanes: 1 and 17, standards, 2 to 5, samples taken at 10 cm from the vent center (lane 2, surface; lane 3, 0-10 mm; lane 4, 10-20 mm; lane 5, 20-30 mm); 6 to 9, amples taken at 123 cm (lane 6, surface; lane 7, 0-10 mm; lane 8, 10-20 mm; lane 9, 20-30 mm); 10 to 12, samples taken at 165 cm (lane 10, 0-10 mm; lane 11, 10-20 mm; lane 12, 20-30 mm); 13 to 16, samples taken at 235 cm(lane 13, surface; lane 14, 0-10 mm; lane 15, 10-20 mm; lane 16, 20-30 mm). (B) DGGE pattern of the samples taken in September 1996. Lanes: 1 and 18, standards, 2 to 5, samples taken at 30 cm from the vent center (lane 2, surface; lane 3, 0-5 mm; lane 4, 8-13 mm; lane 5, 16-26 mm); 6 to 9, samples taken at 117 cm (lane 6, surface; lane 7, 0-5 mm; lane 8, 8-13 mm; lane 9, 16-26 mm); 10 to 13, samples taken at 117 cm (lane 10, surface; lane 11, 0-5 mm; lane 12, 8-13 mm; lane 13, 16-26 mm); 14 to 17, samples taken at 200 cm (lane 14, surface; lane 15, 0-5 mm; lane 16, 8-13 mm; lane 17, 16-26 mm).

The pH did not correlate significantly with the numbers of DGGE bands ($r = 0.28$; $P = 0.079$ [data not shown]). In September, a trend of decreasing numbers of bands when approaching the vent center was not discernible and the DGGE banding pattern appeared to be more homogeneous. In addition, there was no correlation between temperature and numbers of DGGE bands ($r = 0.2$; $P = 0.45$; data not shown).

One dominant band was present in every region and almost every depth at both sampling times (Fig. 6 A and B; band number 1 and number 5). However, its dominance was less pronounced at 200 cm and 235 cm distance. The position of the band relative to the two

standards was the same at both sampling times. Other bands seemed to be restricted to certain depths or zones, such as band number 2, number 3 and number 4 in June (Fig. 6A), and number 6 in September (Fig. 6B).

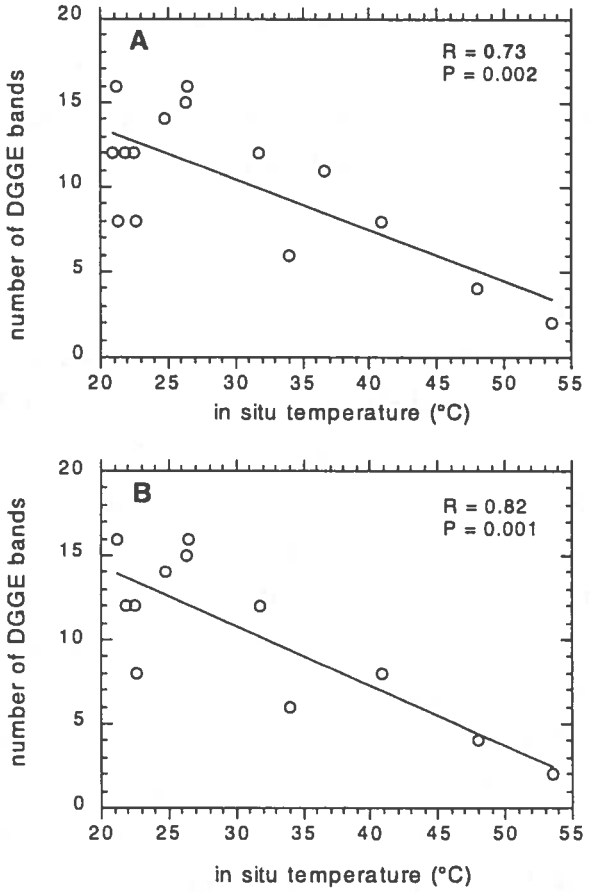

Figure 7. Correlation of the *in situ* temperature with the number of DGGE bands in each lane for June 1996, including water samples (A) and excluding water samples (B). The Pearson correlation coefficient, r, and its significance, P, are shown.

DISCUSSION

Total cell counts and MPN counts. There are only a few studies where the fraction of culturable sub-populations of natural communities growing autotrophically or with single carbon sources was related to the total cell counts (e.g., 39, 43, 53). The low numbers found in the present study fall within the range of values reported previously, although the fraction might be increased when complex media are used (19, 22). However, since typically less than 1% of the total prokaryotic populations in natural habitats may generally be cultivable (2), the present numbers might represent a substantial fraction of the culturable organisms in this habitat. In addition, since total cell counts do not differentiate between active and inactive cells, a

substantial fraction of the total cell counts might consist of dead cells (57). Although we tried to maximize the viable cell counts by incubating at the *in situ* temperature (48), we certainlyunderestimated the absolute numbers of the respective physiological groups by not providing the appropriate growth media. In addition, the association of cells in clumps or with particles can not be ruled out, although we vigorously vortexed the samples to disaggregate and dislodge the cells. Clumping would also lead to an underestimation, since the MPN evaluation assumes that only one cell initiated the growth in the highest dilution (3). Cells may also have been present in higher dilutions, but were not detected due to the insensitivity of the detection methods used (43, 45, 53).

The high total cell numbers at 200 cm and 235 cm as well as the relatively high abundance of DIRB and SRB at 235 cm distance were probably related to the moderate environmental conditions, e.g., neutral pH and temperatures of about 20° C, in these sediments and to the presence of diatoms and other primary producers such as SOB (see below). The organic matter produced by autotrophic organisms could be used by the anaerobic heterotrophs, a possibility supported by their confinement to the upper sediment layers. Similar observations have been made in a marine microbial mat (46) and in other marine sediments (24). In addition, organic matter (such as seagrass fragments) transported by bottom currents from the surrounding area to the vent site could form the basis of a phytodetrital food chain (44). The high numbers of SRB at 235 cm distance correlated well with sulfate reduction rates, which were also highest in this zone and peaked in the upper 2 cm at the studied vent (56) and at similar vents in Palaeochori Bay (10). The low numbers of SRB in the cores from 30 cm and 123 cm were probably related to the low pH in the sediments, which is known to be inhibitory to sulfate reducers (50). The presence of thermophilic SRB in this zone (36) could be related to the presence of microniches (17, 50).

The relatively high numbers of DIRB in the brownish zone at 235 cm distance compared to SRB indicate that organic matter degradation by dissimilatory iron reduction is likely to be an important process in the hydrothermally influenced sediments of Palaeochori Bay. This hypothesis is supported by the co-occurence of the highest numbers of DIRB and high concentrations of Fe(III) (56). The absence of DIRB closer to the center of the vent could be explained by low concentrations of Fe(III) in these zones due to the reduced conditions at the sediment surface. Temperature was not likely to be an important parameter in restricting the occurrence of DIRB in these zones since dissimilatory Fe(III)-reducing bacteria which are able to grow at temperatures up to 74°C were isolated (37).

In addition to phototrophic organisms, i.e., diatoms and cyanobacteria, the green-brownish transition zone also contained a high numbers of aerobic SOB (Fig. 5B). These organisms are capable of autotrophic growth and therefore most likely contributed to primary production. The slight increase in temperature indicates that this zone was affected by the

hydrothermal processes. The reduced sulfur compounds contained in the hydrothermal fluid, mainly H_2S, would provide the necessary electron donor for the SOB. However, SOB could also use sulfide produced by SRB. The observation that numbers of SOB at 200 cm from the center were also high in deeper sediment layers could be related to the increased penetration depth of O_2 at this site. At 200 cm distance from the vent center, O_2 penetration reached up to 9 mm sediment depth, whereas O_2 did not penetrate into the sediment at 30 cm and 117 cm distance from the vent center. The nature of the electron acceptor for SOB below the oxygen penetration depth remains unclear, since anaerobic SOB using nitrate as electron acceptor were present in much lower numbers. One explanation might be that the oxygen penetration depth is temporally variable, e.g., influenced by tidal effects on hydrothermal fluid discharge (1, 16). In the German Wadden Sea (North Sea), although aerobic *Thiomicrospira* populations were homogeneously distributed in the upper cm of the sediment, only the populations which were in the O_2-penetrating zone seemed to be metabolically active (6). The lower abundance of SOB at 30 cm and 117 cm distance from the vent center is probably related to the elevated temperatures and the lack of O_2 as electron acceptor in the sediments.

Since the outflowing hydrothermal fluid has an elevated salinity relative to ambient seawater (44), we were concerned whether we would miss additional cultivable SOB using the standard medium. However, with the exception of the water above the sediment surface, both high and normal salinity media showed comparable numbers, although there may have been differences among the types of SOB present in the dilutions (7). The significantly higher numbers of SOB in the water above the sediment surface in the first core at 117 cm distance from the vent center compared to the second core at 117 cm are probably not related to the difference in salinity, but to absence of the white precipitate in the second core, since the same trend was found for the total cell numbers. This result indicates that the precipitate was a suitable habitat for certain bacteria, such as SOB and a vibrio, which was further identified to be an *Arcobacter* species by using an oligonucleotide probe specific for this genus (18).

Recently, Taylor and Wirsen (42) described the formation of filamentous sulfur by a highly motile vibroid chemoautotrophic strain of H_2S-oxidizing bacterium in a continuos flow, H_2S-enriched seawater reactor. The filaments resembled the precipitate at deep-sea hydrothermal vents (42) and at the vent site investigated in the present study. Since this vibrio belongs also to the genus *Arcobacter* within the ε-Proteobacteria (52), it is possible that the vibroid *Arcobacter* species detected in the present study is involved in the formation of the white precipitate. As argued by Taylor and Wirsen (42), precipitate formation could be a strategy for retainment in environments characterized by active fluid motion. The precipitate would also favor the growth of other SOB, due to the fact that it stabilized the gradient between the sulfidic brine and the oxygenated seawater. In addition, the H_2S is rapidly transformed into the more stable form of sulfur, which could still be used as an electron donor (42).

DGGE analysis. DGGE allowed a general assessment of the spatial distribution of bacterial populations and the bacterial community structure at this particular submarine hydrothermal vent. The bacterial populations were neither vertically nor horizontally homogeneously distributed, as has been described for, e.g., soil bacterial communities (13). Inhomogenous distribution is most likely due to changes in the physicochemical parameters that occurred on a relatively small scale. For June, a change in the bacterial community structure, i.e., in the number of populations and their relative abundance, was observed along the transect from the center of the vent to the surrounding area. This trend was not apparent in September, however (see below). If it is assumed that the intensity of the PCR product is proportional to the abundance of the template (see e.g., references 29, 33), the changes observed in June support the hypothesis that extreme environmental conditions will lead to a community structure with a limited number of dominant populations, since fewer, but stronger bands were found at the locations closer to the vent center. This finding is in accordance with results from other studies at different deep-sea hydrothermal vents in which molecular methods were used (27, 30, 34). An acidothermal soil from a thermal area in New Zealand also contained a limited microbial diversity compared to 'normal' soil (49). However, previous studies have not investigated how the community structure might change with a decreasing influence of the hydrothermal fluid.

In June, we found the highest diversity of bacterial populations as indicated by DGGE bands at a distance of 165 cm and 235 cm. However, the community structure at the two distances differed. At 165 cm, one population seemed to dominate as indicated by the strong band, whereas at 235 cm, no such dominant population was found(Fig. 6A). This result could be explained by the more moderate environmental conditions at 235 cm, which probably allowed more populations to coexist rather than favoring a few populations. Interestingly, Thiermann and coworkers observed a general decrease in faunal diversity towards the hydrothermally active area at a similar vent system in Palaeochori Bay (44). In addition, the faunal community in the strongly hydrothermally influenced sediments was found to be uneven and dominated by tolerant, opportunistic species (44).

The significant correlation between temperature and the numbers of DGGE bands found in the June samples suggests that temperature was an important environmental parameter affecting the bacterial community structure at the vent site. Temperature has also been found to be a key factor in determining the distribution of bacterial populations in the outflow of a hot spring (14) and it has been demonstrated that the microbial diversity of hot spring mats decreased as the temperature of the environment increased (21). However, total cell numbers and numbers of culturable hyperthermophiles increased in deeper layers (≥ 10 cm) in the hot sediments of a shallow submarine vent (22). Thus, the trend described above might change when the deeper, hyperthermophilic microbial community is analyzed. These populations are obviously present in Palaeochori Bay, albeit in unknown numbers (9).

Although PCR-DGGE has the advantage that it circumvents selective and potential ineffective cultivation, it also has limitations. First, only numerically dominant populations will be detected by DGGE (29, 39). This limitation precludes determination of changes in species richness at this vent site, since numerically smaller populations, which would contribute to species richness, might have been present, but were not detected. In addition, bacteria specific to this habitat may not contain the signature sites necessary for efficient amplification with the bacterial primers used and thus would have been not included in the analysis. An insufficient or preferential disruption of cells would also distort the view of the community composition (47). Although our microscopic observations indicated that complete lysis was achieved, the recovery might still have been reduced by degradation or adsorption of nucleic acids upon their release from cells to matrix material contained in the sediment. Such a process might have influenced the trend, since the sedimentary environment changed concurrently. Furthermore, PCR biases, e.g., a selective amplification of certain DNA fragments or differential amplification efficiencies, can not be excluded (15, 40, 47). As we did not know the amount of DNA attributable to bacteria and whether this proportion would change along the transect, we were concerned that the amount of template DNA would have an effect on the observed changes in the banding pattern. However, a PCR bias related to the amount of template DNA can be excluded, since the banding pattern of the DGGE and the relative intensities of the bands did not change when we used serial dilutions (up to 1,000 fold) of the template DNA (data not shown). An increased proportion of Archaea towards the center of the vent might actually be responsible for the lack of variation in total cell counts along the transect. However, it is also important to note that two communities could contain the same quantity of cells, but be structured differently.

The trend of a more uneven community structure with increasingly extreme environmental conditions was not discernible in the DGGE banding pattern from September, however. One reason might be the sampling interval, since the cores were sliced at a finer resolution in September than in June. This would suggest that the community changes are less obvious on a scale less then 1 cm. However, a storm occurred a few days prior to the sampling. Thus, another explanation could be the disturbance of the microbial community caused by resuspension of the upper sediment layers due to strong bottom currents caused by the wave action. Such a disturbance would also account for the more homogenous DGGE banding pattern in September and for the lack of a negative correlation between temperature and numbers of DGGE bands. In addition, it would explain the presence of *Thiomicrospira* spp. in deeper sediment layers with unfavorable growth conditions close to the center of the vent as found by DGGE analysis (7). Thus, it could be speculated that longer periods of calm conditions are necessary for the establishment of distinct bacterial populations along the natural occurring physicochemical gradients at this vent site.

The presence of the bands number 1 and number 5 at the two sampling dates might indicate a certain degree of stability of the bacterial community over time, despite the highly dynamic changes that occur in this environment. That these bands were present in almost every zone and depth irrespective of the different physicochemical conditions, further suggests that these bands belong to a population with a broad tolerance to environmental conditions. Judged from the intensity of the bands, it could be inferred that the preferred habitat for this population was between the center of the vent and 165 cm and therefore in the more strongly hydrothermal influenced sediments. However, as it has been demonstrated that different sequences can have the same melting properties in DGGE (14, 29), this band does not a priori belong to the same population in every case. Other bands, e.g., bands number 2, 3, 4, and 6, appeared to be more confined to certain depth and zones, suggesting that they belong to populations that are more specialized.

Conclusions. The data indicate that the changing physicochemical conditions at the vent site affected bacterial distribution and community structure. The highest cell numbers, the greatest diversity of dominant populations, and an even community structure, were found in a transition zone from the strongly hydrothermally-influenced sediments to normal sedimentary conditions. Closer to the vent center cell numbers were significantly lower and the community structure was dominated by few populations, probably due to the harsh environmental conditions in these regions. This trend is likely to apply also to deep-sea hydrothermal vents, where sharp physicochemical gradients are known to occur (4, 5, 26). DGGE seems to be an appropriate tool to address this question in future studies. The trend of a more uneven community structure with increasingly extreme environmental conditions, however, was not observed at the second sampling time. Resuspension of the upper sediment layers due a storm prior to the sampling most likely was responsible for this observation. In future studies, the influence of such disturbances on the dynamics of the microbial community should be studied in greater detail. The relatively high numbers of DIRB in the mesophilic zone indicated that in addition to sulfate reduction, ferric iron reduction was an important pathway for the anaerobic degradation of organic matter at the vent site. The results of the MPN series for SOB indicate that chemoautotrophy based on reduced sulfur compounds adds to primary production at the vent site, most notably in the transition zone and possibly in the white precipitate. The detection of sulfur-oxidizing *Thiomicrospira* spp. among the predominant populations as identified by DGGE (7) and the indication that the vibroid *Arcobacter* sp. is a sulfur-oxidizing bacterium, further supports this assumption.

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2

Identification of 16S rDNA-Defined Bacterial Populations at a Shallow Submarine Hydrothermal Vent near Milos Island (Greece)

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ABSTRACT

In a recent publication (S. M. Sievert, T. Brinkhoff, G. Muyzer, W. Ziebis, and J. Kuever, Appl. Environ. Microbiol. 65:3834-3842) we described the spatio-temporal changes in the bacterial community structure of a shallow-water hydrothermal vent in the Aegean Sea near the isle of Milos (Greece). Here we report on the identification and phylogenetic analysis of the predominant bacterial populations and their distribution at the vent site by sequencing DNA molecules ("bands") excised from denaturing gradient gels. In total, 91 unique bands, most likely corresponding to unique populations, could be identified. From these 22 yielded unambigous sequences. The data indicate the presence of a wide spectrum of bacterial lineages at this vent site, but only a low percentage of sequences matched closely with cultivated organisms. The sequences of two bands were nearly identical (>98% similarity) to *Caldicellulosiruptor lactoaceticus*, an extremely thermophilic cellulolytic bacterium isolated from a hot spring in Iceland. Many sequences, e.g., the sequence of the most prominent band, showed affiliation to the *Cytophaga-Flavobacterium-Bacteroides* phylogenetic branch, which is known to contain many polymer degrading members. Another set of sequences was affiliated with the *Acidobacterium* cluster, which contains up to now mainly cloned sequences retrieved from very different environments. This is the first report of their occurrence at a marine hydrothermal site. Other sequences were related to recently published sequences from Obsidian Pool (a hot spring in Yellowstone National Park) that could not be affiliated with known bacterial divisions (Hugenholtz, P., C. Pitulle, K. L. Hershberger, and N. R. Pace, J. Bact. 180:366-376, 1997), indicating that similar bacteria are also important in marine hydrothermal systems. In addition, sequences related to chloroplasts of diatoms as well as to the sulfur-oxidizing chemolithoautotrophic genus *Thiomicrospira* were identified. The results presented here suggest, that apart from organotrophy, primary production by photosynthesis and chemosynthesis are important processes in this environment.

INTRODUCTION

Information about the microbial community structure of hydrothermal vent systems is necessary in order to gain a more thorough understanding of the functioning of these unique ecosystems and their impact on the surrounding environment. Vent associated microorganisms form the basis for the foodweb at such localities (10, 20, 25, 41, 43) and may also be involved in the microbial mediated transformation and precipitation of elements (19, 23). Selective enrichment cultivation is not considered to be a suitable tool to characterize microbial communities (2, 31, 38, 59), and several studies have used methods based on the analysis of 16S rRNA sequences for studying the bacterial communities at deep-sea vent sites (13, 14, 29, 30, 33, 40). These studies demonstrated that only a few spezialized bacterial populations dominate the microbial communities under the extreme physicochemical conditions found at the vent sites. By using denaturing gradient gel electrophoresis (DGGE) Muyzer et al. (33) identified four phylotypes in samples taken from two vent sites at the Mid-Atlantic-Ridge (MAR). Two of these phylotypes were closely related to sulfur-oxidizing *Thiomicrospira* spp. which were frequently isolated from a variety of vent sites, including the MAR (22, 47, 48, 64). At another MAR vent site, Polz et al. (22) found that the putative sulfur-oxidizing epibiont of a shrimp dominated the microbial community. At a hydrothermal vent system located on Loihi Seamount, Hawaii, a midplate volcano, one of the two operational taxonomic units dominating the fairly diverse community affiliated with the sulfur-oxidizing bacterium *Thiovolum* sp. (30). These results substantiated the earlier assumption that chemolitho(auto)trophy dependent on reduced sulfur compounds is an important process at vent sites (10, 20, 21, 25, 65). However, in these studies no attempts were made to examine the spatial distribution of the bacterial populations and changes in the community structure. Furthermore, the environment was not physicochemically characterized. Yet, hydrothermal vents offer a variety of habitats for metabolically diverse microbes due to sharp physical and chemical gradients (4, 5, 20, 24).

We have used a shallow submarine hydrothermal vent in the Aegean Sea near the island of Milos (Greece) to investigate the relationship between changes in physicochemical parameters and bacterial population distributions by using DGGE of PCR-amplified 16S rRNA gene fragments (49). In the present study we report on the identification of dominant 16S rRNA defined bacterial populations along a transect from the center of the vent to the surrounding sediment. Several bands were excised from the gels and sequenced to obtain information about the phylogenetic affiliation of the dominant populations and to make inferences about the trophic structure of the microbial communities at the vent site.

MATERIALS AND METHODS

Sampling. The study site was a solitary gaseous hydrothermal vent located at a water depth of 8 m in Palaeochori Bay (24°31.220' E; 36°40.391' N)(49), which is a sandy bay off the south-eastern coast of the island of Milos (Greece) in the Aegean Sea In the bay, seagrass beds of *Cymodocea nodosa* (depth 6 m to 20 m) and *Posidonia oceanica* from (depth 10 m to 40 m) were observed (1, 9). For a more detailed site description including physicochemical parameters see Sievert et al. (49). Sediment cores were taken with polycarbonate tubes by SCUBA divers along a transect from the center of the almost circular vent to the surrounding area at 10 cm, 123 cm, 165 cm and 235 cm distance from the vent center in June 1996 and at 30 cm, 117 cm and 200 cm distance from the vent center in September 1996.

Sediment cores were immediately sub-sampled by slicing the extruded sediment. In June 1996 the first 30 mm of every sediment core were subsampled at 10 mm intervals. In September 1996 the layers from 0 to 5 mm, from 8 to 13 mm and from 16 to 26 mm were sub-sampled. At both sampling times the first sample of each core consisted of water from just above the sediment surface which was sampled with a sterile syringe. In the case of cores from 123 cm (June 1996) and 117 cm (September 1996) this upper sample consisted of a white precipitate, which had a thickness of 2 to 5 mm.

Nucleic acid extraction. Subsamples from sliced sediment cores were immediately deep-frozen in liquid N_2 and stored at -80°C. DNA was extracted from sediment and water samples by a method originally described by Zhou et al. (66) and modified by Sievert et al. (49).

PCR amplification of 16S rDNA fragments. PCR amplifications were performed as described by Muyzer et al. (33). The primer pair GM5F and 907R is specific for the 16S rDNA of the domain *Bacteria* and amplifies a 550 bp long fragment for DGGE analysis (from nucleotide positions 357-907 corresponding to the *Escherichia coli* numbering (8)). Amplification products were first analyzed on agarose gels before further characterization by DGGE analysis or DNA sequencing.

DGGE analysis of PCR products. DGGE was performed as described by Brinkhoff and Muyzer (33). Gels of 1 mm thickness comprising 6% (wt/vol) polyacrylamide and a denaturant gradient from 20-70% were run for 20 h at a constant voltage of 100 V in 1 x TAE electrophoresis buffer (10 mM Tris, 20 mM sodium acetate, 1 mM EDTA, pH 8.3) using the D-Gene™ system (Bio-Rad Laboratories, Inc.). After electrophoresis, the gels were stained with ethidium bromide, and processed as described before (49).

Sequencing of DGGE bands. Selected DGGE bands were excised from the DGGE gels, reamplified by PCR with the primer pair GM5F and 907R and rerun on a DGGE gel to verify the purity and the position relative to the original band, as described previously (11). Before being sequenced the PCR products of the excised bands were purified by using the

Qiaquick Spin PCR purification kit (Qiagen Inc., Chatsworth, Calif.). The *Taq* Dyedeoxy Terminator Cycle Sequencing kit (Applied Biosystems, Foster City, Calif.) was used to sequence the 16S rDNA fragments with the primer pair GM5F and 907R. The sequence reaction mixtures were electrophoresed on an Applied Biosystems 373S DNA sequencer.

Comparative analysis of 16S rRNA sequences. Sequences were added to the 16S rRNA sequence data base of the Technical University Munich (Germany) using the software program package ARB (55). Sequences were first aligned automatically using ARB_ALIGN, then checked by eye and corrected manually. Only sequences at least 90% complete were used for tree construction. Partial sequences obtained from DGGE were inserted into the reconstructed tree by applying the parsimony criteria without allowing for changes in overall tree topology. Tree topologies were further evaluated by performing maximum parsimony, neighbor joining, and maximum likelihood analysis.

RESULTS

Number of unique populations. Fig. 1 shows two DGGE gels performed with samples from June 1996 (Fig. 1A) and September 1996 (Fig. 1B), respectively. The lower panel shows all bands that could be visualized on the DGGE. The number of unique bands having the same electrophoretic mobility was estimated fro both gels and these were subsequentyl referred to as operational taxonomic units (OTU's). In total we counted 95 OUT's, i.e., 51 OTU's for June (Fig. 1A) and 44 OTU's for September (Fig. 1B). However, four of the OTU's in September were identical to OTU's from June, reducing the total number of OTU's to 91. It is likely that this number of OTU's is an underestimation of the actual diversity, since bands with the same electrophoretic mobility can have different sequences. However, in all cases where we sequenced bands with the same electrophoretic mobility, we found that the sequences were nearly identical (see below). In total, about 80 bands were excised from the both gels. From these, 36 bands resulted in unambiguous sequences that were used for the phylogenetic analysis. The other excised bands either resisted reamplification by PCR probably due to a low amount of DNA or yielded ambiguous sequences. This could be due to the presence of more than one sequence in a particular band (45). Among the 36 sequences were 22 unique sequences (numbers 1 to 22), which amounted to 24% of the OTU's detected using DGGE.

Distribution of populations along the transect. The distribution of the sequenced bands shows that the sequences correspond to bacterial populations in different zones and sediment depths as well as different sampling times (Fig. 1A and B). However, fewer sequences were derived from the outer zones at both sampling times. This might be related to the higher complexity of the DGGE-profiles in these regions (49), leading to a higher probability that particular bands contained more than one sequence (36). In general, it was observed that the

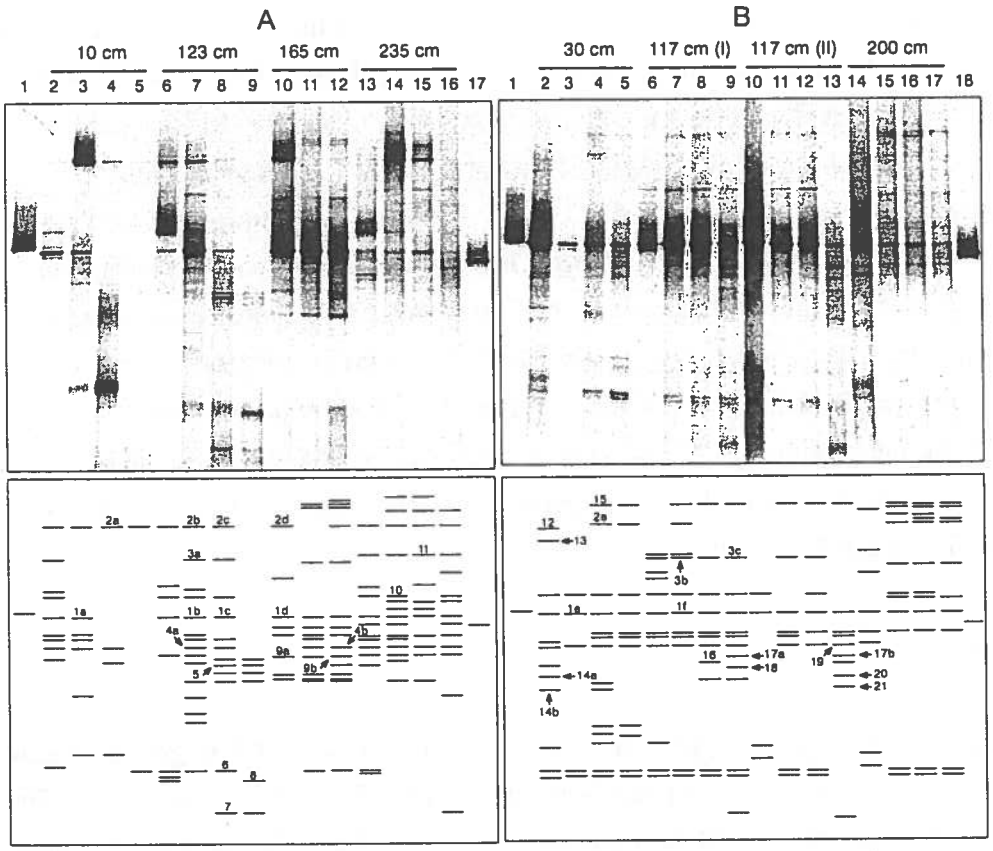

Figure 1. DGGE analysis of 16S rDNA fragments obtained after PCR amplification with the bacterial primer pair GM5F-GC-clamp and 907R of genomic DNA from environmental samples and standards with a known melting behavior for June 1996 (A) and September 1996 (B). In the upper panels the actual DGGE is shown, whereas in the lower panel a sketch showing the bands that could be identified on each DGGE is depicted. The numbers indicate the excised and sequenced bands and will be discussed in the text. The environmental samples were taken at specific locations along a transect from the vent center towards the surrounding area. The two cores at 117 cm distance in September were taken 1 week apart. During the first sampling (117 cm I) a white precipitate on the sediment surface was present, whereas during the second sampling (117 cm II), it was absent. No PCR-product could be obtained from the overlying water at 165 cm. (A) DGGE pattern of the samples taken in June 1996. Lanes: 1 and 17, standards, 2 to 5, samples taken at 10 cm from the vent center (lane 2 surface; lane 3, 0-10 mm; lane 4, 10-20 mm; lane 5, 20-30 mm); 6 to 9, samples taken at 123 cm (lane 6. surface; lane 7, 0-10 mm; lane 8, 10-20 mm; lane 9, 20-30 mm); 10 to 12, samples taken at 165 cm (lane 10, 0-10 mm; lane 11, 10-20 mm; lane 12, 20-30 mm); 13 to 16, samples taken at 235 cm(lane 13, surface; lane 14, 0-10 mm; lane 15, 10-20 mm; lane 16, 20-30 mm). (B) DGGE pattern of the samples taken in September 1996. Lanes: 1 and 18, standards, 2 to 5, samples taken at 30 cm from the vent center (lane 2, surface; lane 3, 0-5 mm; lane 4, 8-13 mm; lane 5, 16-26 mm); 6 to 9, samples taken at 117 cm (lane 6 surface; lane 7, 0-5 mm; lane 8, 8-13 mm; lane 9, 16-26 mm); 10 to 13, samples taken at 117 cm (lane 10 surface; lane 11, 0-5 mm; lane 12, 8-13 mm; lane 13, 16-26 mm); 14 to 17, samples taken at 200 cm (lane 14, surface; lane 15, 0-5 mm; lane 16, 8-13 mm; lane 17, 16-26 mm).

bacterial populations were neither vertically nor horizontally homogenously distributed along the transect at both times (49). This was most likely due to the concurrently changing environmental conditions, which favored the growth of specific populations. However, there were distinct differences between the two sampling times. In June, it was found that the bacterial community structure changed from an even community structure to an uneven community structure from the surrounding area to the vent center (49). This trend was not found in September and this was attributed to a disturbance of the microbial communities due to resuspension of the upper

sediment layer caused by storm previous to the sampling (49). Despite these differences there were also similarities. The population belonging to DGGE band ML-1a-f (see Fig. 1) seemed to be the dominant phylotype at both sampling times and was present in almost all zones and depths. Judging from the intensity of the band it could be inferred that the preferred habitat for this population was up to 165 cm from the center of the vent and therefore in the more strongly hydrothermal influenced sediments (49). The population belonging to DGGE band ML-2 was also present at both times. However, while band ML-2 was clearly stronger in the upper layers and constituted an important component of the microbial community in June, this population seemed to be of minor importance in September. The population belonging to DGGE band ML-10 was also present at both sampling times. This band was excised from one profile and its sequence was almost identical to an isolated *Thiomicrospira* sp. (7). By using hybridization analysis with a specific probe it was demonstrated that all bands running at the same position in the gel belonged to sulfur-oxidizing *Thiomicrospira* populations (7). Other populations seemed to be more restricted by zone, depth, or sampling times. Thus, DGGE bands ML-3a and ML-3b and c were only present in the zone at 123 cm from the vent center in June and at 117 cm from the vent center in September, respectively. Band ML-11 was only detected at 235 cm distance. The bands ML-4, ML-5, ML-9, ML-10, and ML-11 and the bands ML-20, and ML-22 to ML-26 were also mainly present at 123cm and 165 cm from the center of the vent in June and at 177 cm from the center of the vent in September, respectively. In addition, they seemed to be restricted to the deeper sediment layers. Interestingly, it was observed that for the samples from June that bands in the lower part of the DGGE gel were mainly present in the zones with a stronger influence from hydrothermal fluid, i.e., at 10, 123 and 165 cm from the center of the vent. DGGE bands ML-12 to ML-14b were only present in the water above the sediment at 30 cm from the center of the vent in September.

Phylogenetic affiliation. The sequences analyzed, their phylogenetic position, and their putative physiology inferred from cultivated organisms most closely related to the respective sequences, are summarized in Table 1. Eleven sequences were affiliated to the *Cytophaga-Flavobacterium* (CF) cluster of the *Cytophaga-Flavobacterium-Bacteroides* (CFB) phylum. Among these were six sequences that came from the dominant band (ML-1a-f). It was suggested that this band belongs to one population with a broad tolerance to environmental conditions. However, as it has been demonstrated that different sequences can have the same melting properties in DGGE (11, 34), it had yet to be shown that these bands are in fact the same. We excised and sequenced bands with the same electrophoretic mobility from different zones and depth. The sequences were in all cases identical or at least extremely highly related (> 99% similarity). Following the suggestions of Fox et al. (12), we grouped all these sequences into one 'rRNA superspecies' and only one representative is shown as ML-1. The slight differences between the various sequences are statistically not significant and could be either due

Table 1. Summary of the 16S rRNA sequences derived from excised DGGE-bands depicted in Figure 1.

DGGE-band No.	Putative Division	Database match (≥90% similarity)	% Similarity	Group	Inferred metabolism
1a-f	CFB[a]	Deep-sea hydrothermal vent clone VC2.1 Bac22	96	*Cytophaga* spp.	Organotrophy?
2a-e	Cyanobacteria and chloroplasts	*Odontella sinensis*	98	*Bacillariophyta*	Photosynthesis
3a-c	CFB[a]			*Cytophaga* spp.	Organotrophy?
4a-b	Spirochetes				Organotrophy?
5	Proteobacteria (δ)			*Desulfobacteriaceae*	Sulfate reduction?
6	Firmicutes			*Synergistes*	-
7	candidate division OP8				-
8[b]	candidate division OP9	Clone OPB 46	94		-
9a-b	Acidobacterium				-
10	Proteobacteria (γ)	*Thiomicrospira* strain Milos T-2	99	*Thiomicrospira*	chemolithoautotrophic sulfur-oxidation
11	CFB[a]	*Cytophaga fermentans*	91	*Cytophaga* spp.	Organotrophy?
12	Cyanobacteria and chloroplasts	Chloroplast (*Emiliana huxleyi*)	96	*Haptophyceae*	Photosynthesis
13	Cyanobacteria and chloroplasts	Chloroplast (*Emiliana huxleyi*)	97	*Haptophyceae*	Photosynthesis
14a[b]	Gram-positive bacteria	*Caldicellulosiruptor lactoaceticus*	97	*Caldicellulosiruptor*	Organotrophy, fermentative degradation of polysaccharides
14b[b]	Gram-positive bacteria	*Caldicellulosiruptor lactoaceticus*	99	*Caldicellulosiruptor*	see above
15	CFB[a]			*Flexibacter*	Organotrophy?
16	Acidobacterium				-
17a-b				*Flexistipes*	-
18	Acidobacterium				-
19	Proteobacteria (δ)			*Desulfobacteriaceae*	Sulfate reduction?
20	Acidobacterium				-
21	Acidobacterium				-

[a] CFB, Cytophaga-Flexibacter-Bacteroides; [b] inferred thermophily

to inherent errors in the sequence determination or to real differences (12). The closest relative of ML-1 is a sequence from a deep-sea hydrothermal vent clone (VC2.1Bac22; accession number AF068798) sharing a sequence similarity of 96% (Fig. 2). Three sequences, i.e., ML-3a-c that derived from one and two bands in June and September, respectively, formed another group in the CF-cluster (Fig. 2). The sequences from September were almost identical (> 99% similarity) and both have a similarity of 98% to the sequence from June. These sequences had no close relatives in the database (similarity <85%) and thus their exact phylogenetic position within the CF cluster remains unclear. Two other sequences within the CF cluster, i.e., ML-11 and ML-16, shared a sequence similarity of 92% to *Cytophaga fermentans* and of 88% to *Flexibacter maritimus*, respectively (Fig.2). Similarly to ML-1, the sequences that derived from ML-2 in dif-

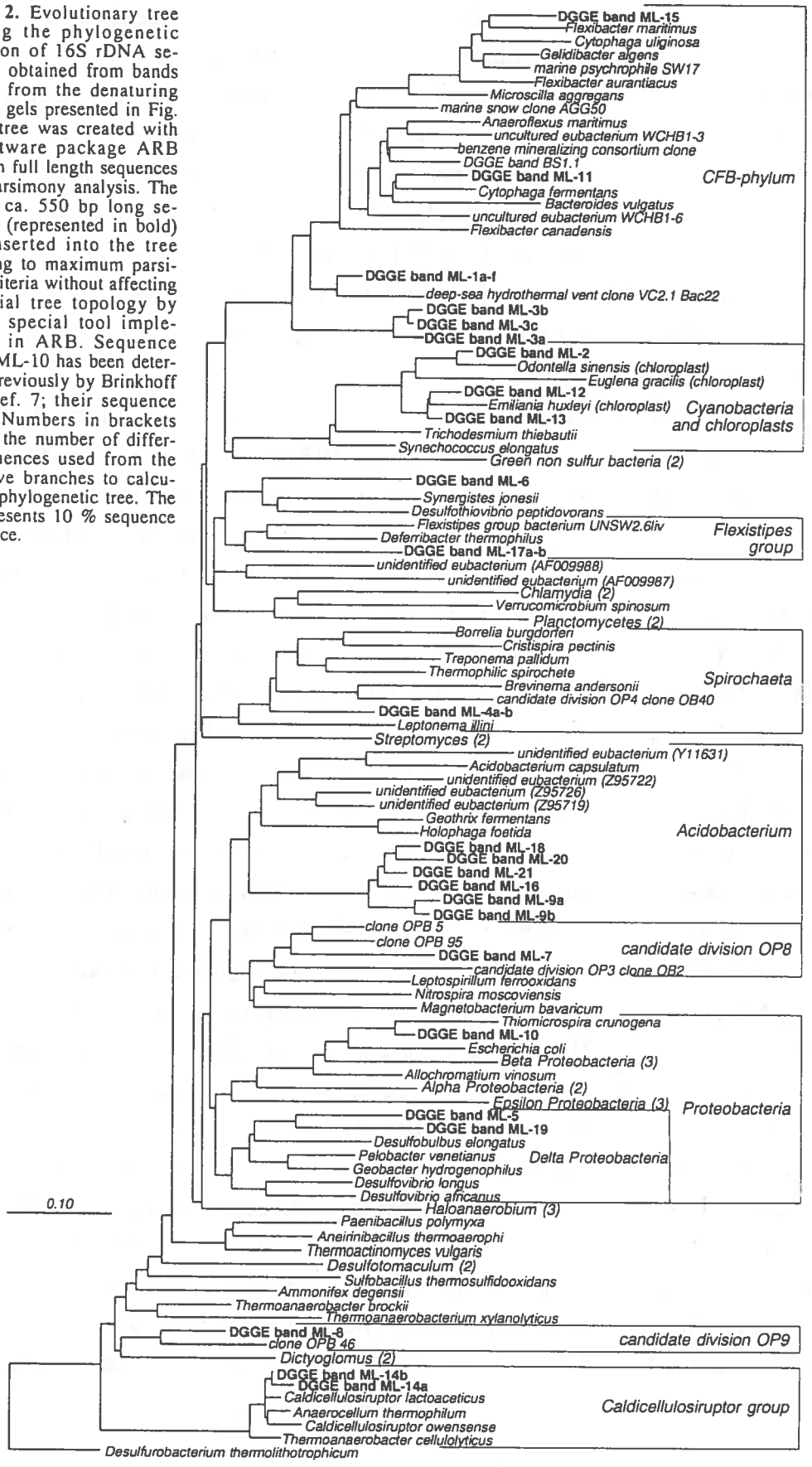

Figure 2. Evolutionary tree showing the phylogenetic affiliation of 16S rDNA sequences obtained from bands excised from the denaturing gradient gels presented in Fig. 1. The tree was created with the software package ARB based on full length sequences using parsimony analysis. The partial, ca. 550 bp long sequences (represented in bold) were inserted into the tree according to maximum parsimony criteria without affecting the initial tree topology by using a special tool implemented in ARB. Sequence DGGE ML-10 has been determined previously by Brinkhoff et al. (ref. 7; their sequence ML-1). Numbers in brackets indicate the number of different sequences used from the respective branches to calculate the phylogenetic tree. The bar represents 10 % sequence divergence.

ferent profiles, i.e. ML-2a-e, were identical and only one representative sequence of this 'rRNA superspecies' is depicted. They were affiliated to chloroplasts of diatoms and the closest full length sequence relative is the chloroplast sequence of the marine diatom *Odontella sinensis* with a sequence similarity of 98% (Fig. 2). Based on the 415 nt overlap between partialsequences of band ML-2, *Navicula salinicola* (37), and *Amphora delicatissima* (37), band ML-2 forms a cluster with these diatoms and is most closely related to *Navicula salinicola* (data not shown). Two other chloroplastic sequences, i.e. band ML-12 and 13, that were only present in September have *Haptophyceae* as their closest relatives (97% similarity) to the chloroplastic 16S rRNA sequence of *Emiliana huxleyi* and *Orchrosphaera neapolitana* (Fig. 2). Both described species are marine and planktonic algae.

Another set of sequences (ML-9a-b, ML-16, ML-18, ML-20, ML-21) formed a group that was affiliated to the *Acidobacterium* cluster (Fig. 2). All sequences derived from September (ML-16, ML-18, ML-20, ML-21) formed a group of highly related sequences (between 96 and 97% similarity), and they were more distantly related to the sequence from June (ML-9a-b; 93% similarity).Three sequences were found which showed affiliation to the Proteobacteria (Fig. 2). One sequence (ML-10) was almost identical to *Thiomicrospira* sp. strain Milos T-2 within the γ-subdivision of the Proteobacteria (7). *Thiomicrospira* sp. strain Milos T-2 was isolated from the same vent site (7). Two other sequences, i.e., band ML-5 and 19, were loosely affiliated to the δ-subdivision of the Proteobacteria with a similarity of 84% to *Desulfobulbus elongatus*. Two sequences, i.e., ML-14a-b, were closely related *to Caldicellulosiruptor lactoaceticus* (almost 99% sequence similarity; Fig. 2), a thermophilic, cellulolytic, and anaerobic bacterium that was isolated from a hot-spring on Iceland (28). This affiliates with the *Bacillus/Clostridium* subphylum of Gram-positive bacteria (42). Although *C. lactoaceticus* stains Gram-negative, it has been demonstrated that members of the genus *Caldicellulosiruptor* have a Gram-positive type cell wall (16). Bands ML-14a and b differed only in two unambiguous base positions. Other sequences, i.e., ML-7 and ML-8, were related to recently published sequences from Obsidian Pool, a hot spring in Yellowstone National Park, that could not be affiliated with known bacterial divisions (18). The closest match in this case was observed with the sequence of DGGE band ML-8 to clone OPB46 of the candidate division OP9 (94% sequence similarity; Fig. 2). Band ML-7 was related to clones of candidate division OP 8. However, as the similarity was low (85%) its placement is uncertain. Two sequences that were identical in two profiles (ML-4a-b) fell into the *Spirochetes* cluster (83% similarity to a thermophilic spirochete; Fig. 2). One sequence (band ML-6) was affiliated to the group containing *Synergistes jonesii* and *Desulfothiovibrio peptidovorans*, albeit only distantly (82% similarity to *D. peptidovorans*; Fig. 2). The same was true for ML-17a-b, that was loosely affiliated to the *Flexistipes* group and shared 79% sequence similarity with *Deferribacter thermophilus* (Fig. 2).

DISCUSSION

DGGE has been successfully applied in a variety of habitats to analyze microbial communities (31, 32). One of the advantages of this technique is, that it allows the simultaneous analysis of large number of samples. This made it the ideal tool to analyze the bacterial communities along an environmental gradient at the studied vent site, since changes in the composition of the bacterial communities and in the bacterial community structure could readily be detected by comparing the different profiles (49). However, it is important to realize that this method as any other detection method has limitations (32). All molecular methods based on the extraction of nucleic acids face the problem to achieve an efficient and equal lysing of cells. An insufficient or preferential disruption of cells would distort the view of the community composition (63). Our microscopic observations indicated that complete lysis was achieved (49). In fact, that we recovered sequences from phylogenetically diverse bacteria including Gram-positives, that are thought to be extra resistant to lysing (63), gives us confidence that this bias was likely to be small. This finding also indicates that the primers we used were in fact appropriate to analyze the bacterial communities. However, we can not exclude that bacteria specific to this habitat may not contain the signature sites necessary for efficient amplification with the bacterial primers used and thus would have been not included in the analysis. Furthermore, PCR biases, e.g., a selective amplification of certain DNA fragments or differential amplification efficiencies, can not be ruled out (63). Limitations that are specific to the DGGE analysis include, that only numerically dominant populations are detected (32) and that the diversity might be overestimated due to the presence of multiple rrN operons with sequence microheterogeneity in some bacteria (35). It is also important to note, that the phylogenetic information that can be obtained by sequencing excised bands is restricted, due to the relatively small size of the separated 16S rRNA fragments. This becomes increasingly important if the sequence has only a low similarity to known sequences (17).

DGGE allowed us to identify some of the dominant populations, and their distribution at this hydrothermal vent. The phylogenetic analysis of the bands indicated a wide diversity within the domain *Bacteria*. Most populations seemed to present new lineages with no close culturable relatives. The actual diversity might be even higher since we sequenced only 28% of the unique bands occurring at this vent site. Similar findings have been made in a variety of environments including deep-sea hydrothermal vents (30) and terrestrial hot springs (18). This shows, that molecular methods, despite their biases and pitfalls, are less cumbersome than culturing methods and thus indispensable to evaluate the actual composition and structure of microbial communities.

In previous investigations of microbial communities at geothermal systems it was found that although these communities contained a high number of OTU's, the communities were

dominated by few populations (18, 29, 30, 61). However, we could demonstrate that this uneven community structure was only present near the vent center. In a zone that was less influenced by the hydrothermal fluid a diverse range of bacterial populations was present in approximately equal abundance (49). However, this trend was only apparent in June, but not in September. This was attributed to a disturbance of the microbial communities by a resupension of the upper sediment layer due to a storm previous to the sampling in September (49). The sequencing of bands in the present study further supported this hypothesis. The bands corresponding to the populations that are closely related to *C. lactoaceticus* were only present in the water overlying the sediment at a distance of 30 cm from the center of the vent. However, the *in situ* temperature at the surface was about 30°C (49), and clearly below the minimal growth temperature of *C. lactoaceticus* (50°C) (28). The temperature, which increased with depth and was about 60°C at 3 cm (49), would probably allow the growth of this population. Furthermore, *C. lactoaceticus* is an obligate anaerobe (28) and oxygen was absent from the sediment, whereas it waspresent in the water above the sediment (49). Additional support for the disturbance hypothesis comes also from the different distribution pattern of *Thiomicrospira* spp. in June 1996 and September 1996 (7) and the lack of correlation between the number of bands in each layer and the *in situ* temperature (49). Moreover, the low intensity of the bands corresponding to the chloroplastic sequences might indicate that these photosynthetic populations were dispersed due to the resuspension of the upper sediment layer. Despite the apparently highly dynamic nature of this environment, the presence of identical (ML-1 and ML-2) or highly related sequences (ML-3a-c), as well as the detection of *Thiomicrospira* sp. (7) at both sampling times, indicating the presence of key stone species.

Members of CFB accounted for 23% of the OTU's and were the most prominent among the phylotypes detected. If it can be assumed that the relative intensity of bands in DGGE can be correlated to the actual abundance of the corresponding populations (34, 35), then it can be inferred that the population belonging to band ML-1 is a predominant member of the microbial community particularly in zones more strongly affected by hydrothermal fluid at this vent site. That this population was most closely related to a clone that was derived from an *in situ* growth chamber deployed at a deep-sea hydrothermal vent provides further support for the assumption that it is a indigenous and probably thermophilic population. However, because of the highly conserved nature of the 16S rRNA gene, sequence identity does not necessarily mean that the corresponding organisms belong to the same species or have similar phenotypes (12, 39, 54). Thus, it is also possible that the highly related sequences of the 'rRNA superspecies' ML-1 represent distinct populations that could occupy different ecological niches along the environmental gradient at the studied hydrothermal vent (39, 60). In this respect it has to be kept in mind, that we only determined approximately one third of the total 16S rRNA gene sequence. Although this partial sequence contains a highly variable part, it is possible that more differences

could be detected by determining the whole sequence. Populations related to the CF-cluster have also been reported to be important in other marine sediments, e.g., North Sea (26), and Black Sea (45). Inference of the physiology of these OTU's from their phylogeny, is difficult, but since all cultured members of CF-cluster are heterotrophic and many members are known to degrade polymers (44), it seems likely that the populations belonging to these OTU's perform a similar metabolism. However, it is possible that some OTU's represent lineages with new metabolic capabilities.

The sequences that were related to phototrophic algae could in principle originate from three sources: (i) through sedimentation of planktonic species from the water column, (ii) *in situ* growth of benthic species on the sediment surface, and (iii) from epiphytic species that were transported to the hydrothermal vent on, e.g., seagrass fragments. With regard to sequences related to chloroplasts of diatoms we could exclude the first possibility. The sequence had species belonging to the pennate genera *Navicula* and *Amphora* as its closest relatives and both are confined to growing on surfaces (46). While it could still be possible that these species were epiphytic, we have good reasons to believe that the sequence originated from populations that were growing *in situ* on the sediment. The presence of the genera *Amphora* and of the species *Navicula salina* could be microscopically verified in samples from different zones along this transect (Economou-Amilli, pers. comm.), and we could also find pennate diatoms of unknown affiliation thriving in the white precipitate and at the center of the vent after microscopic inspection on site (unpublished data). That the population belonging to band M2 was predominantely present in the upper sediment layer further supports this assumption. It is interesting, that these populations seemed also to be active near the center of the vent, where the *in situ* temperature at the sediment surface was between 30 and 40°C. Most diatoms cannot grow at these temperatures, although the closest relatives of band ML-2 originating from hypersaline mats in Guerrero Negro, Baja California (Mexico), were able to grow at 37°C (Garcia-Pichel, pers. comm.). Thus, it is suggested that the diatoms belonging to band ML-2 contributed to the production of autochtonous organic matter at this vent site by carrying out photosynthesis. The populations belonging to band ML-12 and 13 probably originated from planktonic species, since all known *Haptophyceaea* are planktonic. However, unknown benthic forms might exist.

The finding that *Thiomicrospira* spp. constitute an important component of the microbial community (7), suggests that besides photosynthesis, chemosynthesis is contributing to the primary production at this vent site. That we could isolate the population that seems to be dominant at the vent site further demonstrates, that it is in principle possible to cultivate ecological relevant species. It was important, however, to dilute the inocolum before starting the enrichments. The isolation of other chemolithoautotrophic sulfur-oxidizing bacteria from similar or even higher dilutions (unpublished data), gives further support for the importance of chemosynthesis in this habitat and demonstrate a high diversity of sulfur-oxidizing bacteria. In

addition to the cultivated species, we have also indications, that a new sulfur-oxidizing, chemolithoautotrophic *Arcobacter* sp., that seems to be prevalent at certain deep-sea vents (56, 57), might also be of importance at the studied vent site (49).

Populations affiliated with the Acidobacterium cluster were also abundant. This cluster, which has been considered a new kingdom (3), now contains only three cultured representatives, i.e. *Holophaga foetida*, *Geothrix fermentans*, and *Acidobacterium capsulatum*, but many uncultured forms that were obtained by molecular analysis from a variety of environments (3, 27). However, this is the first report of their occurrence at a marine hydrothermal site. Inferences about their physiology cannot be made at present and must await the cultivation of related bacteria.

A population affiliated with the newly discovered candidate division OP9 (18), was also detected. Members of this new line of descent within the domain *Bacteria* have so far only been found in a terrestrial hot spring, and the presence of related populations at a marine hydrothermal site extends the known habitat range for this group and gives further evidence for the creation of a new division for these bacteria. Based on the finding that the G+C-content of the 16S rRNA gene of clone OPB46 was higher than 60 mol%, a thermophilic nature of this population was inferred (18). The partial sequence of band ML-8 had a G+C-content of 58 mol%, and in the corresponding region OPB46 had a G+C-content of 59 mol%. Thus, it could be inferred that band ML-8 also belongs to a thermophilic population. This would be in accordance with the finding that the band was present in lower part of the DGGE gel where it encountered a higher concentration of denaturant. Two other bands (ML-6 and ML-7) were also detected in the lower part of the DGGE gel and thus may also belong to thermophilic populations. That ML-7 had clones as its closest relatives that were detected in a hot spring supports this assumption. A clear thermophily could be inferred for ML-14a and b, since the sequences of these bands were almost identical to *Caldicellullosiruptor lactoaceticus*. As this bacterium was isolated from a terrestrial hot spring, this provides a further example of a connection between terrestrial and marine geothermal systems. Other populations seemed to be only distantly related to known phylotypes or bacteria. However, since we only have a partial sequence their exact phylogenetic position remains elusive. It is interesting, however, that ML-4a-b are affiliated with Spirochetes, since the first anaerobic bacterium isolated from a deep-sea hydrothermal vent was a Spirochete (15). The detection of a spirochete like sequence by DGGE indicates that these organisms might play an important role in this environment.

The presence of populations that are loosely affiliated to sulfate-reducing bacteria within the δ-subdivision of the Proteobacteria, could be an indication that sulfate reduction is an important electron acceptor for the terminal oxidation of organic matter in this habitat. Sulfate reducers were most abundant at 235 cm from the center of the vent (49). From the highest dilution a bacterium that was most closely related to *Desulfobacter halotolerans* ((6); 97% 16S

rRNA similarity) could be isolated (unpublished data). *Desulfobacter* spp. have a narrow substrate spectrum and are thought to be specialists for the degradation of acetate (62). Thus, their relatively high numbers suggest that acetate, as one of the final end products of anaerobic degradation of organic compounds, is an important substrate in this habitat. Unfortunately, no isolates were obtained from the zones from which the present sequences were derived, and thus it is not known whether or not bacteria related to *Desulfobulbus* were present in the MPN's.

Implications for the trophic structure and similarities to other geothermal systems. The data presented in this paper suggest that, although autotrophic as well as heterotrophic populations could be detected, the microbial community at this vent site was net heterotrophic. Potential substrates used by the microbial community could be (i) organic matter produced at the vent site through photosynthesis by diatoms and trough chemosynthesis by sulfur-oxidizing bacteria, e.g., *Thiomicrospira*, as well as (ii) seagrass fragments from the surrounding seagrass meadows (1). This would provide an explanation for the presence of populations that degrade macromolecules such as polysaccharides, e.g., organisms that affiliate with the CF-phylogenetic branch and *Caldicellulosiruptor lactocaceticus* related organisms. It has been suggested before, that the occurrence of photosysnthesis at this shallow-water vent site might lead to a phytodetrital based food-chain (58). This might be a major difference from most deep-sea hydrothermal vents, were the autochtonous organic matter is produced by chemosynthesis rather than photosynthesis and the input of allochtonous organic matter is low. Thus, at these vents without appreciable sediment cover autotrophic populations seem to be predominant (19), although no rigorous proof for this hypothesis has been presented (24). However, there might be similarities to deep-sea vent sites with high sedimentation rates of organic matter derived from the euphotic zone, such as Guaymas Basin (Mexico). At this site about 300-400 m of diatomaceous sediment rich in organic matter overlies the vents (51). This could lead to a higher proportion of heterotrophic organisms relative to autotrophic populations (52), as observed at the present vent.

An important similarity between the shallow-water vent site and deep-sea vents is that *Thiomicrospira* spp. are important components of the microbial communities (7, 33) and that populations related to the dominant phylotype at the shallow-water site seem to be present at deep-sea vents. It is noteworthy, however, that we did not detect any similarities in the OTU's in the present study to the OTU's that were identified at an active hydrothermal vent on Loihi seamount (30). One important difference, apart from the organic matter concentration and its source, that could account for this, could be the temperature that was found to be low, i.e. near ambient sea water temperature, at Loihi (30), whereas at the studied vent site appreciable higher temperatures were measured (49).

It is interesting, however, that there were similarities between this marine shallow-water hydrothermal vent and terrestrial hot-springs in Yellowstone National Park, i.e., Obsidian Pool

(OP), and Iceland. Besides the presence of OP clone related sequences and the finding of populations that are closely related to *Caldicellulosiruptor lactoaceticus* we could also isolate a thermophilic sulfate-reducing bacterium which is phylogentically related to the cluster *Thermodesulforhadus/Desulfacinum* in the δ-subdivision of the Proteobacteria (50), which were also found to be abundant in Obsidian Pool (18). We do not know the reasons for this smiliraty. However, it is tempting to suggest that it might be related to the source of organic matter. Terrestrial hot springs and this shallow submarine vent receive both allochtonous organic matter from the surrounding area, including vascular plant remnants, in addition to autochtonously produced organic matter, which might lead to similar microbial communities. At least in hot springs on Iceland many polymer degrading populations seem to be present (53). However, so far nothing is known about the metabolism of the organisms of the candidate divisions OP8 and 9.

Conclusion. In conclusion, we have demonstrated a fairly high number of unique populations at this vent site, although the community was dominated by only few populations closer to the vent center. In addition, we could identify important members of the microbial community and delineate microbial processes that might be of importance at this vent site. Future investigations should aim in revealing more detailed information about the microbial communities, e.g., quantification of specific populations and studying their fine scale distribution by using fluorescent *in situ* hybridisation, and to measure and quantify the microbial mediated processes. In this respect, it would be of utmost importance to know the relative contribution of chemosysnthesis and photosysnthesis as producers of organic matter. In addition, in future work it should be tried to culture the relevant organisms that were outlined in this publication. Finally, as this study presents the first molecular analysis of a bacterial community of a marine shallow-water hydrothermal vent, it should also provide a framework with which to compare similar environments in the future.

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3

Relative abundance of *Archaea* and *Bacteria* along a thermal gradient of a shallow-water hydrothermal vent quantified by rRNA slot-blot hybridization

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ABSTRACT

We applied slot-blot hybridization of rRNA with domain specific oligonucleotide probes targeting the 16S rRNA of *Archaea* and *Bacteria* to assess the relative abundance of these domains along a thermal gradient at a shallow submarine hydrothermal vent near Milos Island (Greece). Highest prokaryotic rRNA concentrations (defined as the sum of bacterial and archaeal rRNA) were found in the uppermost sediment surface (0-20 mm), and decreasing strongly with depth. This indicates that the microbial activity was mainly occuring in the surface layer of this hydrothermal vent. Furthermore, rRNA concentrations were higher in regions closer to the vent, suggesting that the hydrothermal activity stimulated the microbial activity. *Archaea* seemed to be a minor component of the microbial community at this vent site, even in the zones with higher temperatures. *Bacteria* made up at least 78% (average 95%) of the prokaryotic rRNA. However, along the steepest temperature gradient the proportion of archaeal rRNA was increasing. Nevertheless, even in the hottest sediment layer where a quantification was possible (*in situ* temperature 82 °C) archaeal rRNA made up only 11.9% of the prokaryotic rRNA. This suggests that *Archaea* were generally of minor importance at this vent site and were probably restricted to a narrow niche. The factors that allow *Bacteria* to dominate in a high temperature environment that was once believed to be the realm of *Archaea* remain elusive.

INTRODUCTION

The composition of microbial communities of high temperature hydrothermal vents was until recently mainly studied by cultivation based techniques. Because of the frequent isolation of *Archaea* from these habitats it was assumed that *Archaea* dominate these communities (5, 26). The first cultivation independent support for that hypothesis came form the analysis of lipids extracted from sulfide structures of a black smoker (11). However, the application of molecular-biological methods revealed a quite different picture. Although it could be shown that the diversity of *Archaea* was unexpectedly high in terrestrial and marine hydrothermal systems (3, 4, 6, 27), it seemed to be the *Bacteria* that dominated the microbial communities in most of these environments (9, 12, 21, 27). However, in none of these studies was the ratio of the two domains related to the *in situ* temperature nor was it investigated how this ratio would change along a thermal gradient. This is surprising, since temperature is the most obvious parameter that could determine the relative abundance of *Bacteria* and *Archaea* at hydrothermal habitats. In addition, most of these studies have used molecular techniques that are not a priori quantitative.

In the present study we have studied the relative abundance of two domains of life, i.e. *Archaea* and *Bacteria* (28), along a thermal gradient at a shallow-water hydrothermal vent. We used slot-blot hybridization of rRNA directly extracted from the sediments to quantify the relative abundance of *Archaea* and *Bacteria* with domain specific probes. This technique has been successfully applied in a variety of environments to estimate the relative abundance of specific groups of microorgansims (18-20, 22, 23, 25). Concurrently with the sampling, the *in situ* temperature of the respective samples was determined.

MATERIAL AND METHODS

Study Site. The study site was a solitary gaseous hydrothermal vent located in 8 m water depth in Palaeochori Bay (24°31.220' E; 36°40.391' N), a sandy bay in the south-eastern part of the island Milos in the Aegean Sea (Greece). For a more detailed site description see Sievert et al. (24).

Sampling and temperature measurements. Sampling was carried out along a transect from the center of the almost circular vent to the surrounding area. Sediment cores (polycarbonate tubes with an inner diameter of 37 mm) were taken by SCUBA divers at specified distances from the vent center in June 1997. Vertical profiles of temperature were determined *in situ* up to 12 cm sediment depth in 1 cm increments along the same transect. The sensors were attached to a micro manipulator mounted on a tripod. The instruments were self-contained and held in watertight housings. The signals were transferred to and stored by a 12 bit data logger.

Slicing of sediment cores. Sediment cores were immediately subsampled by extruding the sediment from the polycarbonate tubes and slicing each core. The following depth intervals were sampled: 0-2 mm, 2-7 mm, 7-12 mm, 12-17 mm, 17-27 mm, 27-37 mm, 37-57 mm, 57-77 mm, 77-97 mm, and 97-117 mm.

RNA extraction Subsamples (5 cm^3) from sliced sediment cores were immediately deep-frozen in liquid N_2. For long-term storage, the samples were kept at -80 °C. RNA was isolated directly from the sediments by bead-beating, phenol extraction, and ethanol precipitation based on methods described elsewhere (18, 22, 25). The extracted RNA was stored at -80 °C until further analysis. The quality of the RNA was checked by polyacrylamide gel electrophoresis.

Oligonucleotides. Oligonucleotide were purchased from Biometra. The probes used where S-*-Univ-1392-a-A-15 (25), which targets most known forms of life, S-D-Bact-0338-a-A-18 (1)), which is specific for *Bacteria*, S-D-Arch-0915-a-A-20 (2) targeting *Archaea*.

Quantification of domains in environmental samples. For quantitative rRNA hybridization experiments, RNA extracts from natural samples and from suitable reference organisms (*Escherichia coli* and *Methanobacterium bryantii*) were denatured with 1.5% glutaraldehyde and loaded onto nylon membranes (Magna Charge, Micron Separations, Westborough, Mass.) using a slot blotting apparatus (Minifold II, Schleicher & Schüll, Keene, N.H.) as described previously (25). Because the amount of extractable RNA from environmental samples was in some cases very low, i.e., below the detection limit of the gel electrophoresis, we could not blot equal amounts of RNA. When possible approximately 50 ng RNA was blotted in triplicate. However, in some cases triplicate blotting was not possible due to the low amount of RNA.

After blotting, the membranes were baked at 80 °C for 1 h. Membranes were further prehybridized at 40 °C for 1 h with 10 ml of hybridization buffer (0.9 M NaCl, 50 mM $NaPO_4$ pH 7.2, 5 mM EDTA pH 7.2, 10X Denhardt's solution, 0.5 mg ml^{-1} polyadenosine, 0.5% SDS) followed by the addition of 16S rRNA oligonucleotide probes (see below) end-labeled with ^{32}P. After overnight hybridization at 40 °C, membranes were washed twice at 40 °C for 1 h with washing buffer (1% SDS, 1X SSC). Membranes were then transferred to the same buffer preequilibrated at the high stringency washing temperature (40 °C for S-*-Univ-1392-a-A-15 , 54 °C for S-D-Bact-0338-a-A-18, 56 °C for S-D-Arch-0915-a-A-20), washed twice for 15 min, removed, and air dried.

The dried membranes were exposed to Storage PhosphorImager screens (Molecular Dynamics, Sunnyvale, Calif.). Subsequently, the hybridization signal intensity was measured with a PhosphorImager (Molecular Dynamics) and quantified with the software package ImageQuant (Molecular Dynamics) relative to suitable reference RNA. For each probe an individual set of membranes was prepared to which a reference dilution series of reference RNA

and the environmental samples were applied. In addition, one membrane was prepared with the dilution series of all reference RNA's for the universal hybridization. This membrane was used to quantify the reference RNA of *M. bryantii* according to an *E. coli* standard (Boehringer, Mannheim, Germany).

RESULTS

The profiles of the *in situ* temperature illustrates that the studied vent system was well suited to study the relative distribution of *Archaea* and Bacteria along an environmental thermal gradient (Fig. 1). It has been shown before that temperature was an important parameter in determining the bacterial community structure (24) and it was expected that it would also influence the relative distribution of *Archaea* and *Bacteria.*

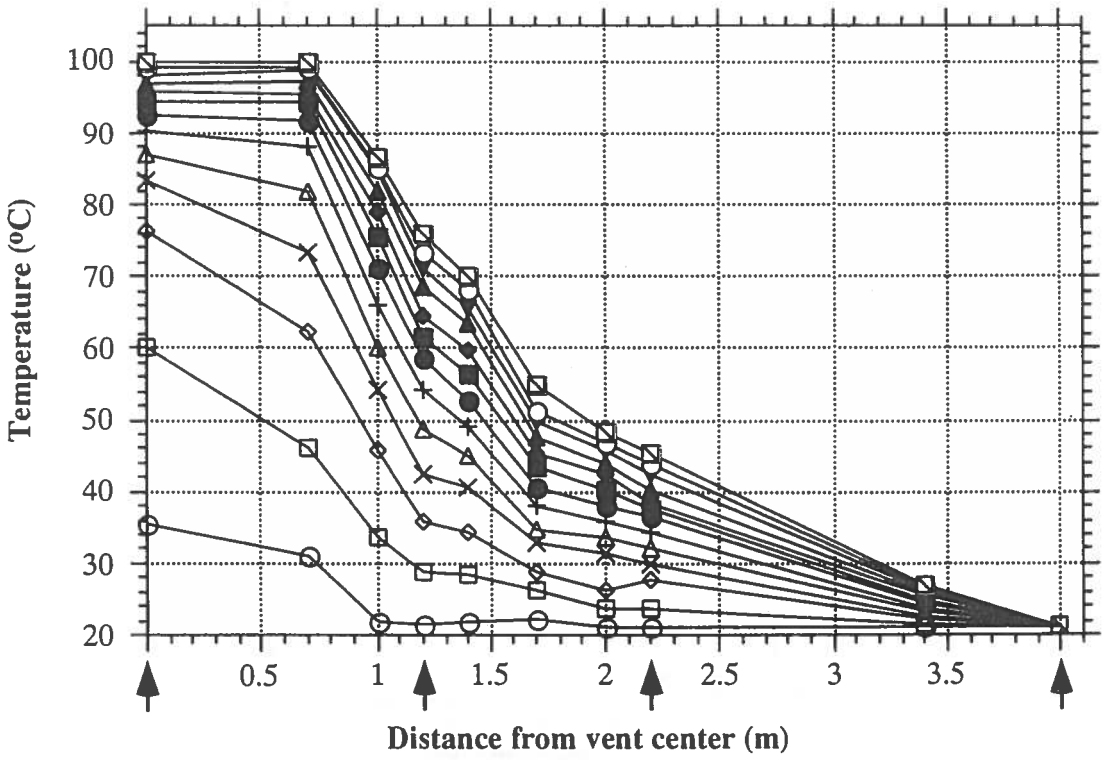

Figure 1. Profile of *in situ* temperature along a transect from the center of the vent to the surrounding area at the following sediment depth: 0 cm (○), 1 cm (□), 2 cm (◊), 3 cm (x), 4 cm (Δ), 5 cm (+), 6 cm (●), 7 cm (■), 8 cm (◆), 9 cm (▲), 10 cm (▼), 11 cm (⊞), and 12 cm (⧅). The arrows mark the position where the sediment cores for the molecular analysis were taken.

Four cores along the transect which varied in their temperature gradient with sediment depth were selected to address this question. Apart from temperature, other physicochemical parameters have previously been determined at this vent (24).

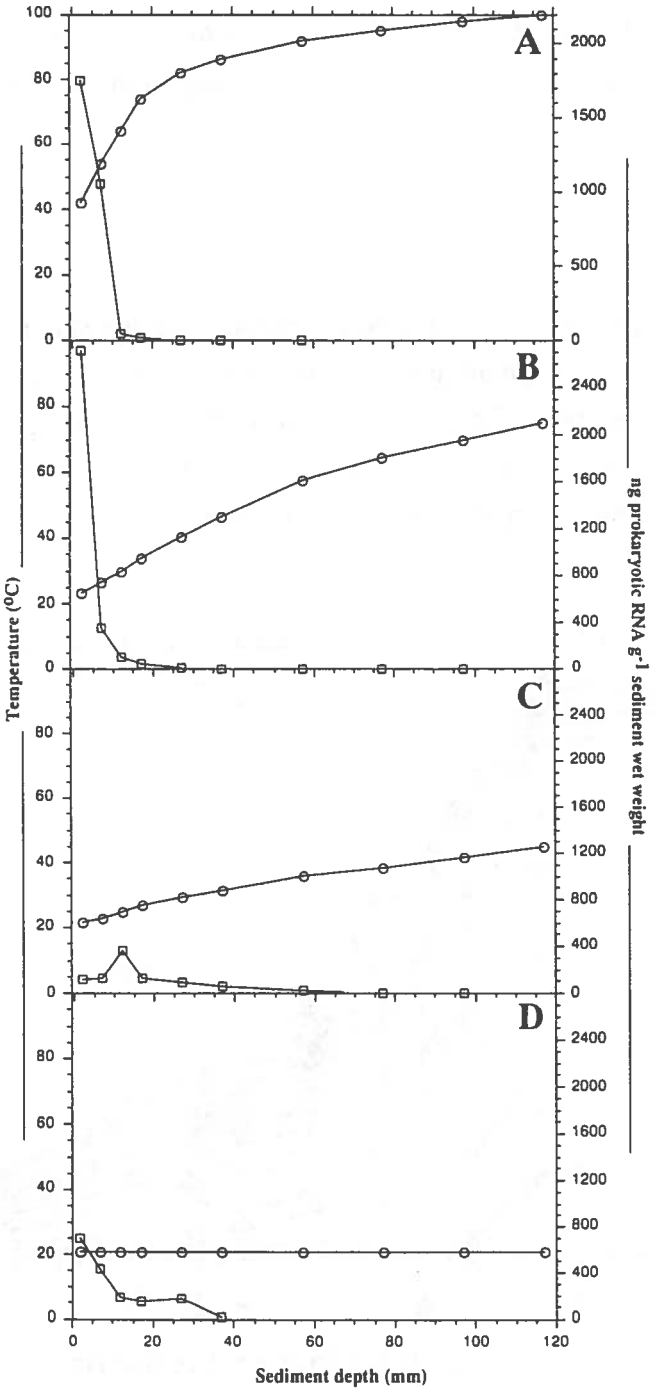

Figure 2. Profiles of temperature (O) and the concentration of prokaryotic rRNA (sum of bacterial and archaeal rRNA) (□) with sediment depth in the four different cores along the transect: A = center; B = 1.2 m; C = 2.2 m; D = 4 m.

As a general feature it could be observed that the amount of prokaryotic rRNA (defined as the sum of bacterial and archaeal rRNA) was rapidly decreasing with sediment depth in all cores, although the decrease seemed to be more pronounced in the zones with the steepest temperature gradient (Fig. 2). In the deeper sediment layers of the cores generally only very low

Table 1. Profile of temperature, prokaryotic rRNA, relative abundance of *Archaea*, and the ratio of *Bacteria* to *Archaea* in the different cores along the transect: A = center; B = 1.2 m; C = 2.2 m; D = 4 m. na = not analyzed, - = not calculated.

A

Depth (mm)	Temperature (°C)	Prokaryotic rRNA (ng/g sediment)	Relative Abundance of *Archaea* (% of total rRNA)	Ratio *Bacteria/Archaea*
2	42	1749.3	0.7	139
7	54	1051.3	1.9	51
12	64	48.0	7.3	13
17	74	14.7	7.8	12
27	82	2.2	11.9	7
37	86	< 1	-	-
57	92	< 1	-	-
77	95	< 1	-	-
97	98	na	-	-
117	100	na	-	-

B

Depth (mm)	Temperature (°C)	Prokaryotic rRNA (ng/g sediment)	Relative Abundance of *Archaea* (% of total rRNA)	Ratio *Bacteria/Archaea*
2	24	2714.8	1.1	88
7	27	354.8	3.5	28
12	30	99.4	4.8	20
17	34	45.0	5.2	18
27	41	17.5	22.2	3
37	47	< 2	-	-
57	58	< 2	-	-
77	65	< 2	-	-
97	70	< 2	-	-
117	75	< 1	-	-

C

Depth (mm)	Temperature (°C)	Prokaryotic rRNA (ng/g sediment)	Relative Abundance of *Archaea* (% of total rRNA)	Ratio *Bacteria/Archaea*
2	22	111.6	3.2	30
7	23	122.8	2.7	36
12	25	371.1	2.3	43
17	27	123.9	2.7	36
27	30	97.8	3.4	29
37	32	53.1	4.9	19
57	36	29.6	8.2	11
77	38	< 2	-	-
97	42	< 2	-	-
117	45	< 2	-	-

D

Depth (mm)	Temperature (°C)	Prokaryotic rRNA (ng/g sediment)	Relative Abundance of *Archaea* (% of total rRNA)	Ratio *Bacteria/Archaea*
2	21	703.2	2.4	41
7	21	435.4	3.0	32
12	21	182.2	3.8	25
17	21	153.0	2.5	38
27	21	176.7	2.4	40
37	21	26.9	4.6	21
57	21	na	-	-
77	21	na	-	-
97	21	na	-	-
117	21	na	-	-

very low amounts of rRNA could be detected (< 1 ng g^{-1} sediment wet weight) and a quantification of the two domains was not attempted in these cases. Interestingly, the upper sediment layers of the inner two cores contained a higher amount of prokaryotic rRNA than the outer two cores. The lowest concentrations of prokaryotic rRNA were found at 2.2 m distance.

Bacteria constituted the most important component of the prokaryotic community in all regions and depth, making up at least 78% (average 95%) of the prokaryotic rRNA (data not shown). *Archaea* constituted only a low fraction of the prokaryotic community (Table 1). However, there was a trend of an increasing archaeal contribution to prokaryotic rRNA with increasing temperature at the center of the vent and in the core from 1.2 m distance from the center (Table 1A and B). The increase was from 0.7% at the surface to 11.9% at 27 mm sediment depth at the center (concurrently the temperature increased from 42 °C to 82 °C) and from 1.1% at the surface to 22.2% at 27 mm sediment depth 1.2 m distance (here the temperature increased from 23.5 °C to 40.5 °C). Such a trend was not apparent in the cores from 2.2 m and 4 m distance (Table 1C and D).

DISCUSSION

We have quantified the relative abundance of *Archaea* and *Bacteria* at a shallow-water hydrothermal vent by using the hybridization of environmental rRNA with domain specific oligonucleotides targeting the 16S rRNA. Although this method like any other molecular method has the advantage that it circumvents selective and potential ineffective cultivation, it also has limitations. 16S rRNA targeted probes are widely used in molecular ecology. However, their sequences are based on known sequences in the database and thus new populations that may not contain the target signature sites are not detected. This limitation is more severe in environments where many unknown or new populations are suspected, such as hydrothermal vents (3, 4, 6, 10, 12, 16, 27). The first step in all studies of microbial community analysis by molecular methods is the extraction of nucleic acids from the environment. Especially in sediments and soils this poses some challenges, and much remains to be learned about the extraction efficiencies of nucleic acids from these kind of samples. In addition, it is unknown whether there is a difference in the lysing efficiency of archaeal and bacterial cells, e.g., due to the difference in cell wall composition and structure (18). It is possible that the much lower yield of archaeal rRNA from sediments as compared to pelagic environments is associated with these two biases (22). Another potential caveat that affects quantification is the unknown relationship between the abundance of rRNA to actual cell numbers, since the content of rRNA per cell varies among species and with growth rate (7, 15). Thus, it is possible that certain organisms contribute proportional more to the total rRNA than the their cell number would suggest and vice versa. Whether there is a systematic difference in rRNA content per cell between *Archaea* and *Bacteria* is not known. The universal probe used in the present study has been shown to overestimate the contribution of *Archaea* consistently about twofold (19). Thus, the data presented here are conservative in respect to the contribution of bacterial rRNA and the actual contribution of *Archaea* may be even lower.

If it is assumed that rRNA is correlated with microbial activity, most of the microbial activity at the studied hydrothermal vent seems to be present in the first 10-20 mm of the sediments in all zones. This has also been observed by others, although in these cases the decrease was not as steep (18, 22, 23). However, a similar drastic decrease of rRNA has been observed in sediment samples from a deep-sea vent site in Guayamas Basin (17). The analysis of lipids extracted from sediments at a deep-sea hydrothermal vent in the Guayamas Basin also revealed that the active microbial biomass was concentrated in the upper first centimeter and decreased drastically with depth (8). If an average rRNA content of 3 fg $cell^{-1}$ is considered (22), the calculated cell numbers in the upper layers are in the range of total cell counts that have been determined earlier at the same vent (24). In the lower layers their is a discrepancy, which might be related to the fact that a higher percentage of cells was not active and thus contained less rRNA. Interestingly, we found higher concentrations of rRNA in the zones closer to the vent center. This indicates that the standing stock of active prokaryotic cells was higher at these sites than in the outer zones. This is surprising considering that the highest total cell numbers were found in the outer zone (24). This suggests that the percentage of active cells was higher in the inner zones compared to the outer zones, and it might be related to stimulated microbial activities due to the higher temperatures and certain constituents of the hydrothermal fluid.

Based on the hybridization analysis *Archaea* seemed to be of minor importance for the microbial community at this vent site, even in the zones with higher temperatures. This finding was somewhat surprising, but is in line with recent findings by others. As there are *Bacteria* known that can grow at 80-90 °C, but none that can grow above 97 °C, one may argue that the temperature limit where *Archaea* will dominate over *Bacteria* may not have been reached in our investigation. However, in this case one would expect an increase in rRNA at the depth were the temperature seems to be optimal for the *Archaea* that were isolated from this environment, i.e., at around 90-95 °C (14). Even in this layer, however, was the rRNA concentration attributable to *Archaea* very low.Nevertheless, the trend of an increasing contribution of archaeal rRNA with sediment depth in the two inner cores might be seen as an indication for a greater importance of *Archaea* at higher temperatures. The values reported for the lower layers, i.e. 11.9% and 22%, are at the upper range of values reported for sediments so far (18, 22, 23), supporting this assumption. By using whole cell hybridization with the same domain specific probes as used in the present study, it was shown that *Archaea* constituted up to 77% of the microbial community in sulfide structures of a black smoker (calculated from ref. 9). However, this number was found in the supposedly coldest, outer part of the structure, whereas the lowest percentage of *Archaea* (1.3 and 1.4%) was found in the presumably hottest, inner part (data in ref. 9). Because the absolute yield of archaeal rRNA was quite low in the present study, the actual numbers of archaeal cells are also expected to be small. This suggests, that the hyperthermophilic *Archaea* were occupying only a narrow niche. In future studies, it should be

worthwhile to investigate the ecological role of these *Archaea* in more detail, e.g., to reveal the causes of their high diversity (6), despite their apparently low numbers. It would have been interesting to see whether the proportion of *Archaea* would have continued to increase at even higher temperatures. However, the amount of rRNA that we could extract from these layers was too low to make reasonable quantifications. This problem has been noted before by Hugenholtz et al. (12), who used the amplification of 16S rDNA by PCR because the amount of rRNA was too low to evaluate the relative abundance of *Archaea* and *Bacteria* directly. However, the ratio of *Bacteria* to *Archaea* they found for three different hot springs (80, 75, and 1, respectively) are comparable to ours and also suggest that the dominance of *Archaea* in high temperature environments is rather the exception than the rule. In line with this, Guezennec et al. (8) also found that *Archaea* contributed insignificantly to the total microbial biomass at a deep-sea hydrothermal vent in the Guayamas Basin, even in sediments with high *in situ* temperatures, and by using domain specific rRNA probes Jannasch and Wirsen (unpublished, cited in ref. 13) found a circa 10:1 ratio of *Bacteria* to *Archaea* at various locations at the same site.

Overall, the data presented in this paper indicate that the main microbial processes at this hydrothermal vent are occurring in the upper sediment layers and that these processes are principally carried out by *Bacteria* rather than *Archaea*. Furthermore, the hydrothermal activity seemed to stimulate the microbial activity as indicated by the higher prokaryotic rRNA concentrations in the regions more strongly affected by the hydrothermalism. However, more studies are required to investigate the ecological roles of *Archaea* and *Bacteria* in high temperature environments in more detail and to unravel the forces that govern their relative abundances.

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4

Distribution and Diversity of Sulfur-Oxidizing *Thiomicrospira* spp. at a Shallow-Water Hydrothermal Vent in the Aegean Sea (Milos, Greece)

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ABSTRACT

A shallow-water hydrothermal vent system in the Aegean Sea close to the island of Milos (Greece) was chosen to study the diversity and distribution of the chemolithoautotrophic sulfur-oxidizing bacterium *Thiomicrospira*. Cell numbers in samples from different regions around a solitary vent decreased towards the center of the vent (horizontal distribution), as well as with depth (vertical distribution), corresponding with an increase in temperature (from ca. 25 °C to 60 °C) and a decrease in pH (from ca. pH 7 to 5). *Thiomicrospira* was one of the most abundant culturable sulfur-oxidizers and was even dominant in one region. Phylogenetic analysis of *Thiomicrospira* spp. present in the highest most-probable-number (MPN) dilutions revealed that most of the obtained sequences grouped in two new closely related clusters within the *Thiomicrospira* branch. Two different new isolates, i.e., Milos-T1 and Milos-T2, were obtained from high-dilution (10^{-5}) enrichments. Phylogenetic analysis indicated that isolate Milos-T1 is related to the recently described *Thiomicrospira kuenenii* and *Hydrogenovibrio marinus;* isolate Milos-T2 grouped together with the MPN sequences of cluster 2. The predominance of strain Milos-T2 was indicated by its identification in several environmental samples using hybridization analysis of DGGE patterns, and by sequencing of one of the corresponding bands, i.e., ML-1, from the DGGE gel. The results shown in this paper support earlier indications that *Thiomicrospira* species are important members of hydrothermal vent communities.

INTRODUCTION

Hydrothermal vents are located at tectonically active regions all over the world; the most spectacular being those at the deep-sea floor, where very unusual light-independent ecosystems based on chemolithoautotrophic sulfur-oxidizing bacteria (see, e.g., references 16, 17) exist. However, hydrothermal vents have also been found in several shallow-water areas (see, e.g., references 15, 26, 28, 30, and 31). These shallow-water vents differ from their deep-sea counterparts mainly by the presence of light. Accordingly, the biology of these ecosystems is different too; e.g., at shallow-water vents photosynthetic organisms, such as benthic microalgae and cyanobacteria, can be present (see, e.g., references 9, 26).

Offshore the Greek island Milos in the Aegean Sea shallow-water vents were found at depths ranging from the littoral zone down to 115 m (9). A rich macrofauna depending on endosymbiotic bacteria is absent at the Milos vents (9, 29). Until now the only sulfur-oxidizing bacteria (SOB) described for the hydrothermal vents around Milos were thiobacilli and *Achromatium volutans* at the vent sites < 30 m water depth (9) and *Thioploca* at a vent at 46 m water depth (11). These identifications were based on microscopical observations. In case of *Achromatium volutans* and *Thioploca* this is well suited because of the particular morphological features of these organisms. For thiobacilli, however, microscopical identification is more problematic, because the rod-shaped morphology might easily be confused with, e.g., the recently described rod-shaped isolates of *Thiomicrospira* (5, 6).

Members belonging to the genus *Thiomicrospira* are chemolithoautotrophic bacteria, which use reduced sulfur compounds as electron donors and obtain carbon from CO_2 (7, 19). Although *Thiomicrospira* spp. were detected in and isolated from several different environments (see, e.g., references 3 and 34), their ecological importance remained obscure for most habitats. Several isolates were obtained from intertidal mud flats (3, 18, 35, 36). However, in a recent study it was found that *Thiomicrospira* constituted only a minor fraction of the total sulfur-oxidizing bacterial community in this habitat, pointing to a minor role of these microorganisms (4). On the other hand, an analysis by molecular methods, i.e., denaturing gradient gel electrophoresis (DGGE) and sequencing of PCR amplified 16S rDNA fragments, demonstrated that *Thiomicrospira* spp. were dominant community members of hydrothermal vent sites at the Mid-Atlantic Ridge (21).

Here, we present the results of an investigation on the diversity and distribution of *Thiomicrospira* populations at a shallow-water hydrothermal vent off Milos. For this study, a combination of molecular and microbiological methods was used in an attempt to understand the ecological importance of these microorganisms.

MATERIALS AND METHODS

Sampling. Samples were taken in June and September 1996 and in June 1997 at a solitary gaseous hydrothermal vent, located at a water depth of 8 m in Palaeochori (24°31.220' E; 36°40.391' N), a sandy bay in the south-eastern part of the Greek island Milos in the Aegean Sea. The mean composition of the discharged gases from the different seeps was 80.5% CO_2, 1.2% H_2S, 0.8% CH_4 and 0.4% H_2 (10). The reduced hydrothermal fluid has an increased salinity of up to 58‰, compared to 39‰ of the ambient seawater (29).

Sampling was carried out along a transect from the center of the almost circular vent to the surrounding area. Sediment cores were taken with polycarbonate cylinders (36 mm in diameter), by SCUBA divers, along the transect at several distances from the vent center. In June 1996, samples were taken at 10, 123, 165, and 235 cm distance from the vent center, and in September 1996, samples were taken at 30, 117, and 200 cm. The distances for sampling were chosen according to different physicochemical parameters around the vent, e.g., increasing temperature and a decreasing pH towards the center and with sediment depth (25). For the 200- and 235-cm distances, moderate environmental conditions were described (25 °C and pH 7.0 in all layers). For the 117-, 123- and 165-cm distances, the temperature and pH were moderate at the surface (25 °C, pH 6.5), but increased and decreased with depth, respectively (40 °C, pH 5.5 at 3 cm sediment depth). For the cores taken at 117 and 123 cm the surface consisted of a white precipitate, that was 2 to 5 mm thick. This conspicuous white precipitate was formed on the sediment surface and increased in thickness under calm weather conditions. In September 1996, a second core was taken at 117 cm distance, one week later than the other samples, at which time no white precipitate was present. At 10 and 30 cm distance from the center, respectively, temperature values of about 30 °C at the surface and 60 °C at 3 cm sediment depth were measured, while the pH values were between 5.7 at the surface and about 5.0 at 3 cm sediment depth. After sampling, the cores were brought to the shore, sliced immediately, and further processed for MPN counts and DNA extraction.

Subsampling of sediment cores. For all samples taken in June 1996, the following layers were investigated: water directly from above the sediment surface and sediment samples from depths of 0 to 10, 10 to 20, and 20 to 30 mm depth. For samples taken in September 1996, cores were sliced at a finer resolution as follows: water directly from above the sediment surface, and sediment from depths 0 to 5, 8 to 13, and 16 to 26 mm depth. Water from just above the surface was sampled with a syringe. Sediment cores were subsampled by extruding the sediment from the polycarbonate tubes and slicing each core. Dilutions for the MPN series were performed directly after slicing. Subsamples of the same layers for DNA extraction were frozen immediately in liquid N_2 until used in further processing.

MPN counts of sulfur-oxidizing bacteria. The MPN technique was used to estimate the abundance of SOB in the different sub-samples taken in September 1996. Sediment cores were subsampled as described above and serial diluted (1:10 steps) with mineral medium without a substrate. Between every dilution step, the samples were vigorously shaken on a vortex apparatus to dislodge the bacteria from the sediment particles. From each dilution three replicate tubes containing growth medium were inoculated and incubated at their approximate in situ temperature. Mineral medium with 20 mM thiosulfate (added by sterile filtration to the autoclaved medium) as sole electron donor was used. The composition of the mineral medium was (per 1,000 ml of H_2O): 29 g of NaCl, 1 g of $(NH_4)_2SO_4$, 1.5 g of $MgSO_4$ x 7 H_2O, 0.42 g of $CaCl_2$ x 2 H_2O, 0.5 g of K_2HPO_4, 0.7 g of KCl, 0.05 mg of vitamin B_{12}, and 1 ml trace element solution with EDTA (32). Bromthymolblue was added as pH indicator at a concentration of 4 mg $liter^{-1}$. K_2HPO_4 was autoclaved separately and added to the medium after autoclaving.

With the second core taken at a distance of 117 cm, a MPN series, in which the salinity of the medium was increased to 55‰ by adding NaCl, was performed. This salinity was similar to the salinity of the outflowing brine (29). This parallel MPN series was carried out to investigate whether there were SOB specifically adapted to the higher salt concentration.

The cultures were incubated in the dark, to avoid growth of phototrophic bacteria, and at their approximate in situ temperatures (see Fig. 1). Growth was monitored by observing a color change of the pH indicator and by microscopy. The presence of *Thiomicrospira* cells in the MPN cultures was determined by using a *Thiomicrospira*-specific primer set in a PCR with cells taken directly from the cultures (3). The numbers of SOB and *Thiomicrospira* cells were determined by using the MPN index of the American Public Health Association (1) in three parallel determinations.

Isolation and cultivation of bacteria. High-dilution enrichments were performed with samples taken in June 1996 and June 1997, with the intention of obtaining the most abundant SOB. The medium and the culture conditions were the same as for the MPN cultures. Aliquots from the tubes were transferred on solid agar plates, containing the same culture medium and 1% (wt/vol) agar (DIFCO). All known *Thiomicrospira* species and strains isolated so far form intensively yellow colored colonies due to sulfur precipitation. Additionally, they all produce acid. Colonies with these characteristics were chosen for identification with a *Thiomicrospira* specific PCR (3). The obtained positive colonies were transferred at least three times before being considered pure.

Nucleic acid extraction. DNA was extracted from sediment and water samples by the method described by Zhou et al. (37), and modified as described by Sievert et al. (25).

Oligonucleotides used for PCR. The oligonucleotides TMS128F and TMS849R are specific for the 16S rDNA of bacteria belonging to the genus *Thiomicrospira* (3). With these

primers, about 700-bp 16S rDNA fragments are obtained. The primers have been used for the identification of *Thiomicrospira* isolates and for the detection of bacteria belonging to this genus in the MPN cultures. The PCR products obtained from the highest MPN dilutions in which *Thiomicrospira* could be detected were sequenced, to determine the phylogenetic affiliation of the respective organisms.

Oligonucleotides GM3F and GM4R are specific for the 16S rDNA of members of the domain *Bacteria* and were used as primers in a PCR to amplify the nearly complete (1,500 bp) genes of the new isolates. The PCR products obtained with these primers were used for sequencing.

The primer pair GM5F and 907R amplifies the 16S rDNA of members belonging to the domain *Bacteria* and was used to obtain 550-bp fragments for DGGE analysis. The sequences of both primer pairs (GM3F and GM4R, GM5F and 907R) have been published before by Muyzer et al. (21).

PCR amplification of 16S rDNA fragments. PCR amplifications were performed as described by Muyzer et al. (21). A touchdown PCR (12) was performed for primer pair GM5F plus 907R (annealing temperature from 65 to 55 °C in 20 cycles). For primer pair TMS128F plus TMS849R (annealing temperature of 44 °C), and GM3F plus GM4R (annealing temperature of 40 °C) no touchdown PCR was used. Amplification products were analyzed as described by Muyzer et al. (21), before being subjected to further characterization by DGGE analysis or DNA sequencing.

DGGE analysis of PCR products. DGGE was performed with the D-Gene™ system (Bio-Rad Laboratories, Inc.). The protocol as described by Brinkhoff and Muyzer (3) was used: 1 mm thick, 6% (wt/vol) polyacrylamide gels, 1 x TAE electrophoresis buffer (pH 8.3), 20-70% denaturant, and an electrophoresis time of 20 h at a constant voltage of 100 V. After electrophoresis, the gels were stained with ethidium bromide and photographed as described before (22).

Hybridization analysis of blotted DGGE patterns. DGGE patterns were transferred to nylon membranes (Hybond-N$^+$, Amersham, Little Chalfont, United Kingdom) by electroblotting and hybridized with the digoxigenin-labeled *Thiomicrospira*-specific probe TMS849R, as previously described (3).

Sequencing of PCR products. PCR products were purified by using the Qiaquick Spin PCR purification kit (Qiagen Inc., Chatsworth, Calif.). The *Taq* Dyedeoxy Terminator cycle-sequencing kit (Applied Biosystems, Foster City, Calif.) was used to sequence the 16S rDNA fragments. Sequencing primers for the nearly complete 16S rDNA of bacterial isolates were GM3F, GM1F, and GM4R (8). Sequencing primers for *Thiomicrospira* 16S rDNA fragments obtained from MPN cultures with the specific primer pair were the same *Thiomicrospira*-specific oligonucleotides, TMS128F and TMS849R (3). One band in the DGGE

gel, which hybridized with the *Thiomicrospira* specific probe TMS849R, was excised from the gel, reamplified with the primer pair GM5F and 907R, and sequenced with the *Thiomicrospira*-specific primer TMS849R. The sequence reaction mixtures were electrophoresed on an Applied Biosystems 373S DNA sequencer.

Comparative analysis of 16S rRNA sequences. The 16S rRNA sequences were aligned to those obtained from the Ribosomal Database Project (RDP; 20) and GenBank (2). Sequence alignments were prepared with the sequence editor SEQAPP (14). Phylogenetic trees were created using the neighbor joining algorithm with maximum likelihood correction as implemented in the test version of PAUP 4 developed by Swofford.

Nucleotide sequence accession numbers. The sequences obtained in this study are available from EMBL Nucleotide Sequence Database under accession numbers AJ237757 to AJ237769.

RESULTS

MPN counts of SOB and *Thiomicrospira* cells. Numbers of cultivable aerobic chemolithoautotrophic SOB and *Thiomicrospira* cells counted by MPN serial dilution were variable for the different zones and layers (Fig. 1). Where *Thiomicrospira* spp. were present, they accounted for between 2 and 100% of the total numbers of cultivable SOB, which indicates that *Thiomicrospira* spp. belonged to the dominant SOB in this habitat. Generally the numbers of SOB varied between not detectable and 1.4×10^6 cells g (wet weight) of sediment^{-1}. *Thiomicrospira* cell numbers varied between not detectable and 2.7×10^5 cells g (wet weight) of sediment^{-1}.

In the first zone, at 30 cm distance from the vent center, no or only low numbers of SOB up to 2.4×10^2 cells g (wet weight) of sediment^{-1} could be obtained. *Thiomicrospira* cells were not detectable in the MPN cultures of this zone (Fig. 1A).

In the second zone, at a distance of 117 cm from the vent center, the numbers of SOB and *Thiomicrospira* cells were different for the two investigated cores. The surface of the first core (core I, Fig. 1B) contained the white precipitate mentioned above, which was absent at the surface of the second core (core II, Fig. 1C). Core II was used for the MPN counts with the increased salinity. Whereas the cell numbers of SOB obtained for the three sediment layers for core II were only slightly lower than those obtained for core I, the number of *Thiomicrospira* spp. were appreciable lower in the sediment samples of core II than of core I. This is also indicated by the finding that *Thiomicrospira* spp. constituted a much lower fraction of the total numbers of SOB in core II compared to core I. The highest numbers for SOB and *Thiomicrospira* at 117 cm distance were obtained with the sample from the water above the

sediment surface of core I, which contained the white precipitate (4.1 x 10^4 and 1.9 x 10^4 cells ml^{-1} water, respectively [Fig. 1B]).

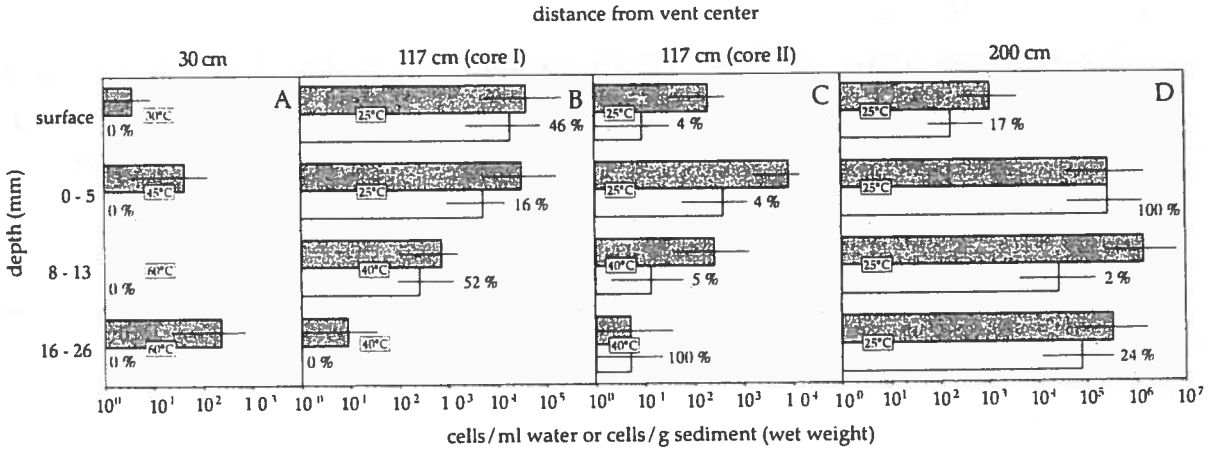

Figure 1. MPN counts of chemolithoautotrophic SOB (dark bars) in sediment samples from the shallow-water hydrothermal vent system off Milos. The *Thiomicrospira* cell number (light bars) were determined by enzymatic amplification of the 16S rDNA using genus-specific primers. The error bars indicate the 95% confidence intervals. Percentages next to the *Thiomicrospira* bar are the *Thiomicrospira* numbers given as percentages of total SOB. The incubation temperatures are also given.

For the third zone, at 200 cm distance from the vent center, the lowest numbers of SOB and *Thiomicrospira* cells were obtained for the water above the sediment surface (Fig. 1D). Highest numbers of SOB (1.4 x 10^6 cells g^{-1}) were found in the second sediment layer (8-13 mm deep), while the highest numbers of *Thiomicrospira* cells (2.7 x 10^5 cells g^{-1}) were found in the first sediment layer (0-5 mm deep). Here *Thiomicrospira* species were also one of the dominant SOB, as they accounted for between 2 and 100% of the total numbers of SOB. Comparison of the different zones revealed generally the highest numbers for SOB and *Thiomicrospira* cells in the sediment layers of the third zone.

Detection of *Thiomicrospira* by DGGE and hybridization analysis. DGGE profiles of 16S rDNA fragments obtained from sediment and water samples of June and September 1996, respectively (Fig. 2A and C), were hybridized with a probe specific for the genus *Thiomicrospira* (Fig. 2B and D). Positive signals were obtained with the bands from two isolates and with bands from samples of different zones and layers. The results for the samples from June 1996 are described first. For the first zone, at 10 cm from the vent center, a signal was obtained for the water above the sediment (Fig. 2B, lane 3). For the second zone (123 cm from the vent center) signals were obtained for the water above the sediment and the first sediment layer (Fig. 2B, lanes 7 and 8). No signals were obtained for the third zone (Fig. 2B, 165 cm from the vent center). For the fourth zone (235 cm) a signal was obtained for the water above the sediment (Fig. 2B, lane 14) and a weak signal for the first sediment layer (Fig. 2B, lane 15).Positive signals were also obtained for the bands from two *Thiomicrospira* isolates and

from samples taken in September 1996. For the first zone, at 30 cm from the vent center, signals were obtained for the first, second, and third sediment layer (Fig. 2D, lanes 4, 5, and 6), but not

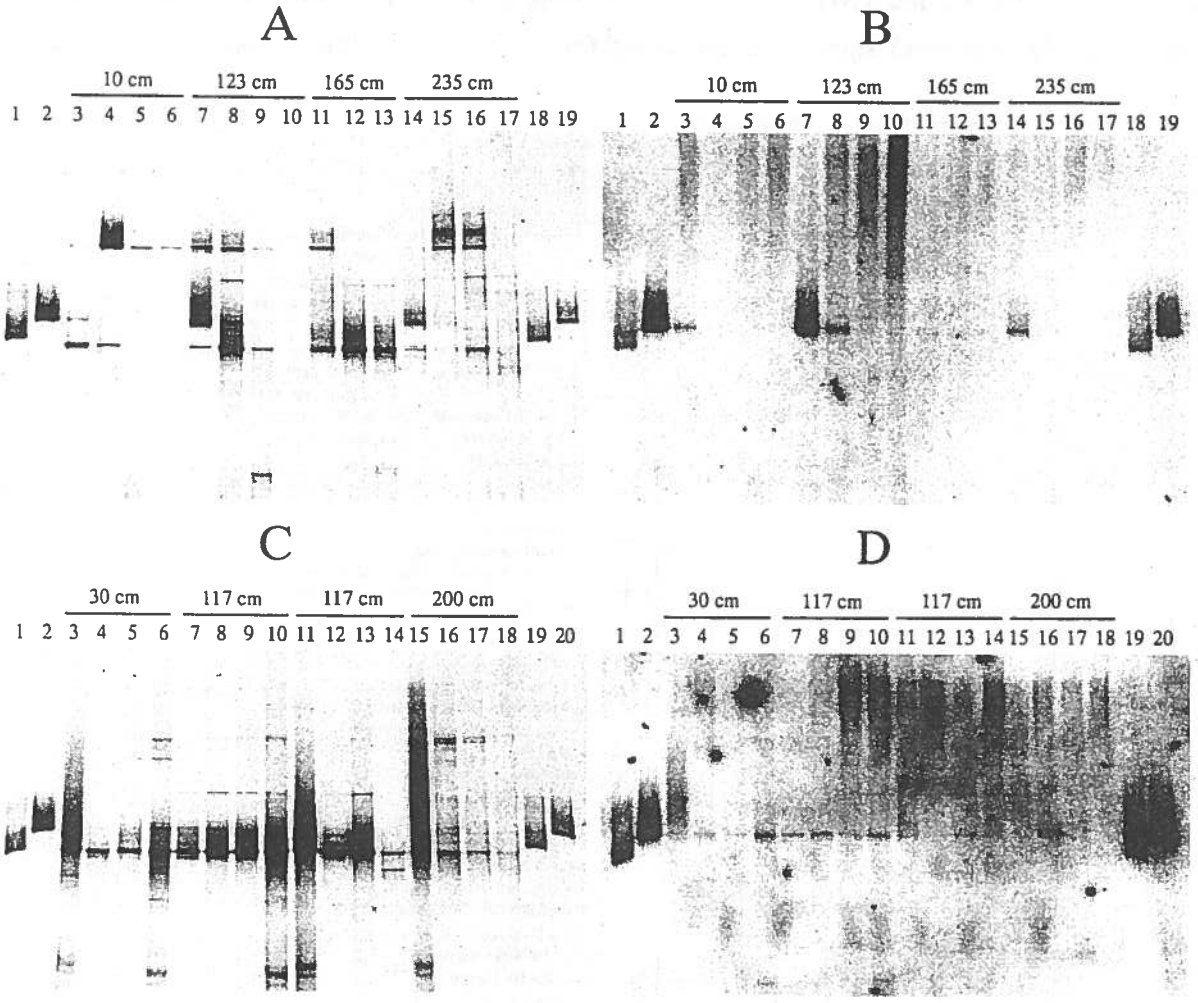

Figure 2. Hybridization analysis of DGGE profiles of 16S rDNA fragments obtained with primers specific for *Bacteria* and template DNA from environmental samples taken along a transect from the shallow-water hydrothermal vent system off Milos and *Thiomicrospira* isolates obtained from the same location. (A) DGGE pattern of samples taken in June 1996. Lanes: 1 and 18, isolate Milos T-1; 2 and 19, isolate Milos T-2; 3 to 6, samples taken at 10 cm distance from the vent center (lane 3, surface; lane 4, 0 - 10 mm deep; lane 5, 10 - 20 mm deep; lane 6, 20 - 30 mm deep); 7 to 10, equivalent samples taken at 123 cm from the vent center; 11 to 13, samples taken at 165 cm from the vent center (lane 11, 0 - 10 mm deep; 12, 10 - 20 mm deep; 13, 20 - 30 mm deep); 14 to 17, equivalent samples taken at 235 cm distance to those taken at 10 cm distance. (B) Hybridization analysis of the pattern in panel A with a *Thiomicrospira* specific digoxigenin-labeled probe. (C) DGGE pattern of samples taken in September 1996. Lanes: 1 and 19, isolate Milos T-1; 2 and 20, isolate Milos T-2; 3 to 6, samples taken at 30 cm from the vent center (lane 3, surface; lane 4, 0 - 5 mm deep; lane 5, 8 - 13 mm deep; lane 6, 16 - 26 mm deep); 7 to 10, equivalent samples taken at 117 cm distance (sediment core number 1); 11 to 14, equivalent samples taken at 117 cm distance (sediment core number 2); 15 to 18, equivalent samples taken at 200 cm distance. (D) Hybridization analysis of the pattern in panel C with the *Thiomicrospira* specific probe.

for the water above the sediment (Fig. 2D, lane 3). For the two cores taken in the second zone (117 cm distance), slightly different results were obtained. For the first core (Fig. 2D, lanes 7-10), signals for the surface (lane 7), and all sediment layers (lanes 8-10) were obtained (although for the second layer [lane 9] only a weak signal was found). For the second core (lanes 11-14), only weak signals were obtained for the surface and the second sediment layer

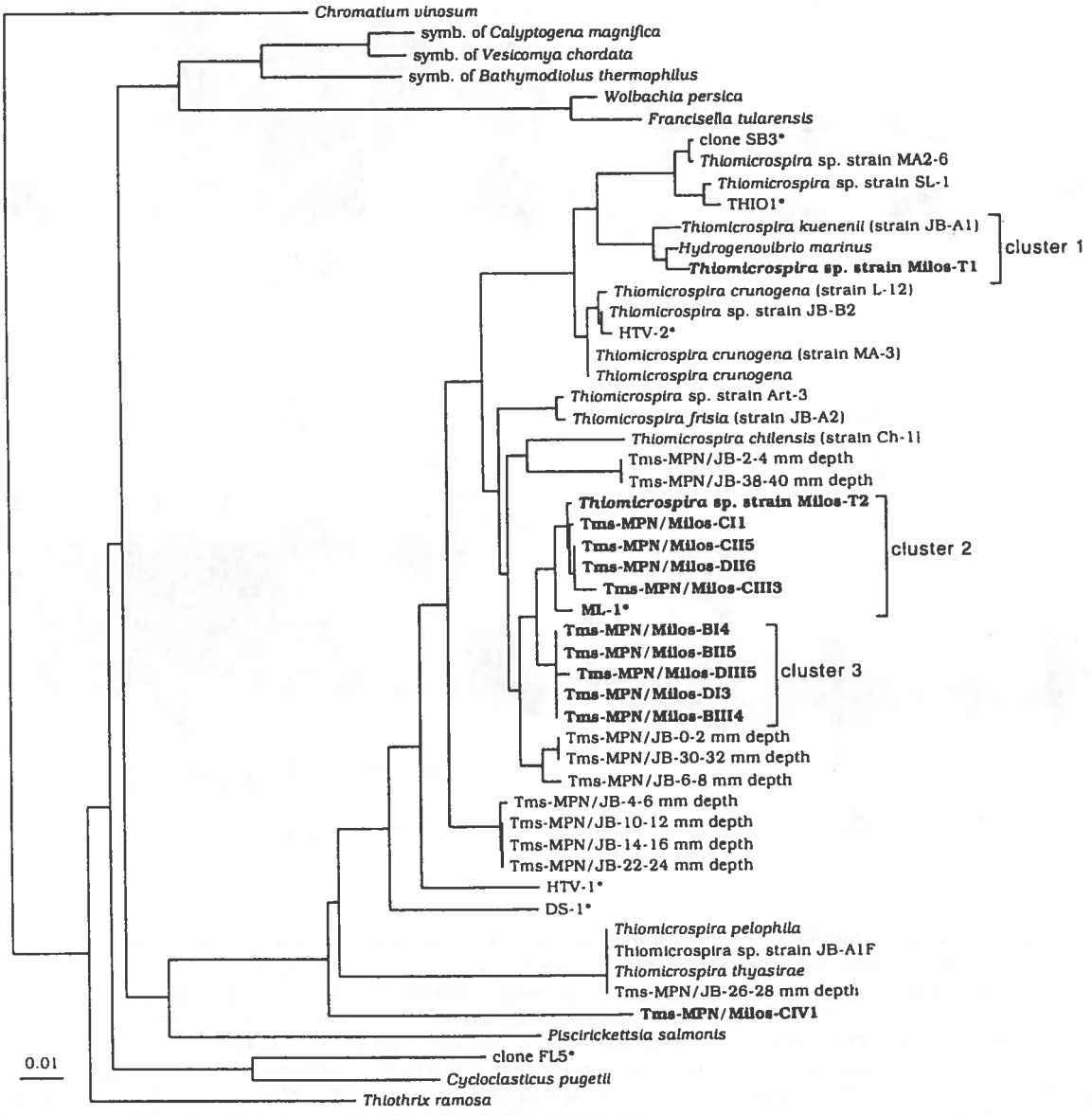

Figure 3. Neighbor joining tree showing the phylogenetic affiliation of the *Thiomicrospira* isolates (strain Milos-T1 and Milos-T-2), and of *Thiomicrospira* spp. present in MPN cultures (indicated by the annotation Tms-MPN/Milos), both from the shallow water hydrothermal vent site near Milos. ML-1 is the sequence from the excised DGGE band, which had the same position in the gel as the band of *Thiomicrospira* sp. strain Milos-T2. Tms-MPN/JB sequences were obtained from *Thiomicrospira* spp. present in MPN cultures of an intertidal mud flat of the Jadebusen bay, Germany, and are published elsewhere (3). The tree is created after comparison of partial (700-nucleotide) 16S rRNA sequences. Sequences marked with an asterisk are molecular isolates. The sequences determined in this study are in bold; A to D refer to the different zones around the vent (30 cm, 117 cm [core I], 117 cm [core II], and 200 cm [Fig. 1]); Roman numerals I to IV refer to the different layers (from surface to 16-26 mm deep); and Arabic numbers refer to the different MPN dilutions.

(lanes 11 and 13, respectively). For the third zone (200 cm from the vent center), a signal was only obtained for the first sediment layer (lane 16). A signal from the water above the sediment might have been obscured by a poor PCR, as indicated by the smear in lane 15 (Fig. 2C). All hybridization signals obtained with the *Thiomicrospira*-specific probe run in both gels at the same position, as the bands belonging to strain Milos T-2 (Fig. 2B, lanes 2 and 19, and Fig. 2D, lanes 2 and 20) which indicates identical or nearly identical sequences.

Phylogenetic analysis of *Thiomicrospira* from MPN cultures. Although the *Thiomicrospira* species from the highest dilutions of the MPN counts performed in September 1996 were not isolated, partial sequences of their 16S rDNA could be determined by using the *Thiomicrospira*-specific primer pair for amplification and sequencing. With the exception of the sequence Tms-MPN/Milos-CIV1 all other sequences from the MPN cultures were very similar (ca. 99%) and grouped in two clusters, i.e., clusters 2 and 3 (Fig. 3). Sequence Tms-MPN/Milos-CIV1, obtained from the MPN culture from the deepest sediment layer (16-26 mm depth) of the second zone (117 cm distance from the vent center) and cultured at 55‰ salinity, was very different from all others and was shown to be the deepest branching *Thiomicrospira* sequence known so far. For one MPN culture (Tms-MPN/Milos-DIV5), a mixed sequence was obtained, indicating that more then one *Thiomicrospira* sequence type was present in this culture.

Characterization of *Thiomicrospira* isolates. Two isolates, which were both obtained from 10^{-5} dilutions of the enrichments, gave a positive signal with the *Thiomicrospira* specific PCR. Strain Milos T-1 was received from a sample taken in June 1996 from the upper sediment layer, underneath the white precipitate, at 123 cm from the vent center. Strain Milos T-2 was isolated directly from the white precipitate in June 1997. While isolate Milos T-1 is vibrio shaped, like most other *Thiomicrospira* spp., strain Milos T-2 is rod-shaped like two recently described new *Thiomicrospira* species, *T. frisia* (5) and *T. chilensis* (6).

Phylogenetic analysis confirmed the affiliation of the isolates to the genus *Thiomicrospira* (Fig. 3). The two new isolates are not closely related to each other and are located in different subclusters of the *Thiomicrospira* lineage. Strain Milos T-1 is related to *Thiomicrospira kuenenii* and *Hydrogenovibrio marinus* (Fig. 3, cluster 1). The latter differs from *Thiomicrospira* spp. only in the ability to use hydrogen as electron donor (23). Strain Milos T-2 grouped with sequences from the MPN cultures and the excised DGGE band ML-1, all obtained during this study (Fig. 3, cluster 2). Band ML-1, which runs in the DGGE gel at the same position as the band from strain Milos T-2, and hybridized with the *Thiomicrospira* specific probe was excised from a DGGE gel and subsequently sequenced. Comparison of the sequence of the excised DGGE band ML-1 with that of strain Milos T-2 resulted in a 1-bp difference out of a total of 470 bp (99.8% sequence similarity).

DISCUSSION

The high abundance of *Thiomicrospira* cells in some zones of the shallow-water hydrothermal vent off the isle of Milos can probably be attributed to the presence of high concentrations of reduced sulfur compounds and CO_2 (10), which both favor the growth of chemolithoautotrophic sulfur-oxidizing bacteria. The distribution pattern of *Thiomicrospira* as determined by the MPN counts probably reflects the influence of environmental parameters, such as temperature, pH, and oxygen concentration. These environmental parameters were determined concurrently along the same transect (25). Temperatures of about 30 to 60 °C, and pH values between 5.7 and 5.0 were measured for the layers of the first zone (30 cm from the center of the vent). This might explain the low numbers of SOB in general and the absence of *Thiomicrospira*.At 117 cm from the center of the vent, the numbers of SOB and *Thiomicrospira* cells were decreasing with depth (Fig. 1B). While the environmental conditions of this zone are moderate at the sediment surface (25 °C, pH 6.5), temperatures of about 40 °C and pH values of 5.5 and lower at the deeper layers, as well as a decreasing oxygen concentration with depth, seemed to inhibit the growth of SOB and *Thiomicrospira*. The numbers of SOB as determined in the MPN count with an increased salinity of 55‰ (Fig. 1C) were slightly lower for the sediment layers, than were those obtained with 30‰ salinity, even though the higher value reflected the in situ concentration of the out flowing brine. This indicates that SOB adapted to a higher salinity were not dominant. The finding that *Thiomicrospira* spp. constituted a lower fraction of the total numbers of SOB in the sediment samples from core II than in those from core I might be an indication that *Thiomicrospira* spp. were more strongly affected by the increased salinity. However, this could also be related to natural variability. The second possibility is supported by the observation, that bands hybridizing with the *Thiomicrospira*-specific probe were less abundant and weaker for the samples from core II as compared to core I (Fig. 2 B and D). The significant lower numbers of SOB and *Thiomicrospira* cells determined for the surface layer of the MPN counts with the increased salinity might be due to the absence of the white precipitate of the respective sample (25).

At 200 cm from the center of the vent, moderate conditions (25 °C, pH 7.0) and a deeper oxygen penetration seemed to allow the presence of higher numbers of SOB and *Thiomicrospira* cells also in deeper layers (Fig. 1D). The predominance of *Thiomicrospira* cells in the first sediment layer of this zone indicates that the environmental parameters favored the growth of members of this genus. It is worth noting, however, that *Thiomicrospira* spp. were not the only SOB present in the highest positive dilutions (24). At 200 cm, *Thiomicrospira* cells were also found in deeper layers in nearly the same order of magnitude as in the upper layers, while oxygen was not present (25). This phenomenon was described earlier for this genus in sediment

of an intertidal mud flat (4). However, in the latter habitat *Thiomicrospira* populations were found to be metabolically active only in the oxic part of the sediment.

Comparison of the results of the hybridization patterns with the MPN results showed differences for the distribution of *Thiomicrospira*, especially for the first zone, i.e., at 10 cm and 30 cm from the vent center. No *Thiomicrospira* cells were detected here with the MPN counts. On the other hand, hybridization analysis of both DGGE patterns gave positive signals for this zone (Fig. 2B and 2D). The physicochemical parameters should actually allow growth of *Thiomicrospira* spp. at the surface of this region like it is indicated by the positive band from the samples taken in June 1996 (Fig. 2B, lane 3). However, the presence of *Thiomicrospira* in the sediment samples taken in September 1996 (Fig. 2D, lanes 4 to 6) is in contradiction to the environmental parameters. It is unlikely that *Thiomicrospira* spp. grew in these layers since the in situ temperature (40-60 °C) was at or above the upper growth limit of known species (7) and oxygen, which is needed as electron donor (7), was not present (25). That these *Thiomicrospira* populations were not detected by the MPN is probably a result of their special adaptation to the extreme environmental conditions that were not imitated in the MPN. This possibility would be supported by the finding of the sequence Tms-MPN/Milos-CIV1, which was obtained from the MPN-cultures with a salinity of 55‰. Its phylogenetic position and the extreme environment where the sequence was obtained from (temperature around 40°, pH 5.0-5.5, no oxygen) might mean, that the corresponding organism has a different physiology compared to known *Thiomicrospira* species. However, the position of the *Thiomicrospira*-positive bands for the samples at 30 cm from the center of the vent were at the same position in the gel as the band for the isolate Milos-T2, which argues against a unique population. Most likely resuspension of the upper sediment layers due to a storm, previous to the sampling in September 1996, was responsible for the occurrence of *Thiomicrospira* populations in deeper sediment layers (25). This would also account for the observation that bands belonging to *Thiomicrospira* spp. were confined to the upper sediment layers and the water above the sediment in June 1996 (Fig. 2B, lanes 3, 7, 8, 14, and 15), which is in agreement with the known physiological capabilities of this genus (7, 19), whereas they were more homogeneously distributed with no clear trend in September 1996 (Fig. 2D).

By using a PCR-DGGE-hybridization assay, it was found that a particular bacterial population representing 0.1% of the total community could still be detected (27). In the present study we could detect *Thiomicrospira* populations, even though their contribution to total cell numbers (determined by acridine orange direct counts [25]) were well below 0.1%. Different explanations, which are not mutually exclusive, could account for this. First, we do not know the extent to which *Archaea* contributed to the total cell counts. Thus, the contribution of *Thiomicrospira* cells to the total bacterial cells might be higher. Second, the association of cells in clumps or with particles, as well as the insensitivity of the detection method (33) might have

lead to an underestimation of the *Thiomicrospira* cell numbers. In addition, it is conceivable that not only one but different and highly related *Thiomicrospira* populations were present in the highest dilutions. Third, a bias towards *Thiomicrospira* might have been caused by the DNA extraction and/or the PCR, i.e., *Thiomicrospira* cells were more efficiently lysed and/or their 16S rRNA might have been specifically over-amplified compared to other sequences. Furthermore, not all cells stained with acridine orange might have contained enough DNA for amplification. The hybridization analysis of DGGE patterns with the environmental samples from June and September 1996 showed that all bands, which gave positive signals, run at the same position in the denaturing gel as those from the new isolated *Thiomicrospira* strain Milos T-2. Based on these data it can be derived that this is apparently the dominant *Thiomicrospira* sequence type of this ecosystem. This was confirmed by the isolation of this organism from a high-dilution enrichment of a sample taken in June 1997 and additionally the sequence similarity of a band excised from a DGGE gel, having 99.8% sequence homology to strain Milos T-2. No bands corresponding to the band of strain Milos T-1 were found in the DGGE patterns, and no similar sequence was obtained from the MPN cultures, even though strain Milos T-1 was also isolated from a high dilution enrichment, like strain Milos T-2. This finding can not be explained by the present data.

Almost all sequences presented in this study show differences among each other lower than 2%. Therefore physiological adaptations to the different zones of the studied vent system might be possible without greater differences in the 16S rDNA. An indication for this is, that all sequences obtained from the MPN cultures of the second zone (117 cm distance), grown at a salinity of 30‰ are identical. On the other hand, most sequences obtained from the MPN counts of the same zone, grown at a salinity of 55‰ formed another cluster. This might reflect an adaptation of specific *Thiomicrospira* populations to a higher salinity. This would be similar to the finding that highly related cyanobacterial populations are adapted to different temperatures according to their occurrence in a thermal gradient along an outflow of a hot spring (13). It was generally observed that the more moderate conditions in the outer zone of the vent correlated with a higher phylogenetic diversity of the obtained *Thiomicrospira* sequences from this zone. This reflects a trend found also for the total bacterial community (25).

Due to the presence of light, chemolithoautotrophic SOB were found to be not the only primary producers of the shallow-water vent system off Milos (9, 29). However, results from this study clearly indicate that even though differences between the deep-sea and the shallow-water hydrothermal vent systems exist, *Thiomicrospira* is in both cases an important member of the sulfur-oxidizing community and has to be taken into account in further ecological and microbiological investigations of these ecosystems.

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Halothiobacillus kellyi sp. nov., a mesophilic obligately chemolithoautotrophic sulfur-oxidizing bacterium isolated from a shallow-water hydrothermal vent in the Aegean Sea and emended description of the genus *Halothiobacillus*

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ABSTRACT

A new chemolithoautotrophic mesophilic sulfur-oxidizing bacterium was isolated from a sediment sample taken from a shallow-water hydrothermal vent in the Aegean Sea with thiosulfate as the electron donor and CO_2 as carbon source. Based on the almost complete sequence of the 16S rRNA gene, strain Milos-BII1^T forms a phylogenetic cluster with *Thiobacillus hydrothermalis, Thiobacillus neapolitanus, Thiobacillus halophilus* and *Thiobacillus* sp. W5, all of which are obligately chemolithoautotrophic bacteria. Because of their phylogenetic relatedness and their physiological similarities it was proposed to transfer these organisms to a new genus within the γ-subdivision of the Proteobacteria, the genus *Halothiobacillus* gen. nov. (Kelly, D. P., and A. P. Wood, Int. J. Syst. Microbiol., in press). The strain described here as Milos-BII1^T represents a new species of this genus, *Halothiobacillus kellyi*. The cells were Gram-negative rods and highly motile. The organism was obligately autotrophic and strictly aerobic. Nitrate was not used as electron acceptor. Chemolithoautotrophic growth was observed with thiosulfate, tetrathionate, sulfur, and sulfide. For strain Milos-BII1^T growth was observed between pH values of 3.5 and 8.5 with an optimum at pH 6.5. The temperature limit for growth was between 3.5 °C and 49 °C, with an optimum growth temperature between 37 °C and 42 °C. Growth occurred between 0 and 2 M NaCl with an optimum NaCl concentration between 400-500 mM. The average maximum specific growth rate on thiosulfate was 0.45 h^{-1} for strain Milos-BII1^T.

INTRODUCTION

The genus *Thiobacillus* contains a wide range of Gram-negative, rod-shaped, colorless sulfur bacteria, which have in common the ability to use reduced sulfur compounds as electron donors for autotrophic growth (15, 19). In contrast to the original description of an obligately autotrophic sulfur-oxidizing organism given by Beijerinck (1, 2) several species growing heterotrophically or mixotrophically were also named *Thiobacillus*, because they could use reduced sulfur compounds as electron donor and produced sulfate as end product. The different physiological properties and the range of DNA base composition (45–70 mol% G + C) already indicated the need for a reclassification of members of this genus. This was even more obvious after the sequence data for the 16S rRNA genes showed that the species of the genus *Thiobacillus* fall into the α-, β-, and γ-subdivisions of the Proteobacteria (19). Since then several species of this genus have been reclassified (10, 14, 23). Because of their phylogenetic relatedness and their physiological similarities, Kelly and Wood (17) proposed to transfer *Thiobacillus hydrothermalis, Thiobacillus neapolitanus,* and *Thiobacillus halophilus* to a new genus within the γ-subdivision of the Proteobacteria, the genus *Halothiobacillus* gen. nov. In this paper we describe a new species of a marine obligate chemolithoautotrophic sulfur-oxidizing bacterium belonging to the genus *Halothiobacillus*, as proposed by Kelly and Wood (17) based on phylogenetic and physiological data. Together with *Thiobacillus hydrothermalis* (9), its closest relative, this is the second example of a chemolithoautotrophic sulfur-oxidizing bacterium isolated from a marine hydrothermal vent system which does not belong to the genus *Thiomicrospira*. In addition the complete 16S rDNA sequence of *Halothiobacillus neapolitanus* (formerly *Thiobacillus neapolitanus*), the type strain of the proposed genus *Halothiobacillus* was determined, because the earlier sequence was incomplete.

MATERIAL AND METHODS

A mineral medium with 20 mM thiosulfate (added by sterile filtration to the autoclaved medium) as sole electron donor was used for enrichment, isolation and routine culture work. The composition of the mineral medium (in g l^{-1}) was: NaCl (29), $(NH_4)_2SO_4$ (1), $MgSO_4$ x 7 H_2O (1.5), $CaCl_2$ x 2 H_2O (0.42), K_2HPO_4 (0.5), KCl (0.7), vitamin B_{12} (0.00005), trace element solution with EDTA (37) (1 ml l^{-1}). Bromothymolblue was added as pH indicator at a concentration of 4 mg l^{-1}. K_2HPO_4 was autoclaved separately and added to the medium after autoclaving.

Isolation of bacteria. Most Probable Number estimates and enrichment cultures were obtained by inoculating medium with 1 cm^3 sediment from a shallow-water hydrothermal vent system located in the Bay of Palaeochori, Milos, Greece. A detailed description of the MPN

counts, the sampling site and the prevailing environmental conditions is given by Sievert et al. (32). The cultures were incubated at 22 °C in the dark to avoid growth of phototrophic bacteria. After growth was obtained, as indicated by a change in the color of the pH indicator and by sulfur deposition, 1 ml was transferred to 10 ml fresh medium. For isolation of pure cultures 0.1 ml of the enrichment cultures were transferred onto thiosulfate agar plates and repeatedly streaked out. Colonies were transferred at least three times to be considered pure.

Growth experiments. Batch cultures were grown in 500 ml flasks containing 100 ml medium on a rotary shaker at 30 °C in the dark. Routine cultivation of the isolates and utilization of different substrates were investigated in 15 ml tubes containing 10 ml mineral medium. Large-scale cultivation was done in 3 l and 20 l glass carboys supplied with 40 mM thiosulfate, in which the pH was monitored by a sterilized pH-electrode (Ingold, Germany) and readjusted by titration with Na_2CO_3 (1 M) through a personal computer program controlling a peristaltic pump. Volker Meyer at the Max-Planck-Institute for Marine Microbiology developed the program.

The maximum specific growth rate in thiosulfate-medium was determined at 22 °C and 37 °C by direct counts (28), increase in optical density (at 420 nm) or protein concentration. Protein was determined by the Coomassie brilliant blue dye binding technique (3) using a Biorad protein assay kit. Growth kinetics on thiosulfate in continuous cultures were determined in self-constructed chemostats. The pH was controlled and adjusted as indicated above. Dissolved oxygen was supplied in excess at 50-100% air saturation. The oxygen concentration was monitored by an autoclavable oxygen electrode (Ingold, Germany) and adjusted through magnetic valves controlled by the same program.

The optimal pH value and the lowest and highest values tolerated by the isolate were determined by using medium adjusted to different initial pH values (3.5 to 10.0). A color change of the pH indicator upon acidification indicated growth. The optimal pH was determined by measuring the oxygen consumption rate at different pH values. The chemostat was equilibrated under substrate limitation at a dilution rate of $D = 0.1\ h^{-1}$ (22 °C, pH 7.0). The addition of substrate was stopped, the dissolved oxygen concentration adjusted to 100% air saturation and the desired pH value of the medium adjusted. Then the cells were supplied with fresh medium at a dilution rate of $D = 0.4\ h^{-1}$ and the oxygen consumption rate was determined. This procedure was carried out for each pH value from 5.0 to 8.0 at 0.5 unit intervals.

The optimal growth temperature of the new isolate was determined in a thermally insulated aluminum block which was heated electrically to +60 °C at one end and cooled to +3.5 °C with a refrigerated circulation thermostat at the other end. The block contained thirty rows of four holes. Thus, samples could be incubated simultaneously at temperature intervals of 1.5 °C with a maximum of four replicates. The temperature limits of growth were established by

screening for acidification for ten days. The optimal growth temperature was determined within 24 hours after inoculation.

The NaCl-requirement and tolerance of the isolates were tested in medium supplied with 20 mM sodium thiosulfate containing varying NaCl-concentrations (in M: 0, 0.2, 0.4, 0.55, 0.8, 1, 2, 2.5, 3) and incubated at 30°C.

Utilization of inorganic electron donors. The ability to oxidize and grow on different reduced sulfur compounds was tested by using the mineral medium described above supplemented with one of the following compounds: thiosulfate (20 mM), tetrathionate (10 mM), sulfite (1, 3, 5, 10, and 20 mM) thiocyanate (1, 3, 5, and 10 mM), elemental sulfur (0.1% (wt/vol)). The sulfite stock solution was prepared in 50 mM EDTA to prevent autoxidation. Substrate utilization and product formation was monitored according to Rethmeier et al. (28). Sulfite oxidation was tested with a solution of Ellman's reagent (5,5'-dithiobis(2-nitrobenzoic acid); DTNB; 1 g l^{-1} in 50 mM potassium phosphate buffer (pH7.0)). DTNB is reduced by sulfite and thiolgroups to a yellow colored product (maximum absorbance at 412 nm). Growth on sulfide was determined by using 0.2% (wt/vol) slush agar gradients over 8 mM sulfide-containing agar plugs (26). Uninoculated controls showed no pH change and no turbidity. Autotrophic growth on hydrogen was tested on solid mineral medium supplied with bicarbonate (30 mM) incubated in a jar containing a gas mixture of 80% hydrogen and 20% air (vol/vol) and in liquid media (27).

Anaerobic growth. The use of nitrate as an electron acceptor in the absence of oxygen was tested under autotrophic and heterotrophic conditions by using bicarbonate (30 mM) buffered medium supplied with 20 mM KNO_3 and prepared anaerobically. Hungate tubes contained 10 ml liquid medium under a 90%/10% (vol/vol) N_2/CO_2 gas phase and the varied electron donors (see above).

Utilization of organic electron donors. Heterotrophic growth was checked in TB medium without pH-indicator supplied with one of the following compounds: fructose (5 mM), glucose (5 mM), formate (20 and 40 mM), acetate (20 mM), pyruvate (10 mM), lactate (10 mM), casamino acids (0.1 and 0.01% (wt/vol)), peptone (0.1 and 0.01% (wt/vol)), and yeast extract (0.1 and 0.01% (wt/vol)). The same additions in mineral medium with bromothymol blue containing 20 mM thiosulfate were used for testing for an inhibitory effect of organic compounds on thiosulfate oxidation.

Ubiquinone analysis. The ubiquinone fraction was isolated, purified, and identified by B. Tindall (DSMZ Identification Service, Braunschweig, Germany) from cells harvested from large scale incubations as described before (4).

DNA base composition. For determining the G+C content, cells were disrupted and the DNA purified on hydroxyapatite (7). The DNA was hydrolyzed with P1 nuclease and the nucleotides dephosphorylated with bovine alkaline phosphatase (22). The resulting

deoxyribonucleosides were analyzed by HPLC according to Tamaoka and Komagata (36). The instrument was calibrated with non-methylated LAMBDA-DNA (Sigma), G+C content 49.86 mol%. The G+C content was calculated from the ratio of deoxyguanosine (dG) and deoxyadenosine (dA) at the DSMZ (Braunschweig, Germany) according to Mesbah et al. (22).

PCR amplification and sequencing of the 16S rRNA gene. To amplify the almost complete 16S rRNA encoding gene (1,500 bp) of strain Milos-BII1 and *Thiobacillus neapolitanus* (Parker strain X), primers GM3F and GM4R (24) were used in a 35-cycle PCR with an annealing temperature of 40 °C. PCR products were purified by using the QIAquick Spin PCR purificartion kit (Qiagen, Inc., Chatsworth, Calif.) as described by the manufacturer. The *Taq* Dyedeoxy Terminator Cycle Sequencing kit (Applied Biosytems, Foster Cit, Calif.) was used to directly sequence the PCR products according to the protocol provided by the manufacturer. The sequencing primers have been described previously (6). The sequence reaction mixtures were electrophoresed on an Applied Biosystems 373S DNA sequencer.

Comparative analysis of 16S rRNA sequences. The 16S rRNA sequences used in this study in addition to the sequence of strain Milos-BII1 and the newly determined sequence of were: *Thiomonas thermosulfata* (U17839), *Thiomonas cuprina* (U67162), *Acidiphilium acidophilum* (D86511), *Thiobacillus thioparus* (M79426), *Thiobacillus thiooxidans* (M79396, M79397, M79398), *Thiobacillus ferrooxidans* (M79404, M79405, M79406), *Thiobacillus caldus* (Z29975), *Thiobacillus tepidarius* (M79399, M79419, M79420), *Thiobacillus perometabolis* (M79399, M79400), *Thiobacillus novellus* (D32247), *Thiobacillus* sp. W5 (X97534); *Thiobacillus halophilus* (U58020); *Thiobacillus hydrothermalis* (M90662); *Thiobacillus neapolitanus* (M79418, M79419,79420). Sequences that were not included in the 16S rRNA sequence data base of the Technical University Munich using the program package ARB (35) were added from databases. The tool ARB_ALIGN was used for sequence alignment. The alignment was checked by eye and corrected manually. Tree topologies were evaluated by performing maximum parsimony, neighbor joining, and maximum likelihood analysis. Only at least 90 % complete sequences were used for the calculation of different trees. Partial sequences were inserted into the reconstructed tree by applying the parsimony criteria without allowing for changes in the overall tree topology.

Nucleotide sequence accession number. The nearly complete 16S rRNA sequences of strain Milos-BII1 and *Halothiobacillus neapolitanus* (formerly *Thiobacillus neapolitanus*) are available from GenBank under the accession numbers AF170419 and AF1731169, respectively.

RESULTS

Isolation of strain Milos-BII1. Pure cultures were obtained by subculturing single colonies of the lowest dilution of the MPN series and from enrichments. After sequencing of the 16S rRNA genes of these isolates it became obvious that they were all identical, but showed less than 93% similarity to all described *Thiobacillus* spp. (see Table 1). Based on their 16S rRNA sequence, several identical strains were obtained from different zones and sediment depths indicating widespread occurrence at the vent site. However, *Thiobacillus* spp. were only isolated from the low dilutions of the MPN, which could also be seen as enrichment cultures. The higher dilutions of the MPN series lead to the isolation of other sulfur-oxidizing bacteria, e. g. *Thiomicrospira* spp. (5). One isolate, Milos B-II1, originating from sediment underlying a white precipitate (sediment layer 0-5 mm) that formed at a specific region around the vent site (32) was used for further characterization. The in situ temperature in this zone increased from 22 °C at the sediment surface to almost 50 °C in 5 cm sediment depth; the in situ pH was about 6-7 at the sediment surface and remained constant at about 5.5 in the sediment (32).

Morphology. Cells of strain Milos-BII1 appeared single or pairwise as motile rods of 0.4-0.6 μm width and 1.2-2.5 μm in length. The isolate was Gram negative and spore-formation was absent.

Growth conditions. The isolate was strictly aerobic and grew autotrophically on thiosulfate, tetrathionate, sulfur, and sulfide, but not on sulfite and thiocyanate. Growth on thiosulfate lowered the pH to 2.8 to 3.0. Thiosulfate was completely oxidized to sulfate, with a recovery of 90% to 99%. Formation of elemental sulfur was observed on solid media and in liquid media. Sulfite was oxidized to sulfate if the concentration in the medium did not exceed 3 mM. EDTA alone showed no toxic effect. In controls without bacteria sulfite was still present as indicated by the reduction of DTNB. No growth occurred in medium supplemented with any of the organic substrates tested. The oxidation of thiosulfate was not inhibited by any of the organic substrates. Nitrate was not used as a terminal electron acceptor. Hydrogen was not used as an electron donor for autotrophic growth. Addition of vitamin B_{12} was not essential for growth. Maximum specific growth rates on thiosulfate at 37 °C and optimal pH were obtained from the average of three different procedural determinations i.e. direct counts, optical density and protein production. Growth in batch cultures with 20 mM thiosulfate showed a maximum specific growth (μ_{max}) of 0.4 h^{-1} at pH 6.5 and 37 °C, whereas the rate at pH 7.0 and 22 °C was 0.25-0.3 h^{-1}. The maximum specific growth in chemostat cultures with 20 mM thiosulfate was estimated from washout kinetics after raising the dilution rate of the culture in steady state from D=0.2 to 0.4. At pH 7.0 and 22 °C the μ_{max} was between 0.25 and 0.3. Under optimal conditions (pH 6.5 and 37 °C) the values for μ_{max} estimated from washout kinetics were in the range of 0.5 to 0.6. The rates were nearly the same for 100% and 20% air saturated medium.

Table 1. Similarity values (in %) of the 16S rRNA sequences of strain Milos-BII1 and other microorganisms originally classified as *Thiobacillus*.

Strain	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15
1. *Tm. perometabolis*	-														
2. *Tm. thermosulfata*	95.0	-													
3. *Tm. cuprina*	93.7	93.8	-												
4. *Tb. thioparus*	87.7	87.7	89.0	-											
5. *Tb.* sp. W5	85.7	83.1	82.9	83.4	-										
6. *Tb. hydrothermalis*	85.3	83.4	83.6	84.3	90.2	-									
7. *Tb. thiooxidans*	83.0	82.3	82.6	83.2	84.1	83.8	-								
8. *Tb. ferrooxidans*	83.8	84.0	84.1	83.9	84.5	84.5	98.2	-							
9. *Tb. caldus*	83.2	82.5	82.7	83.9	83.6	84.5	94.8	96.3	-						
10. *Tb. tepidarius*	83.7	82.8	83.1	83.5	84.3	83.6	90.2	90.7	89.6	-					
11. *Tb. novellus*	83.0	81.1	81.0	80.3	81.1	81.1	80.4	82.6	80.1	82.1	-				
12. *Ap. acidophilus*	83.2	82.7	83.0	82.0	83.7	84.2	83.5	84.4	84.0	82.6	85.3	-			
13. *Tb. halophilus*	85.2	83.1	83.3	84.1	90.1	98.7	83.8	84.3	84.4	83.5	81.0	83.7	-		
14. Strain Milos-BII1	87.5	84.9	85.1	83.5	91.7	93.4	84.2	85.2	85.1	84.4	81.7	84.2	93.0	-	
15. *Tb. neapolitanus*	85.8	83.3	83.5	83.4	98.5	90.8	84.3	85.0	83.8	84.5	81.2	83.7	90.6	92.2	-

Tm: Thiomonas; Tb: Thiobacillus; Ap: Acidiphilium

The pH range for growth on thiosulfate for strain Milos-BII1 was between 3.5 and 8.5. At a pH of 9.0 no growth was observed. The temperature range for growth was between 3.5 and 49 °C, with an optimum between 37 - 42 °C. Growth was determined by acidification of the medium over a period of 10 days. The isolate showed no specific requirement for NaCl (0-2M), although best growth occurred at NaCl-concentrations between 400 and 500 mM. Nevertheless, it should be noted that the medium without NaCl contained at least 40 mM Na^+ from the addition of sodium thiosulfate.

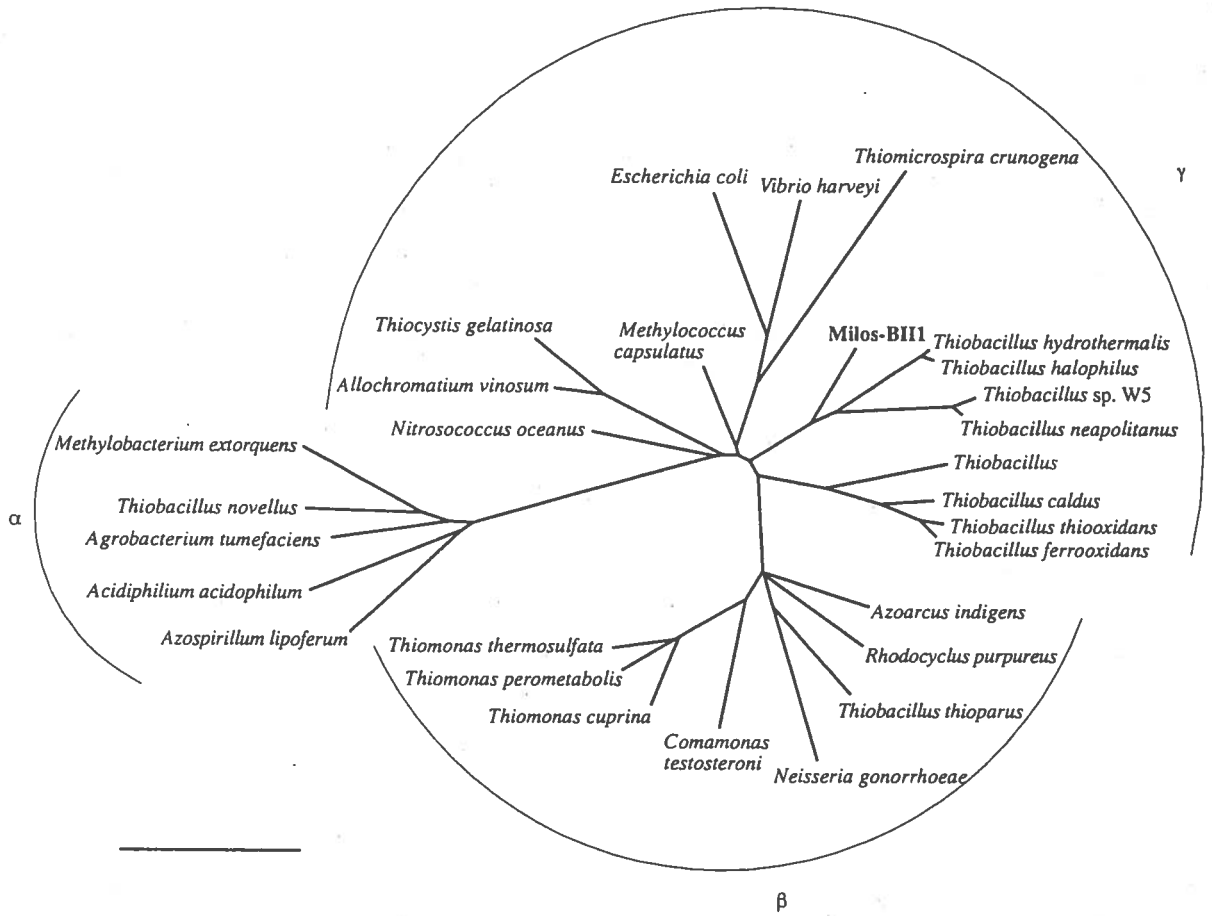

Figure 1. Maximum likelihood tree showing the phylogenetic positions of strain Milos-BII1 and *Halothiobacillus neapolitanus* (Parker strain X). The tree is based on 1,308 positions of nearly full length 16S rRNA sequences from 23 bacteria, with the α-subdivision Proteobacteria as an outgroup. Trees constructed with other tree reconstruction algorithms (neighboor joining and parsimony) resulted in the same overall tree topology. In bootstrap analysis using neighbor joining and parsimony criteria the branching of the proposed Halothiobacillus cluster was supported with values of 100. Partial sequences of *Acidiphilium acidophilum*, *Thiomonas thermosulfata*, *Thiomonas perometabolis*, *Thiobacillus cuprinus*, *Thiobacillus ferroxidans*, *Thiobacillus thiooxidans*, and *Thiobacillus tepidarius* were inserted to the tree by applying parsimony criteria without allowing for changes in the overall tree topologies. The bacteria belonging to the alpha-, beta-, and gamma-subdivison Proteobacteria are indicated by α, β, and γ, respectively. The sequences of *Halothiobacillus* sp. strain Milos-BII1 and *Halothiobacillus neapolitanus* (Parker strain x) have been determined in this study. Scale bar represents 10 substitutions per 100 nucleotides.

DNA base ratio and ubiquinone content. The G + C content for strain Milos-BII1 was 62 ± 0.2 mol%. The isolate contained Q-8 as the only major ubiquinone.

Phylogenetic analysis. The similarity matrix values and the phylogenetic affiliation are shown in Table 1 and Figure 1, respectively. Comparison of the nearly complete 16S rRNA-genes shows that strain Milos-BII1 belongs to the γ-subdivision of the *Proteobacteria* and exhibits less than 93.4% similarity to all other described *Thiobacillus* spp. The closest relative is *H. hydrothermalis* with a value of 93.4%. The new nearly complete sequence of the 16S rRNA gene from *H. neapolitanus* corresponded well to previous results obtained with the incomplete sequence (21).

DISCUSSION

The isolate, strain Milos-BII1 is a chemolithoautotrophic sulfur-oxidizing bacterium. Based on the almost complete sequence of the 16S rRNA gene, strain Milos-BII1 forms a phylo-

Table 2. Morphological and physiological characteristics among *Thiobacillus* spp.[a]

	Strain Milos-BII1	*T. hydrothermalis*	*T. halophilus*	*T. neapolitanus*^{T}	*T.* sp. W5
Shape	rod	rod	rod	rod	rod
Width (μm)	0.4-0.6	0.4-0.5	0.3-0.5	0.3-0.5	0.5
Length (μm)	1.2-2.5	1.2-1.5	1.0-1.2	1.0-1.5	1.0-1.5
Motility	+	+	+	+	+
G+C content (mol%)	62	67.4	64.2	56.0	56
Ubiquinone	Q-8	Q-8	Q-8	Q-8	Q-8
Maximum specific growth rate on thiosulfate(h^{-1})	0.45	0.6	n.d.	0.28	n.d.
Optimum pH	6.5	7.5-8.0	7.0-7.3	6.5-6.9	7.0-7.5
pH range for growth	3.5-8.5	6.0-9.0	n.d.	4.5-8.5	3.5-8.5
Lowest pH produced in thiosulfate medium	2.8	4.8	5.5-6.0	2.8	n.d.
Optimum temperature (°C)	37-42	35-40	30-32	28-32	25-30
Maximum temperature (°C)	48-49	48-49	35-36	39	42
NaCl requirement	-	-	+	-	n.d.
Optimal NaCl concentration (mM)	400-500	430	800-1000	n.d.	n.d.
NaCl tolerance (mM)	2500	2000	4000	>860	n.d.
Obligate chemolithotrophy	+	+	+	+	+
Oxidation of sulfide, sulfur, thiosulfate, and tetrathionate	+	+	+	+	+
Oxidation of thioisocyanate	-	-	-	-	-
Denitrification	-	-	-	-	-

[a] data from: Hutchinson et al. (11,12); Smith and Kelly (33); Kelly and Harrison (15); Durand et al. (9); Wood and Kelly (38); McDonald et al. (21), Visser et al. (37), Kelly et al. (16) and own data. n.d. not determined.

genetic cluster with *Thiobacillus hydrothermalis, Thiobacillus neapolitanus, Thiobacillus halophilus* and *Thiobacillus* sp. W5, which are also obligately chemolithoautotrophic. The similarity values of the 16S rRNA sequences for the new isolate as listed in Table 1 are below 97%. According to the definition of Stackebrandt and Goebel (34), this already indicates that strain Milos-BII1 does not belong to presently described species. Although strain Milos-BII1 shares many physiological properties with *T. hydrothermalis,* there are distinct differences (Table 2). For example, the pH optimum and the lower limit for growth are shifted to lower values. This might indicate an adaptation to a more acidic environment (32). Therefore, we propose that the isolate can be considered as a new species within the aforementioned cluster.

Ecological significance. By its growth characteristics strain Milos-BII1 seemed to be well adapted to the environment from which it was isolated. However, it was apparently not among the dominant sulfur-oxidizing bacteria in this habitat. Strain Milos-BII1 could not be isolated from the highest dilutions of MPN series that showed positive growth. Instead, other sulfur-oxidizing bacteria, e.g. *Thiomicrospira* strains Milos T-1 and T-2 (5), could be isolated. In addition, *Thiomicrospira* populations could be detected in a DGGE analysis of the bacterial community (5), whereas no DGGE bands were found that exhibited the same electrophoretic behavior as the 16S rDNA fragment of strain Milos-BII1 (unpublished data). Results obtained from a competition experiment carried out in a chemostat between strain Milos-BII1 and *Thiomicrospira* strain Milos T-2 indicated that *Thiomicrospira* strain MilosT-2 was superior to strain Milos-BII1 at high dilution rates, i.e. D= above 1.7 h^{-1}. In contrast, at low dilution rates, i.e. low substrate concentrations, strain Milos-BII1 displaced *Thiomicrospira* strain Milos T-2 (unpublished data). In relation to the actual environment this might indicate that the sulfur-oxidizing bacterial community was not limited by substrate availability in situ. This seems reasonable, considering a constant supply of reduced sulfur compounds by the expelled hydrothermal fluid. Another possible explanation might be a limitation of iron or other metals caused by the formation of metal sulfides as a result of high sulfide levels at the vent site. In chemostat studies under iron limiting conditions, *Thiomicrospira pelophila* outcompeted *Thiobacillus thioparus* (18). Both observations could also provide an explanation for the prevalence of *Thiomicrospira* at hydrothermal vent systems in general (5, 13, 24, 30, 31). However, more studies are required to confirm these hypotheses, e.g. by varying other environmental relevant parameters in the chemostat and by enumeration of the respective cells with culture independent techniques such as fluorescent in situ hybridization (FISH). Since both organisms fall into coherent phylogenetic clusters the design of specific probes should be feasible. It is interesting to note that at the hydrothermal vent system in the Fiji Basin, both *Thiobacillus hydrothermalis* and *Thiomicrospira* spp. were present (8).

Similarities between *T. neapolitanus*, *T. hydrothermalis*, *T. halophilus* and strain Milos-BII1. As already has been shown in previous publications about the

taxonomic position of various *Thiobacillus* spp., there is a high level of physiological and phylogenetic similarity between *T. neapolitanus*, *T. hydrothermalis*, and *T. halophilus* (16, 21). As shown in Figure 1, all these organisms form a monophyletic group within the γ-subdivision of the Proteobacteria as supported by a bootstrap value of 100% and can clearly be distinguished from other strains of the genus belonging to the same subgroup or to the α- and β-subdivisions of the Proteobacteria (21). The identity of the different species was confirmed by DNA-DNA hybridization for organisms showing a high level of similarity on the 16S rRNA gene level (16). The organism described in this paper and another obligate chemolithoautotrophic sulfur-oxidizing bacterium tentatively named *Thiobacillus* sp. W5 (36) are phylogenetically and physiologically closely related to these species (see Table 1 and Table 2), although some data and a formal description of the latter is missing. Combining the available physiological data about all species belonging to this cluster, it becomes obvious that they share several properties. They are all motile, obligately chemolithoautotrophic sulfur-oxidizing bacteria containing ubiquinone Q-8. A very unusual feature compared to other *Thiobacillus* spp. is a NaCl-tolerance of all species and a NaCl-requirement of some species. *H. neapolitanus* has not been shown to have a strict NaCl-requirement, but it tolerates high NaCl concentration of more than 860 mM. The available data in this respect for *Thiobacillus* sp. W5 are missing. Together with the phylogenetic analysis of the aforementioned organisms the physiological data suggest that a revision of the genus *Thiobacillus*, as proposed by Kelly and Wood (17) is needed to clarify the confused taxonomic situation. The new nearly complete 16S rDNA sequences confirm the results already obtained by McDonald et al. (21) and Kelly et al. (16). The proposal by Kelly and Wood (17) for the new genus *Halothiobacillus* to accommodate the halotolerant obligate chemolithoautotrophic organisms *Thiobacillus neapolitanus*, *Thiobacillus halophilus*, and *Thiobacillus hydrothermalis* is justified with respect to our data. Their choice, leaving *Halothiobacillus neapolitanus* (NCIMB 8539, DSM 581) as the type species of the genus *Halothiobacillus* is well suited. It was the first one described and the subject of many early studies of the oxidation of reduced sulfur compounds by autotrophic organisms. In addition, historical reasons should be considered, because Alexander Nathansohn (25) isolated a very similar organism from the Bay of Naples, Italy. This species is well suited to reflect the halotolerance of most species of this genus.

Emended description of the genus *Halothiobacillus* (as proposed by Kelly and Wood [17]). The cells of all *Halothiobacillus* species are rod shaped, 0.3-0.6 μm in diameter and 1.0-2.5 μm in length. They stain Gram-negative occur single or in pairs and are motile. Spore formation is absent. All members of the genus are strictly aerobic and grow lithoautotrophically with thiosulfate, tetrathionate, sulfur, and sulfide, but not with thiocyanate as electron donor, and carbon dioxide as carbon source. Heterotrophic growth was never observed. Addition of acetate can increase the amount of biomass produced, if a reduced sulfur

compound is provided as electron donor. Sulfate is the end product of sulfur compound oxidation, but sulfur, sulfite or polythionates may be accumulated, sometimes transiently, by most species. During growth on reduced sulfur compounds the pH decreases from neutrality to a pH as low as 2.5 to 3.0 depending on the species. Optimal growth occurs between pH 6.5 and 8.0 at a temperature of 30 to 42 °C. The optimal NaCl-concentration for growth is 400-500 mM for most strains, but many tolerate much higher concentrations. A requirement for NaCl is found for some species. Carbon dioxide is fixed by means of ribulose bisphosphate carboxylase. Nitrate can be reduced to nitrite, but is not reduced further. On thiosulfate agar, cells produce small white to yellowish, smooth, entire colonies (1-3 mm in diameter) in which sulfur is deposited and acid is produced. Ubiquinone Q-8 is present in the respiratory chain. The G+C content of the DNA of members of this genus varies between 56 to 67 mol%. As determined by a 16S rRNA gene sequence analysis the genus *Halothiobacillus* belongs to the γ-subdivision of the Proteobacteria. Members of the genus can be isolated from freshwater, soil and frequently from marine environments. Traditional enrichment culture techniques seem to favor the isolation of members of this genus, because of their higher acid tolerance compared to other marine sulfur oxidizing bacteria, like *Thiomicrospira* spp. The type species is *Halothiobacillus neapolitanus* (formerly *Thiobacillus neapolitanus,* strain NCIMB 8539 (DSM 581; Parker strain X).

Description of *Halothiobacillus kellyi*. *Halothiobacillus kellyi* (kel´ly.i. M. L. gen. n. *kellyi* of Kelly; named after Donovan. P. Kelly, a British microbiologist who has made important contributions to research on sulfur-oxidizing bacteria and their physiology). Cells are Gram-negative, motile, and rod shaped (0.4-0.6 x 1.2-2.5 μm). *Halothiobacillus kellyi* is strictly aerobic and grows autotrophically on thiosulfate, tetrathionate, sulfur, and sulfide, but not on thiocyanate. Sulfite is oxidized to sulfate, if the concentration does not exceed 3 mM. The organism does not grow heterotrophically. When thiosulfate is used as the primary energy source a transient formation of sulfur occurs. During growth on reduced sulfur compounds the pH decreases from neutrality to around 2.8. Thiosulfate is completely oxidized to sulfate. Autotrophic growth on thiosulfate occurs between pH 3.5 and 8.5 and at a temperature of 3.5 to 49 °C; optimum growth occurs at pH 6.5 and at 37 to 42 °C. The optimal NaCl-concentration for growth is 0.4 to 0.5 mM; growth is possible between a NaCl concentrations of 0 and 2 M. Nitrate is not used as terminal electron acceptor. The average maximum specific growth rate on thiosulfate was 0.45 h^{-1} for strain Milos-BII1^T. On thiosulfate agar, cells produce white to yellowish, smooth, entire colonies (diameter on 1.2% (wt/vol) agar is 1-4 mm) in which sulfur is deposited and acid is produced. Ubiquinone Q-8 is present in the respiratory chain. The G + C content of the DNA is 62 mol%. As determined by a 16S rRNA gene sequence analysis, *Halothiobacillus kellyi* belongs to the γ-subdivision of the Proteobacteria and is closely related to other members of this genus. The strain Milos-BII1 was isolated from a marine shallow water hydrothermal vent system and is deposited at the Deutsche Sammlung von Mikroorganismen

und Zellkulturen GmbH under number 13162, which is the type strain of the species. The GenBank accession number for the nearly complete 16S rRNA gene sequence of *H. kellyi* is AF170419.

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Novel sulfur-oxidizing bacteria from a shallow submarine hydrothermal vent, most closely related to obligate symbionts of invertebrates

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ABSTRACT

Five strains of sulfur-oxidizing bacteria, Milos-OAII2, Milos-OBII5, Milos-ODIII6, Milos-NDII1.1 and Milos-NDII1.2, isolated from MPN cultures at a shallow-water hydrothermal vent in the Aegean Sea are described. All are obligate chemolithoautotrophs, have pH-optima in the neutrophilic range, and grow optimally at marine NaCl-concentrations. They have distinct temperature optima which might reflect an adaptation to the habitat from which they were isolated. Some of the isolates originate from the 10^{-5}-10^{-6} dilutions and thus are likely to be of ecological relevance. Three of the isolates are capable of coupling the oxidation of thiosulfate with the reduction of nitrate to N_2. Based on their 16S rRNA sequences these isolates belong to the γ-subclass of the Proteobacteria. They do not have close relatives among cultured or other free-living sulfur-oxidizing bacteria and thus could not be assigned to recognized groups. However, based on the 16S rRNA sequence the isolates represent the closest free-living relatives of the endosymbionts of *Riftia pachyptila* and other vestimentiferan tubeworms and of several bivalve molluscs. The data suggest, that the isolates and the endosymbionts had a common ancestor and thus provide further evidence that the endosymbiosis was established by a sulfur-oxidizing bacterium. Because of their phylogenetic and physiological similarities the new isolates might be suitable model organisms for physiological and genetical investigations, which are not yet possible with the endosymbionts.

INTRODUCTION

Chemolithoautotrophic sulfur-oxidizing bacteria (SOB) are thought to be the main producers of organic matter at deep-sea hydrothermal vents (26) and also contribute to the production of organic matter at shallow-water hydrothermal vents (4, 10, 52, 56), where light allows primary production through photosynthesis. SOB live either at the interface between the anoxic hydrothermal fluid and oxic seawater (26) or in symbiosis with animals (9, 16, 39). At present, the only validly described chemolithoautotrophic free-living SOB's from marine hydrothermal environments are *Thiomicrospira* spp. (27, 49, 61) and *Thiobacillus hydrothermalis* (15). In addition, ecophysiological studies have also been possible without cultivation with the filament-forming sulfur-oxidizing bacteria of the genus *Beggiatoa* (21, 34, 41). Although the symbionts resisted cultivation, several studies have investigated their physiology (17, 25, 39), biochemistry (33, 39), and phylogeny (12-14, 18, 28, 30). Physiological studies showed that the investigated endosymbionts are chemoautotrophes, fixing CO_2 via the Calvin-Benson cycle and carrying out the energy-conserving oxidation of sulfite to sulfate via the adenylylphosphosulfate (APS)-pathway (39). Furthermore, there is evidence that endosymbiotic bacteria of *Riftia pachyptila* respire nitrate and possibly nitrite in addition to oxygen (24, 25, 33). Furthermore, nitrate respiration has also been demonstrated in the endosymbionts of the bivalve species *Lucinoma aequizonata* and *Solemya reidi* (23, 60), as well as in the ectosymbionts of stilbonematide nematodes (22). Phylogenetic analysis revealed that all putative sulfur-oxidizing endosymbionts form two monophyletic clusters in the γ-subdivision of the *Proteobacteria* (12, 13, 38, 46). To date, it has not been possible to determine the evolutionary origin of the endosymbionts and it seems that the symbiosis evolved independently in different host lineages (12). In some hosts, the symbionts are transmitted vertically (6, 29, 44), while others, including *Riftia pachyptila*, are dependent on the reinfection of larvae from the environment in each generation (7, 19, 20, 31). This indicates, that there must be a stock of free-living SOB that can potentially form a symbiosis (37). *Thiomicrospira* species were suggested to be the closest free-living relatives (12, 13, 38, 45, 46). However, *Thiomicrospira* species do not use the APS-pathway to oxidize reduced sulfur compounds (39) and, with the exception of the wrongly classified *Thiomicrospira denitrificans* (38), cannot reduce nitrate (2). This indicated that there must be other free-living, chemoautotrophic, sulfur-oxidizing bacteria that can potentially form a symbiosis.

In this publication we report on the isolation and characterization of several new types of SOB from a shallow-water hydrothermal vent. These isolates were obtained from MPN dilution series to estimate the number of SOB in different regions at the vent site (4, 52). Some of the isolates originate from the 10^{-5}-10^{-6} dilutions and thus are likely to be of ecological relevance. Phylogenetically all isolates are members of the γ-subdivision proteobacteria and do not have

close relatives among presently cultivated bacteria. However, based on the 16S rRNA sequence the isolates represent the closest free-living relatives of the endosymbionts of vestimentiferan tubeworms including *Riftia pachyptila* and of several bivalve molluscs. Furthermore, some isolates have the capability to oxidize thiosulfate anaerobically and express enzymes indicative of the APS-pathway. The data presented here suggest that the isolates and the endosymbionts had a common ancestor and thus provide further evidence that the endosymbiosis was established by a sulfur-oxidizing bacterium.

MATERIAL AND METHODS

Isolation of bacteria. Most probable number (MPN) cultures were obtained by inoculating mineral medium containing 20 mM thiosulfate (added by sterile filtration to the autoclaved medium) with 1 cm^3 sediment or water from a shallow-water hydrothermal vent system located in the Bay of Palaeochori, Milos, Greece. Subsequently, the sample was serially diluted (1:10 steps) in the same medium. Besides thiosulfate, the medium contained [in g l^{-1}]: NaCl [29], $(NH_4)_2SO_4$ [1], $MgSO_4$ x 7 H_2O [1.5], $CaCl_2$ x 2 H_2O [0.42], K_2HPO_4 [0.5], KCl [0.7], vitamin B_{12} [0.00005], trace element solution with EDTA [1 ml l^{-1}] (59). Bromothymolblue was added as pH indicator at a concentration of 4 mg l^{-1}. K_2HPO_4 was autoclaved separately and added to the medium after autoclaving. Besides aerobic MPN cultures, anaerobic MPN cultures were prepared under strict anaerobic conditions with the same medium as described above, but with NO_3 (10 mM) as electron acceptor and buffered with 15 mM $NaHCO_3$. The cultures were incubated at their approximate in situ temperature in the dark to avoid growth of phototrophic bacteria. For isolation of pure cultures 0.1 ml of the MPN cultures were transferred onto thiosulfate agar plates. Colonies were transferred at least 3 times to be considered pure. However, some strains resisted cultivation on solid media. In these cases, the isolation was carried out by dilution to extinction and repeated transferring of the highest positive dilution. Pure cultures of denitrifiers were obtained by repeated use of deep agar dilution series (59). A detailed description of the MPN procedure, the sampling site and its environmental conditions is given elsewhere (52).

Growth experiments. Batch cultures were grown in 500 ml flasks containing 100 ml medium on a rotary shaker at 30 °C in the dark. Routine cultivation of the isolates and utilization of different substrates were investigated in 15 ml tubes containing 10 ml mineral medium. Large scale cultivation was done in 3 l and 20 l glass carboys supplied with 40 mM thiosulfate, in which the pH was monitored by a sterilized pH-electrode (Ingold, Germany) and readjusted by titration with Na_2CO_3 (1 M) through a personal computer program controlling a peristaltic pump. The program was developed by Volker Meyer at the Max-Planck Institute for Marine Microbiology.

Estimate of the optimal pH value and the lowest and highest values tolerated by the isolate were determined by using medium adjusted to different initial pH values.

The optimal growth temperatures of the new isolates were determined in a thermally insulated aluminum block which was heated electrically to +60 °C at one end and cooled to +3.5 °C with a refrigerated circulation thermostat at the other end. The block contained thirty rows of four holes, so that samples could be incubated simultaneously at temperature intervals of 1.5 °C with a maximum of four replicates. The temperature limits of growth were established by screening for acidification for ten days. The optimal growth temperature was determined within 24 hours after inoculation.

The NaCl-requirement and tolerance of the isolates were tested in medium supplied with 20 mM sodium thiosulfate containing varying NaCl-concentrations (in M: 0, 0.2, 0.4, 0.55, 0.8, 1, 2, 2.5, 3) and incubated at 30 °C for 10 days.

Utilization of inorganic electron donors. The ability to oxidize and grow on different reduced sulfur compounds was tested by using the mineral medium described above supplemented with one of the following compounds: thiosulfate (20 mM), tetrathionate (10 mM), sulfite (3, 5 or 20 mM), thiocyanate (3, 5 or 20 mM), elemental sulfur (0.1% (wt/vol)). Substrate utilization and product formation was monitored according to Rethmeier et al. (48). Growth on sulfide was determined by using 0.2% (wt/vol) slush agar gradients over 8 mM sulfide-containing agar plugs (40). Uninoculated controls showed no pH change and no turbidity. Autotrophic growth on hydrogen was tested on solid mineral medium supplied with bicarbonate (30 mM) incubated in a jar containing a gas mixture of 80% hydrogen and 20% air (vol/vol) and in liquid media (42).

Anaerobic growth. The use of nitrate as an electron acceptor in the absence of oxygen was tested under autotrophic and heterotrophic conditions by using bicarbonate (30 mM) buffered medium supplied with 20 mM KNO_3 and prepared anaerobically. Hungate tubes contained 10 ml liquid medium under a 90%/10% (vol/vol) N_2/CO_2 gas phase and the various electron donors (see below). The consumption of nitrate and thiosulfate and the corresponding formation of intermediate nitrogen species, e.g., nitrite, and of sulfate was followed by HPLC (column RT 100-4,6 Polyspher® IC AN-1). The anions were eluted at a flow rate 1.3 ml min^{-1} at a temperature of 35°C and were detected with an indirect UV-detection system at 254 nm. The eluent had a pH of 4.0 and consisted of 1.5 mM phthalic acid, 1.38 mM Tris, and 300 mM boric acid. For the determination of the gaseous end product of nitrate reduction Hungate tubes with a helium gas phase were used. The gas phase was analyzed with a gas chromatograph.

Utilization of organic electron donors. Heterotrophic growth was checked in mineral medium without pH-indicator supplied with one of the following compounds (27): glucose (5 mM), acetate (20 mM), succinate (5mM), casamino acids (0.1 % wt/vol), peptone and yeast extract (0.1 % wt/vol each), and marine medium 2216E (full strenght and 1/100). The

same additions in mineral medium with bromothymol blue containing 20 mM thiosulfate were used for testing for an inhibitory effect of organic compounds on thiosulfate oxidation.

Ubiquinone analysis. The ubiquinone fraction was isolated, purified, and identified by B. Tindall (DSMZ Identification Service, Braunschweig, Germany) from cells harvested from large scale incubations as described before (3).

DNA base composition. For determining the G+C content, cells were disrupted and the DNA purified on hydroxyapatite (8). The DNA was hydrolyzed with P1 nuclease and the nucleotides dephosphorylated with bovine alkaline phosphatase (35). The resulting deoxyribonucleosides were analyzed by HPLC according to Tamaoka and Komagata (55). The instrument was calibrated with non-methylated LAMBDA-DNA (Sigma), G+C content 49.86 mol%. The G+C content was calculated from the ratio of deoxyguanosine (dG) and deoxyadenosine (dA) at the DSMZ (Braunschweig, Germany) (35).

Enzyme assays. The enzymes sulfite:ferricytochrome-c oxidoreductase (E.C. 1.8.2.1) and APS-reductase (adenosine phosphosulfate-reductase; E.C. 1.8.99.2) were determined for strains Milos-OAII2, Milos-ODIII6, and *Thiomicrospira* sp. strain Milos-T2 as described previously (32, 36). In both assays ferricyanide ($K_3Fe(CN)_6$; 1 mM) served as electron acceptor. Protein was determined by the Coomassie brilliant blue dye binding technique (1) using a protein assay kit (Bio-Rad, Hercules, Calif.).

PCR amplification and sequencing of the 16S rRNA gene. To amplify the almost complete 16S rRNA encoding gene (1,500 bp) of strains Milos-OBII5, Milos-ODIII6, Milos-OAII2, Milos-NDII1.1, and Milos-NDII1.2, primers GM3F and GM4R (38) were used in a 35-cycle PCR with an annealing temperature of 40 °C. PCR products were purified by using the QIAquick Spin PCR purificartion kit (Qiagen, Inc., Chatsworth, Calif.) as described by the manufacturer. The *Taq* Dyedeoxy Terminator Cycle Sequencing kit (Applied Biosytems, Foster Cit, Calif.) was used to sequence the PCR products according to the protocol provided by the manufacturer. The sequencing primers have been described previously (5). The sequence reaction mixtures were electrophoresed on an Applied Biosystems 373S DNA sequencer.

Phylogenetic analysis. DNA extraction, PCR amplification and sequencing of 16S rRNA encoding genes was performed as described previously (3, 38). Comparative sequence analysis was performed using the ARB software program (54). Sequences were aligned automatically using the ARB_ALIGN tool and corrected manually when needed. Tree topologies were evaluated by performing maximum pasimony, neighbor joining, and maximum likelihood analyses. Only nearly complete 16S rRNA sequences were used for the analysis. Nucleotide sequences of strains Milos-OBII5, Milos-ODIII6, Milos-OAII2, Milos-NDII1.1, and Milos-NDII1.2 have been deposited at GenBank under the accesion numbers AF170421, AF170422, AF170423, AF170424, and AF181991, respectively.

Naming of strains. The isolated strains were named with a code indicating the medium from which it was isolated (O = aerobic medium with O_2 as electron acceptor; N = anaerobic medium with nitrate as electron acceptor), location of the sample along the transect from the center of the vent to the surrounding area (A = 30 cm; B = 117 cm; D = 200 cm), the sediment horizon (I = water above sediment surface; II = sediment layer between 0 and 5 mm; III = sediment layer between 8 and 13 mm), and the MPN dilution from which it was isolated.

RESULTS

Isolation of strains. Pure cultures were obtained by subculturing the terminal positive dilutions of the aerobic and anaerobic MPN series. Several strains could be isolated, whose name, isolation source, and various other growth parameters are depicted in Table 1.

Table 1. Physiological characterization of sulfur-oxidizing isolates.

Strain	Milos-NDII1.1	Milos-NDII1.2	Milos-OAII2	Milos-OBII5	Milos-ODIII6
Isolated on (medium)	anaerobic	anaerobic	aerobic	aerobic	aerobic
Isolated from:					
distance from vent center	200 cm	200 cm	30 cm	123 cm	200
sediment layer	0-5 mm	0-5 mm	0-5 mm	0-5 mm	8-13 mm
Dilution factor	10^{-1}	10^{-1}	10^{-2}	10^{-5}	10^{-6}
Temperature range (°C)	8.6 - 44	8.5-39.7	13 - 44	8.6 - 46	8.6 - 37
Temperature optimum (°C)	37.5 – 39.7	33 - 35	37.5 – 39.7	39 - 42	31 - 33
NaCl range (M)	0.2 - 1	0.2 - 1	0.2 - 1	0 - 2	0 - 1
NaCl optimum (M)	0.45	0.45	0.45	0.45	0.45
lowest pH	6.1	5.4	5.5	5.3	6.1
pH optimum	7-8	7-8	7-8	7-8	7-8
Cell size (μm)	0.3 x	0.7 x	0.3 x	0.4 x	0.7 x
	2-3	0.9-1.3	1.5-3.5	1.5-2.6	1.5
Morphology	rod	rod	rod	rod	rod
Motility	-	+	+	+	-
Gram staining	-	-	-	-	-
Ubiquinone Q8	+	n.d.	+	+	+
G+C (mol %)	50.1	n.d.	62.9	63.9	62.5
Nitrate reduction with thiosulfate	+	+	+	n.d.	+
APS reductase activity	n.d.	n.d.	+	n.d.	+
sulfite:ferricytochrome-c oxidoreductase activity	n.d.	n.d.	+	n.d.	+
Precipitation of S^0 when growing on thiosulfate	-	+	+	-	+
Autotrophic growth with:					
Sulfide	+	+	+	(+)	+
Sulfur	(+)	+	(+)	(+)	(+)
Thiosulfate	+	+	+	+	+
Tetrathionate	+	+	+	+	+
Sulfite	-	-	-	-	-
Thiocyanate	-	-	-	-	-
H_2/O_2	-	-	-	-	-
Nitrate reduction with thiosulfate	+	+	+	-	-
Heterotrophic growth	-	-	-	-	-

n.d. not determined, (+) slow growth

Initially the positive cultures were transferred three times in fresh liquid media before isolation attempts were started. In cases where growth on solid media was possible, colonies were transferred three times to be considered pure. When growth on solid media was not possible pure cultures were obtained by repeated transfers of the highest positive dilutions of dilution to extinction series. With the anaerobic cultures two successive deep-agar dilution series were carried out. At this stage only one colony type could be observed. Individual colonies were picked with drawn sterile Pasteur pipettes and transferred in fresh medium. Gas production due to denitrification was evident by the formation of gas bubbles in the agar.

Morphology. The various isolates differed in morphology and size (Table 1; Fig. 1). In some cases, i.e. with the isolates Milos-NDII1.2 and Milos-ODIII6, it could be observed that sulfur globules were associated with the cell (Fig. 1). At present it is not clear whether the sulfur occurs extracellularly or inside the cell. All isolates were Gram-negative and spore-formation was absent.

Growth conditions. All strains had pH optima in the neutral range (Table 1). Marine sodium chloride concentrations supported optimum growth (Table 1). The isolates were either strictly aerobic or faculativeley anerobic with nitrate as electron acceptor (Table 1). They all grew autotrophically on thiosulfate, tetrathionate, sulfur, and sulfide, but not on sulfite, thiocyanate,

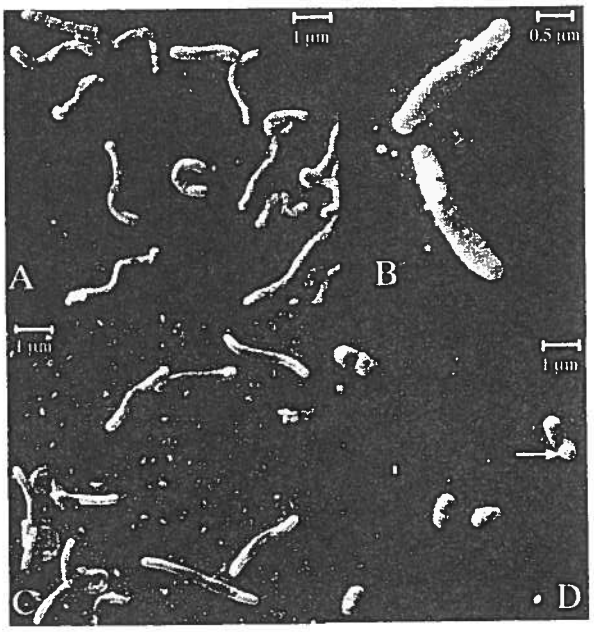

Figure 1. Electron micrographs showing the isolates Milos-OAII2 (A), Milos-OBII5 (B), Milos-NDII1.1 (C), and Milos-NDII1.2 (D). The arrow in (D) indicates a presumed sulfur globule. No picture of strain Milos-ODIII6 is shown, since the cells lysed during the preparation. However, in morphology it resembled strain Milos-NDII1.2.

and hydrogen (Table 1). Growth on thiosulfate lowered the pH to between 5 and 6 (Table 1). Formation of elemental sulfur was observed on solid media and in liquid media. No growth occurred in medium supplemented with any of the organic substrates tested.

Nitrate was used by strains Milos-OAII2, Milos-NDII1.1, and Milos-NDII1.2 as a terminal electron acceptor with N_2 as the end product (Table 1). No intermediate formation of nitrite or ammonium could be observed (data not shown). Thiosulfate was completely oxidized to sulfate after the following stoichometry:

$$5\ S_2O_3^{2-} + 8\ NO_3^- + H_2O \text{-----> } 10\ SO_4^{2-} + 2\ H^+ + 4\ N_2$$

With respect to the temperature range for growth and the temperature optima two groups could be distinguished (Table 1). Whereas strains Milos-OAII2, Milos-OBII5 and Milos-NDII1.1 grew between 8.6 and 46°C and had an optimum growth temperature between 37.5 and 42°C (Table 1), the temperature range of strains Milos-ODIII6 and Milos-NDII1.2 was between 8.5 and 39.7°C and the optimum between 31 and 35°C (Table 1). Whereas two isolates, i.e., strains Milos-ODIII and Milos-OBII5, showed no specific requirement for NaCl (0-2M), the other three isolates did not grow below a concentration of 0.2 M NaCl (Table 1). All isolates grew best at NaCl-concentrations between 400 and 500 mM. Nevertheless, it should be noted that the medium without NaCl contained 40 mM Na^+ from the addition of sodium thiosulfate.

DNA base ratio and ubiquinone content. The G + C content of strains Milos-OAII2, Milos-OBII5, Milos-ODIII6, and Milos NDII1.1 was 62.9 ± 0.3 mol%, 63.9 ± 0.2 mol%, 62.5 ± 0.3 mol%, and 50.1 ± 0.3 mol%, respectively (Table 1). The isolates Milos-OAII2, Milos-OBII5, Milos-ODIII6, and Milos-NDII1.1 contained Q-8 as the only major ubiquinone (Table 1), which is a general characteristic of obligately chemolithoautotrophic sulfur-oxidizing bacteria.

Enzyme activities. All tested organisms showed activity of sulfite:ferricytochrome-c oxidoreductase. The activities for strain Milos-OAII2, strain Milos-ODIII6, and *Thiomicrospira* sp. strain Milos-T2 were (in nmol * min^{-1} * [mg protein]$^{-1}$) 4.2-11.7, 9.7-11.1, and 12.5, respectively. Only strains Milos-OAII2 and Milos-ODIII6 exhibited activities of APS-reductase. The activities were (in nmol * min^{-1} * [mg Protein]$^{-1}$) 10.7-13.2 and 8.6-21.5 for strains Milos-OAII2 and Milos-ODIII6, respectively.

Phylogenetic analysis. Comparative sequence analysis of the 16S rRNA encoding genes of the five isolates showed highest similarities with rRNA sequences from symbiotic, so far uncultered bacteria of various invertebrates (Table 2). Phylogenetic analysis placed four isolates, i.e. Milos-OAII2, Milos-OBII5, and Milos-NDII1.1, within the group of endosymbionts containing the symbionts of *Riftia pachyptila* and other vestimentiferan tubeworms, and of bivalves of the families *Lucinidae*, *Solemyidae*, and *Thiaysaridae* (Fig. 1). Although strain Milos-ODIII6 fell in the same group in some calculations (data not shown), it

Table 2. 16S rRNA sequence similarities (in %) among the new isolates, sulfur-oxidizing ecto- and endosysmbionts and other free-living sulfur-oxidizing bacteria among the gamma subdivision of the Proteobacteria.

Organism	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19	20	21	22	23	24	25	26	27	28	29	30	31	32	33	34
1 *Thiomicrospira crunogena*	-																																	
2 *Thiomicrospira pelophila*	92.0	-																																
3 *Solemya reidi* [a]	87.6	87.7	-																															
4 *Calyptogena sp.* [a] *(Oregon subduction)*	84.0	84.7	86.1	-																														
5 *Calyptogena elongata* [a]	83.5	84.7	85.8	97.3	-																													
6 *Calyptogena magnifica* [a]	83.5	84.5	85.7	95.9	96.5	-																												
7 *Vesicomya chordata* [a]	83.7	84.8	85.9	96.3	96.6	97.4	-																											
8 *Bathyomodiolus thermophilus* [a]	85.6	86.0	87.5	92.5	92.9	92.7	93.5	-																										
9 *Lucinoma aequizonata* [a]	85.5	85.4	90.6	85.8	85.8	85.5	85.7	87.9	-																									
10 *Lamellibrachia columna* [a]	86.7	86.0	91.9	86.4	86.4	86.3	86.4	89.0	93.9	-																								
11 *Riftia pachyptila* [a]	87.0	86.7	91.4	85.7	86.2	86.0	86.0	89.1	93.8	95.9	-																							
12 *Solemya terraerigena* [a]	86.9	86.7	91.1	85.7	85.4	85.4	85.4	87.7	93.4	94.0	94.2	-																						
13 *Andantia Phillipiana* [c]	86.2	85.9	91.2	85.4	85.3	85.3	85.3	88.4	93.5	93.9	94.1	97.5	-																					
14 *Thysira flexuosa* [c]	87.0	86.3	90.4	86.5	85.9	86.0	85.9	87.8	92.9	93.7	94.0	95.0	94.2	-																				
15 *Lucina pectinata* [a]	86.8	87.2	91.0	85.3	85.5	85.5	85.2	87.2	92.5	93.2	94.2	95.4	94.2	93.8	-																			
16 *Solemya velum* [a]	85.4	85.0	90.7	84.0	84.1	84.4	84.6	86.3	90.4	91.9	91.4	90.7	91.2	90.7	91.0	-																		
17 *Solemya occidentalis* [a]	84.3	84.1	89.6	83.7	83.3	83.4	83.8	85.6	90.2	90.0	91.1	90.8	90.6	90.5	91.4	91.2	-																	
18 *Chromatium oxaliferum*	84.4	84.1	88.8	83.7	83.6	84.4	84.0	84.1	87.5	88.3	88.6	88.1	88.0	89.0	89.8	88.4	87.2	-																
19 *Beggiatoa sp. 'Bay of Concepcion'*	83.8	84.3	89.6	84.3	84.9	85.4	85.4	86.3	87.8	87.9	88.2	89.0	89.2	87.4	88.1	88.4	88.3	85.6	-															
20 *Lamprocystis roseopercina*	84.4	84.7	90.2	83.2	83.1	83.9	83.8	85.3	88.7	89.4	90.1	89.1	89.8	88.9	90.1	90.8	88.7	89.2	88.0	-														
21 *Allochromatium vinosum*	84.5	84.8	89.5	83.3	83.3	83.5	83.4	84.8	87.8	88.9	89.0	87.8	88.6	87.4	88.6	89.3	86.4	89.0	87.2	92.7	-													
22 *Thiocystis gelatinosa*	83.4	83.8	88.6	82.4	82.6	82.9	82.7	84.5	87.8	88.3	88.6	87.6	88.4	87.1	88.3	88.0	86.6	88.2	86.4	93.4	94.5	-												
23 *Amoebobacter roseus*	83.8	84.7	89.7	82.0	82.3	82.7	82.5	84.4	88.2	88.6	90.1	88.0	88.8	87.7	89.1	89.1	87.1	88.3	87.5	94.2	93.3	93.7	-											
24 *Rhabdochromatium marinum*	84.7	85.2	89.1	83.0	83.4	83.2	83.2	84.4	88.0	89.1	89.6	88.0	88.2	88.3	89.5	88.9	88.7	89.0	86.9	93.2	92.2	92.2	93.0	-										
25 *Inanidrilus leukodermatus* [b]	85.4	84.9	89.9	84.1	84.9	85.0	84.7	85.2	89.2	89.7	90.1	88.9	88.5	88.4	90.1	89.8	88.5	87.6	88.1	92.2	91.7	90.4	91.4	92.7	-									
26 *Laxus oneistus* [c]	85.3	85.5	89.8	84.8	85.0	85.6	85.2	85.8	89.8	90.2	90.7	89.1	88.4	88.5	90.6	89.2	88.6	87.9	87.3	91.5	90.7	90.1	90.9	92.4	97.2	-								
27 *Thiotrix niveu*	84.6	84.4	87.6	86.4	85.9	86.0	86.0	86.7	87.2	87.6	87.7	86.9	87.4	88.2	87.9	88.9	86.1	86.9	85.8	88.4	88.6	87.2	87.2	87.9	88.4	87.6	-							
28 *Thiotrix ramosa*	83.9	83.4	87.2	87.1	86.4	86.6	86.6	87.4	87.5	88.2	87.9	86.3	86.6	88.1	86.9	88.2	86.6	85.6	85.7	87.4	87.0	86.1	86.4	87.1	88.0	87.0	94.2	-						
29 *Ectothiorhodospira shaposhnikovi*	83.8	84.5	91.4	86.1	86.2	86.0	86.1	87.8	89.4	89.6	89.6	89.3	89.9	88.3	89.6	89.9	88.3	87.3	90.6	90.2	90.2	89.3	89.8	90.1	89.8	89.4	90.0	88.3	-					
30 Milos-OBII5	84.0	84.6	90.5	84.1	84.4	84.5	84.3	86.2	90.3	91.3	90.5	90.7	91.1	89.2	90.5	90.1	89.0	87.5	88.5	89.5	89.9	88.2	88.2	89.1	90.3	89.5	88.4	87.7	91.5	-				
31 Milos-ODIII6	88.3	87.1	90.4	85.9	86.0	86.7	86.2	87.8	90.9	91.5	92.6	91.4	91.0	91.9	91.6	92.0	90.7	89.5	89.1	92.3	90.5	90.1	90.7	90.9	92.7	91.8	88.5	88.7	89.7	90.9	-			
32 Milos-NDII1.1	86.2	85.8	91.5	84.6	84.9	84.8	84.7	86.8	91.9	92.5	91.7	91.3	91.5	91.4	91.4	90.5	89.5	87.5	88.6	88.8	88.7	87.5	87.7	88.2	88.9	88.7	87.1	87.2	88.8	91.3	90.8	-		
33 Milos-NDII1.2	86.2	87.6	91.1	85.1	85.1	85.4	85.2	86.1	90.3	91.8	90.2	91.5	91.4	90.5	90.7	91.8	89.4	88.0	89.1	89.1	88.9	87.9	88.4	88.5	88.9	88.9	87.6	87.4	89.9	91.1	90.2	91.9	-	
34 Milos-OAII2	85.6	85.9	91.2	83.6	84.2	84.0	84.3	86.6	90.7	90.6	90.8	91.0	91.4	90.1	91.6	89.6	89.4	87.7	89.6	90.3	88.6	89.0	89.4	89.3	89.2	89.0	86.6	86.0	89.7	91.2	90.8	94.9	90.7	-

[a] intracellular endosymbiont, host name given; [b] extracellular endosymbiont, host name given; [c] ectosymbiont, host name given

most often affiliated with the branch containing the ectosymbiont of the nematode *Laxus oneistus* and the extracelluar endosymbiont of the oligochaete *Inandrilus leukodermatus*

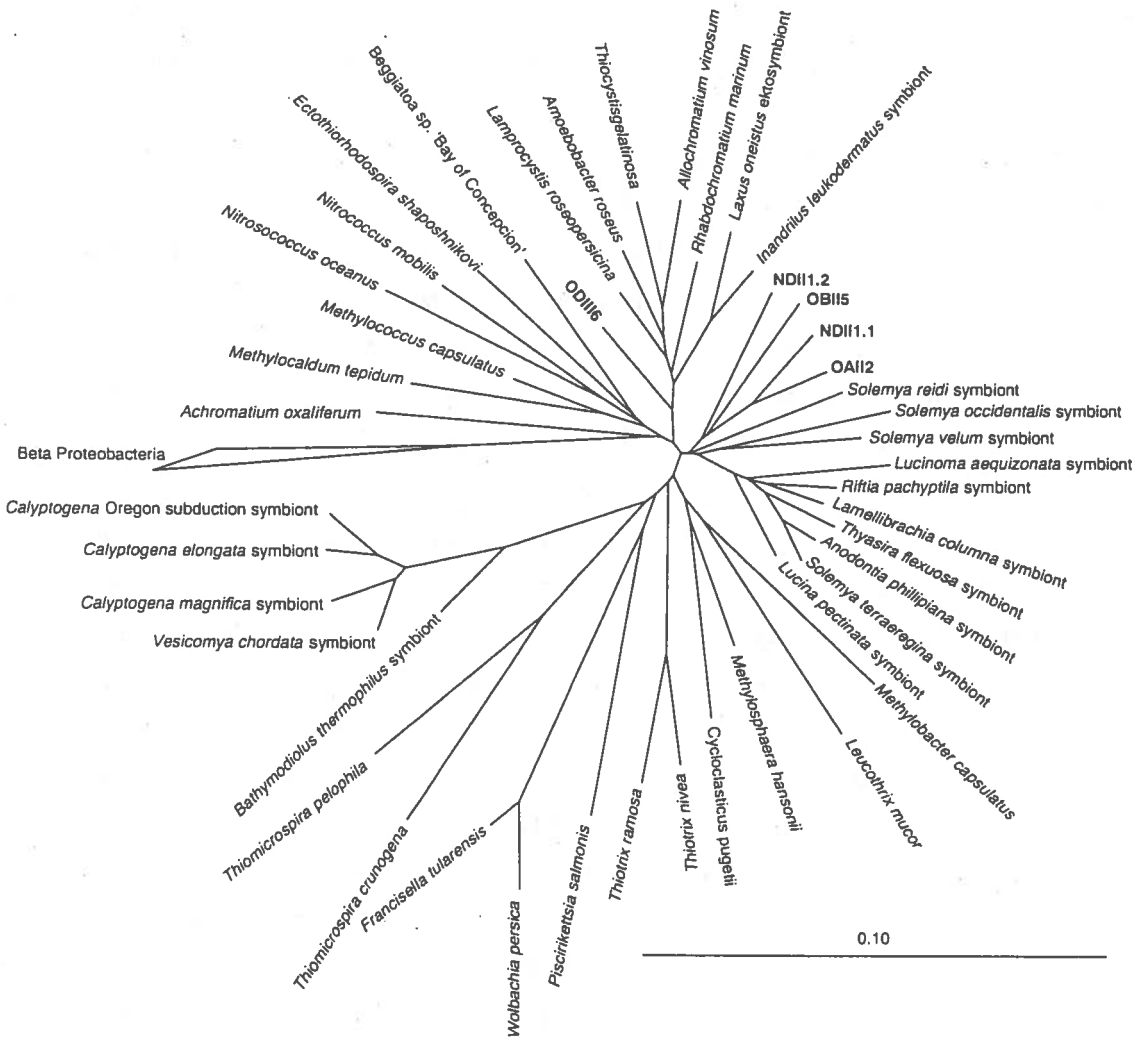

Figure 2. Evolutionary distance tree based on nearly complete 16S rRNA sequences, showing the relationships of the strains Milos-OAII2, Milos-NDII1.1, Milos-OBII5, Milos-NDII1.2, and Milos-ODIII6 (in bold) with sulfur-oxidizing endo- and ectosymbionts of various invertebrates and with other free-living (sulfur-oxidizing) bacteria. Bacteria belonging to the beta-subdivision of the Proteobacteria were used as an outgroup. All other bacteria belong to the γ -subdivision of the Proteobacteria.

DISCUSSION

Since the first discovery of endosymbiotic bacteria in the vestimentiferan tube-worm *Riftia pachyptila* (9, 16) microbiologists were eager to isolate their putative free-living counterparts. Now, nearly two decades later, we describe the isolation and characterization of chemoautotrophic sulfur-oxidizing bacteria (SOB) that based on comparative 16S rRNA sequence analysis and physiological features, are the closest free-living relatives of the endosymbionts of *Riftia pachyptila* and other vestimentiferan tubeworms, as well as several bivalve molluscs. Therefore, the data provide further evidence that the most recent common ancestor of the endosymbionts was a sulfur-oxidizing bacterium. The fact, that the new isolates and the endosymbionts are only affiliated at their roots argues for an early origin of the symbiosis which lead to the speciation between the host and the symbionts and an independent evolution of the free-living forms (12, 38). Thus, the new isolates can be viewed as modern descendants of the last common ancestor of the endosymbionts. There would be two options for a putative common ancestor. It could have been a symbiont from which the free-living forms evolved, or it could have been vice versa with symbionts evolving from a free-living form (39). That some hosts apparently aquire their symbionts from a free-living stock might argue for the second possibility. In this respect it is interesting to ask, whether the environmentally transmitted symbionts, which must go through a free-living stage, are a stable member of the bacterial communitiy or whether they are only transiently viable. This has important implications as it might be difficult to cultivate these symbionts if the latter possibility is correct. This would also mean that no close, truly free-living relatives of the endosymbionts might exist (38).

In contrast to earlier speculations about the role of *Thiomicrospira* as a modern descendant of the last common ancestor of the endosymbionts (12, 13, 38, 45, 46) we do have not only phylogenetic information in support of a close relationship between the symbionts and the new isolates, but also physiological data. The ability to use nitrate as an electron acceptor instead of oxygen seems to be rather widespread among the endo- and ectosymbiotic sulfur-oxidizing bacteria as it has been demonstrated in several host lineages including members of the vestimentiferan tubeworms (24, 25, 33), the bivalve families *Lucinidae* and *Solemyidae* (23, 60), and species of the nematode subfamily *Stilbonematinae* (22). It is important to note, that all of these symbionts are transmitted environmentally (7, 19, 20, 31), suggesting that they have a free-living stage, except the symbionts of the bivalve family *Solemyidae* (6, 29, 44). On the other hand, nitrate respiration coupled to the oxidation of reduced sulfur compounds is rare among the free-living sulfur-oxidizing bacteria. In the γ-subclass of the Proteobacteria, only the so far uncultured large sulfur-oxidizing bacteria of the genera *Beggiatoa* (34), *Thioploca* (43), and *Thiomargarita* (51) have been shown or are believed to carry out this reaction. However,

they are only distantly related to the symbionts and are probably no denitrifiers, but reduce nitrate to ammonia (43).

With respect to sulfur metabolism, the isolates described here seem also to have similarities to the symbionts. All putative sulfur-oxidizing symbionts so far investigated exhibited enzyme activities indicative of the APS-pathway (39). This pathway leads to the formation of ATP through substrate posphorylation. In a first step, the enzyme APS-reductase generates APS from AMP and sulfite. APS is then further oxidized to sulfate by the enzyme ATP-sulfurylase during which APS is converted to ATP. This pathway has only been identified in *Thiobacillus denitrificans*, *Thiobacillus thioparus*, and a marine *Beggiatoa*, but not in *Thiomicrospira* (39). In contrast, the second mechanism of energy conservation during the terminal oxidation of sulfite to sulfate, i.e., the sulfite:acceptor oxidoreductase pathway, has been identified in all thiobacilli and in *Thiomicrospira* (39). Our studies indicate that in the two isolates that were tested both pathways are operating. Taken together, the potential of the new isolates to denitrify and the presence of the APS-pathway provide physiological evidence for a close relationship between the endosymbionts and the here described SOB. Thus, it might be suggested that the new isolates are suitable model organisms for physiological and genetical investigations which are not possible with the endosymbionts.

As the strain Milos-ODIII6 was most closely related to the ectosymbiont of the nematode *Laxus oneistus* (46) it is tempting to suggest that the presence of this particular SOB might be related to such an association. Nematodes of the subfamily *Stilbonematinae*, to which *Laxus oneistus* belongs, are known for their highly specific, obligatory associations with ectosymbiotic bacteria (47) and they have been reported in Palaeochori Bay (57). The finding that this bacterium produces mucilaginous material (see below), could be interpreted as a mean to attach to the surface of potential hosts. The host must be recolonized by the symbionts several times during its life since the nematodes shed their cuticles (46). Thus a key to success is the highly specific recognition between the host and the potential symbionts. Extracellular polysaccharides (EPS) are known to provide such a mechanism (11).

Ecological relevance. Previously isolated SOB from hydrothermal vents were obtained from enrichments of undiluted samples (15, 27, 49, 50, 61). It is most likely that this strategy selects for fast growing, opportunistic strains, which can out compete numerically abundant and ecologically more important populations (53, 58). We used an alternative strategy, the most probable number (MPN) approach, whereby the sample was serially diluted to infinity and so selecting for numerically dominant populations. In addition, this approach favours the growth of abundant, but only slow growing populations. This is exemplified by the strain Milos-ODIII6. This strain was apparently abundant, but would have been overgrown in enrichments due to its slow growth. In fact, in enrichments and low dilution cultures we could always isolate a *Halothiobacillus* species (53). Strain Milos-ODIII6 was also unusual in that it

formed a mucilaginous matrix in which sulfur globules were embedded. Furthermore, it was not possible to obtain growth on a solid substrate. Similar observations have been made by Ward et al. (58), who stressed the importance to dilute the inoculum to get numerically dominant and thus ecological relevant organisms. However, the growth of this particular organism in the highest dilution does not necessarily mean that it is the organism that is primarily responsible for the oxidation of reduced sulfur compounds *in situ*. First, the MPN technique depends on the cultivation of organisms and the relevant organisms might not be cultivable. Secondly, the dilution might lead to the extinction of less abundant but more adapted populations. As a result the growth would be due to the absence of a successful competitor. In this case the high abundance *in situ* might have been caused by a certain, unknown competetive advantage and not *per se* by its ability to use reduced sulfur compounds as electron donor. Thus, MPN results should be viewed with some scepticism, until it has been shown that a particular organism is carrying out this reaction in situ.

The different temperature optima of the isolates might reflect an adaption to the in situ temperature, since the isolates with the highest temperature optima were isolated from the zones closest to the vent center and thus with higher temperatures. Further evidence for this hypothesis comes from two additional isolates, that were obtained from the outer zone and have temperature optima for growth at about 26°C (unpublished data). This emphasizes the importance of incubating the cultures at their in situ temperature to obtain the ecological relevant organisms (58).

Overall, we have found a high diversity within the sulfur-oxidizing bacterial community. Besides the isolates described here we have isolated SOB that belong to the genera *Thiomicrospira* (4) and *Halothiobacillus* (53), both of which have been described at other vent sites before (15, 27, 49, 61). Whether the new isolates are restricted in their occurence to this particular vent site or are also present at other hydrothermal vent systems remains to be determined. In addition, the factors that allow a high diversity of physiological similar organisms at one habitat should also be investigated in more detail in the future.

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Desulfacinum hydrothermale, sp. nov., a thermophilic completely oxidizing sulfate-reducing bacterium from geothermally heated sediments near Milos Island (Greece)

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ABSTRACT

A thermophilic sulfate-reducing bacterium, strain MT-96^T, was isolated from an active marine, shallow-water hydrothermal vent system. It used a large variety of substrates ranging from simple organic compounds to long chain fatty acids as electron donors. Autotrophic growth was possible with H_2 and CO_2 in the presence of sulfate. Sulfate, thiosulfate and sulfite were used as electron acceptors. Sulfur and nitrate were not reduced. Fermentative growth was obtained with pyruvate, but not with fumarate or malate. Substrate oxidation was usually complete leading to CO_2, but at high substrate concentrations acetate accumulated. The oval- shaped cells were 0.8-1.0 μm in width and 1.5-2.5 μm in length. Cells were motile in the early exponential growth phase, but motility rapidly declined in later growth phases. Spores were not produced; cells stained Gram-negative. The temperature limits for growth were between 37 °C and 64 °C, with an optimum at 60 °C. Growth was observed at salinities ranging from 15 to 78 g of NaCl per liter, with optimum growth in the presence of 32 to 36 g NaCl per liter. This might reflect an adaptation to the elevated salinity of the hydrothermal fluid. The G + C content of the DNA was 59.5 mol%. Vitamins or other supplements were not required. Based on the 16S rRNA sequence strain MT-96 belonged to the δ-subdivision of the Proteobacteria. Strain MT-96^T was found to be phenotypically and phylogenetically (<95.3% 16S rRNA sequence similarity) related to *Desulfacinum infernum* and represents a new member of this genus. The name *Desulfacinum hydrothermale* is proposed for this strain; the type strain is MT-96^T.

INTRODUCTION

Most thermophilic dissimilatory sulfate-reducing bacteria are phylogenetically members of the *Clostridium-Bacillus* subphylum of the Gram positive bacteria (30) or a deeply branching lineage within the domain *Bacteria* containing the genera *Thermodesulfobacterium* and *Thermodesulfovibrio* (13, 38). Within the δ-subdivision of the Proteobacteria, which contains all other sulfate-reducing bacteria, only three thermophilic species are described. These are *Desulfacinum infernum* (26), *Thermodesulforhabdus norvegicus* (2) and the incompletely described alkane-oxidizing strain TD3 (27); all were isolated from marine habitats and require marine or brackish water media for cultivation.

Two different habitats could be considered as ideal sites for marine thermophilic sulfate reducers. These are oil reservoirs located several km below the sea floor, which have elevated temperatures and pressures (e.g. 22, 32) or hydrothermal vent systems (15, 16). In addition, a common deep subterranean biosphere might exist that supports the growth of these organisms, as has been suggested by Gold (12). Recently, data were presented that support such an idea (8, 18, 22, 34, but see ref. 32 for a different view). For example, the thermophilic marine sulfate reducer *Desulfacinum infernum* and *Thermodesulforhabdus norvegicus* were isolated from enrichment cultures inoculated with formation water from North Sea oil fields (2, 26). A detailed study of the distribution of thermophilic marine sulfate reducers in North Sea oil field waters and oil reservoirs by using genus-specific fluorescent antibodies showed a clear dominance of the genera *Archaeoglobus* and *Thermodesulforhabdus*, whereas thermophilic *Desulfotomaculum* spp. were only obtained from enrichment (22, 23). In addition, several halophilic or halotolerant *Desulfotomaculum* spp. were isolated from various geothermally influenced terrestrial subsurface habitats (7, 10, 17, 19, 21, 35).

The increased interest in thermophilic sulfate-reducing bacteria has been strongly connected to their role and function during oil recovery, i.e., an enhanced sulfide production and stimulated corrosion due to the activities of sulfate-reducing bacteria. Little is known about the natural role of thermophilic sulfate reducers. Since the isolation of the hydrocarbon degrading stain TD3 from a deep-sea vent site in the Guaymas Basin, the substrate spectra of thermophilic sulfate-reducing bacteria has been extended and now includes alkanes, which were originally thought to be recalcitrant under anaerobic conditions (27). In this paper we describe a thermophilic isolate, MT-96, which was obtained from a shallow submarine hydrothermal vent. The data presented in this communication further suggest that members of the thermophilic genera *Thermodesulforhabdus* and *Desulfacinum* are, indeed, part of a subsurface microbial community, as originally proposed by Nilsen et al. (22).

MATERIAL AND METHODS

Source of organism. Strain MT-96 was isolated from most probable number (MPN) dilution series that were performed to investigate the horizontal and vertical distribution of sulfate-reducing bacteria at a shallow submarine hydrothermal vent located in Palaeochori Bay, which is a sandy bay in the south-eastern part of the Greek island of Milos in the Aegean Sea (29).

Media and culture conditions. The MPN series was performed as described by Sievert et al. (29). Artificial, bicarbonate buffered seawater medium as defined for sulfate-reducing bacteria was used (37); 10 mM acetate as carbon source, non-chelated trace element mixture number 1, vitamins, and a tungstate and selenite solution were added (37). For isolation and routine cultivation acetate was replaced by lactate. Media were reduced with sulfide and prepared as described by Widdel and Bak (37). Pure cultures were obtained by repeated use of deep agar dilution series (37). Substrate utilization was determined by adding the carbon and energy sources from sterile stock solutions, and the preparations were incubated for about 3 weeks. To test the capability for autotrophic growth, cultures were grown with a headspace of 80% H_2-20% CO_2 at an overpressure of 101.29 kPa. The temperature range for growth was determined by incubation in a temperature gradient block from 35 to 80 °C in increments of 2-4 °C. The pH range of growth was determined in mineral medium with pH from 5 to 9. The dependence of growth on the concentration of NaCl was determined in mineral medium with NaCl concentrations from 0 to 82 g NaCl per liter.

Chemical and biochemical characterization. The presence of desulfoviridin was tested as described by Postgate (26). The mol% G + C content was determined by Matthias Nagel (Fachhochschule Bremerhaven, Germany) by HPLC as described previously (4). Lambda DNA was used for final correction of the values.

PCR amplification and sequencing of the 16S rRNA gene. To amplify the almost complete 16S rRNA encoding gene (1,500 bp) of strain MT-96, M40/2 CIV-2.3, and M40/2 CIV-3.2, primers GM3F and GM4R (20) were used in a 35-cycle PCR with an annealing temperature of 40 °C. PCR products were purified by using the QIAquick Spin PCR purification kit (Qiagen, Inc., Chatsworth, Calif.) as described by the manufacturer. The *Taq* Dyedeoxy Terminator Cycle Sequencing kit (Applied Biosytems, Foster City, Calif.) was used to directly sequence the PCR products according to the protocol provided by the manufacturer. The sequencing primers have been described previously (5). The sequence reaction mixtures were electrophoresed on an Applied Biosystems 373S DNA sequencer.

Phylogenetic analyses of 16S rRNA gene sequence data. The sequences were loaded into the 16S rRNA sequence database of the Technical University Munich using the program package ARB (33). The tool ARB_ALIGN was used for sequence alignment. The

alignment was visually inspected and corrected manually where necessary. Tree topologies were evaluated by performing maximum parsimony, neighbor joining, and maximum likelihood analysis. Only sequences with at least 1400 nucleotides were used for the calculation of different trees. The partial sequence of strain M40/2 CIV-3.2 (1268 nucleotides) was added to the reconstructed tree by applying parsimony criteria without allowing changes in the overall tree topology. The strain designations and nucleotide sequence accession numbers are as follows: *Desulfacinum infernum* ACM 3991^T, L27426; *Desulfobacterium niacini* DSM 2650^T, U51845; *Desulfobacter postgatei* DSM 2034^T, M26633; *Desulfohalobium retbaense* DSM 5692^T, U48244; *Desulfomonile tiedjei* ATCC 49306^T, M26635; *Desulforhabdus amnigenus* DSM 10338^T, X83274; *Desulforhopalus vacuolatus* DSM 9700^T, L42613; *Desulfovibrio desulfuricans* ATCC 27774, M34113; *Desulfovibrio gabonensis* DSM 10636^T, U31080; *Desulfovibrio halophilus* DSM 5663^T, U48243; *Desulfuromonas acetoxidans* DSM 684^T, M26634; *Escherichia coli* ATCC 11775^T, X80725; *Syntrophus buswellii* DSM 2612^T, X85131; *Syntrophobacter wolinii* DSM 2805^T, X70905; *Thermodesulforhabdus norvegicus* DSM 9990^T, U25627. The nucleotide accesion numbers of strain TD3 and clone OPB33 are X80922 and AF026987, respectively.

Nucleotide sequence accession numbers. The nearly complete 16S rRNA sequences of strains MT-96, M40/2 CIV-2.3 and the partial sequence of strain M40/2 CIV-3.2 were submitted to GenBank and are available under accession numbers AF170417, AF170418, and AF170420, respectively.

RESULTS

Enrichment and isolation. Serial MPN dilutions were performed using media containing 10 mM acetate as the only carbon source and inocula from different layers of the sediment (29). Strain MT-96 was isolated from the 10^{-2} dilution of a sample that came from the sediment layers between 2 and 3 cm at a distance of 10 cm from the vent (29). The in situ temperature in this sediment layer ranged from 50 to 60 °C and the pH was approximately 5.0 (29). The mean composition of the discharged gases from different seeps in Palaeochori Bay was 80.5 % (vol/vol) CO_2, 1.2 % (vol/vol) H_2S, 0.8 % (vol/vol) CH_4 and 0.4 % (vol/vol) H_2 (6). The chemically reduced hydrothermal fluid had an elevated salinity of up to 58 ‰ compared to 39 ‰ of the ambient seawater (36). After two to three weeks of incubation, formation of sulfide and growth of small oval rods was observed. Following several transfers into liquid media a pure culture was obtained by repeated agar dilution series using lactate as electron donor and sulfate as electron acceptor. Previous experiments showed that lactate allowed faster growth. Besides strain MT-96 which was isolated from a vent site at a water depth of 8 m, two other thermophilic sulfate-reducing isolates were obtained from a vent site that was located

offshore the southeastern part of the island of Milos (Greece) in 112 m water depth (24°32.95' E, 36°40.09' N) using the same procedures as indicated above. These samples were obtained on a cruise with the R/V Meteor during leg M40/2. A MPN series with acetate as single carbon source inoculated with sediment from a horizon 45 – 65 mm below the surface (in situ temperature of 60 to 70 °C) showed growth at the 10^{-2} and 10^{-3} dilutions. From the 10^{-2} dilution strain M40/2 CIV-2.3 and from the 10^{-3} dilution strain M40/2 CIV-3.2 were isolated. A further physiological characterization of both strains was not carried out, but they were included in the phylogenetic analysis since they cluster with MT-96 and *Thermodesulforhabdus norvegicus*. In contrast to the shallow water hydrothermal vent the surface layer of the deeper vent site was covered with large, nonmotile, filamentous sulfur-oxidizing bacteria, which where attached to sediment particles. The in situ temperature at the surface was around 45 °C.

Cell morphology. Cells of strain MT-96 grown with lactate or acetate and sulfate were oval or short rods measuring 0.8-1.0 x 1.5-2.5 μm (Figure 1; Table 1). Cells occurred single or in pairs. Spore formation was absent. Cells were motile only during the early exponential growth phase. In later growth phases motility was only occasionally observed.

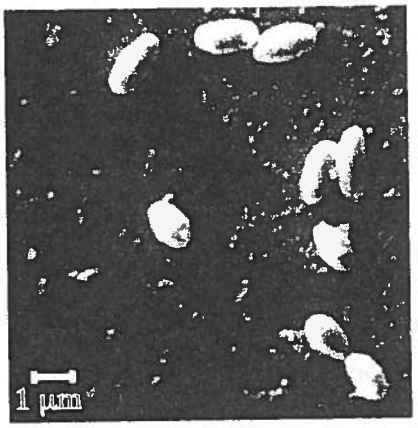

Figure 1. Electron scanning micrograph of strain MT-96^T, showing the typical oval to rod-shaped cell form (bar represents 1 μm).

DNA base ratio. The G + C content of the DNA was 59.5 mol%.

Physiological properties. The optimum growth temperature of strain MT-96 was determined to be 60 °C (Table 1). No growth was observed at temperatures higher than 64 °C and lower than 37 °C. At temperatures < 44°C it was observed that cells aggregated into flocs. The pH range was between 6.0 and 7.5 with an optimum at pH 7.0. Growth was obtained at salinities ranging from 15 to 78 g NaCl per liter; the optimum NaCl concentration was between 32 to 36 g per liter. Vitamins were not essential for growth (six consecutive transfers in vitamin-free medium). Desulfoviridin was absent in strain MT-96. Strain MT-96 used the

Table 1. Comparison of selected characteristics of strain MT-96, *Desulfacinum infernum*, *Thermodesulforhabdus norvegicus*, strain TD3, and *Desulforhabdus amnigenus*.

Characteristic	strain MT-96	*Desulfacinum infernum*	*Thermodesulforhabdus norvegicus*	strain TD3	*Desulforhabdus amnigenus*
Morphology	Oval	Oval	Rod	Rod	Rod
Width x length (μm)	0.8-1.0 x 1.5-2.5	1.5 x 2.5-3	1 x 2.5	0.8-1.0 x 2.0-3.5	1.4-1.9 x 2.5-3.4
Motility	+	-	+(sp)	-/+	-
G + C content (mol%)	59.5	64	51	37.4	52.5
Desulfoviridin	-	-	-	-	-
Salinity optimum (g/l)	32-36	10	16	nr	0
Optimal temperature (°C)	60	60	60	60	37
Oxidation	Complete	Complete	Complete	Complete	Complete
Electron donors:					
$H_2 + CO_2$	+*	+*	-	-	+*
Formate	+*	+*	-	-	+*
Acetate	+	+	+	-	+
Fatty acid : C atoms	3-5, 8, 10, 12,16,18	3-18	4-18	4-8, 12, 16, 18	3-4
Isobutyrate	+	+	nr	nr	+
Isovalerate	+	+	nr	nr	-
Ethanol	+	+	+	-	+
Lactate	+	+	+	-	+
Pyruvate	+	+	+	-	+
Fumarate	-	+	+	-	-
Succinate	-	+	+	-	-
Malate	-	+	+	-	-
Benzoate	-	-	-	-	-
Hexadecane	-	-	-	+	nr
Others	Propanol, butanol, hexanol, alanine	Propanol, butanol, hexanol, alanine		Alkanes	Propanol, butanol
Fermentative growth on: Pyruvate	Pyruvate	Pyruvate	-	nr	-
Electron acceptors:					
Sulfate	+	+	+	+	+
Sulfite	+	+	+	nr	+
Sulfur	-	-	-	nr	-
Thiosulfate	+	+	-	-	+
Nitrate	-	-	-	nr	-
Growth factor requirement	None	vitamins	None	nr	nr

Data obtained from Beeder et al. (2); Ehrenreich (9); Oude Elferink et al. (24); Rees et al. (27); Rueter et al. (28); nr not reported; * autotrophic growth

following compounds as electron donors and carbon sources in the presence of 10 mM sulfate (added substrate concentrations [mM] are given in parentheses): hydrogen plus carbon dioxide

(80%/20%; v/v) (four consecutive transfers without organic substrate; in medium with N_2-CO_2 as headspace no growth was observed), formate (10), acetate (10), propionate (10), butyrate (5), isobutyrate (5), valerate (5), isovalerate (5), octanoate (2.5), decanoate (0.5), dodecanoate (0.5), hexadecanoate (1), octadecanoate (1), ethanol (10), propanol (10), butanol (10), hexanol (5), lactate (10), pyruvate (10), alanine (10), yeast extract (0.1% and 0.01%). The following substrates were tested, but not utilized: fructose (5), glucose (5), sucrose (5), malate (10), fumarate (10), succinate (10), citrate (10), glycerol, (10), glutamate (10), benzoate (2), *p*-cresol (1), hexadecane (1), crude oil (0.001%). Sulfate, sulfite and thiosulfate were used as electron acceptors. Sulfur and nitrate did not serve as electron acceptor. Pyruvate allowed slow growth by fermentation. The minimum doubling time with lactate was 23 h. Increased substrate concentrations resulted in the production of acetate, which was released into the medium. When grown on 20 mM lactate, 2 to 3 mM acetate and 0.5 mM propionate were observed.

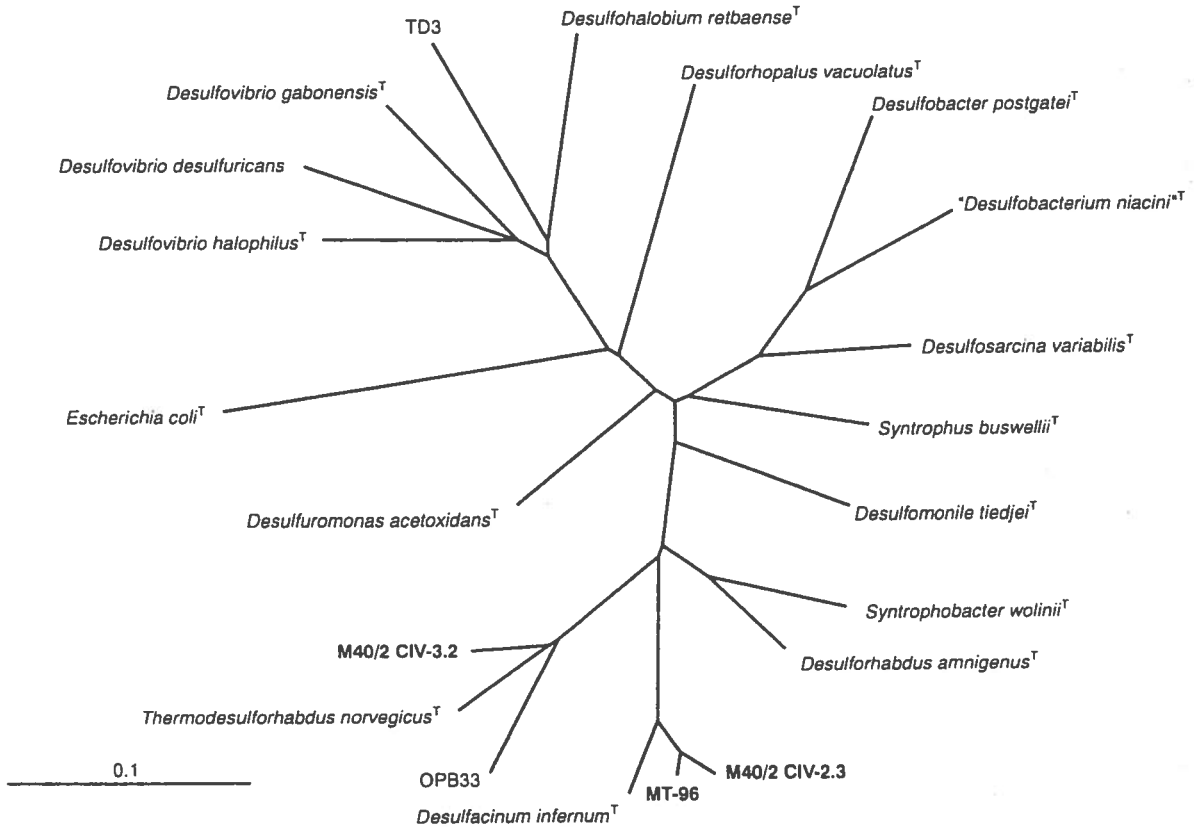

Figure 2. Maximum likelihood tree showing the phylogenetic position of the strains MT-96, M40/2 CIV-2.3, and M40/2 CIV-3.2 based on 1,426 positions of nearly full length 16S rRNA sequences from 20 bacteria and one clonal sequence (OPB33) from the δ-subdivision of the Proteobacteria, *Escherichia coli* (*gamma* subdivision of the Proteobacteria) was used as an outgroup. All trees constructed with other tree reconstruction algorithms (neighbor joining and parsimony) resulted in the same overall tree topology. In bootstrap analysis using neighbor joining and parsimony criteria the branching leading to the *Desulfacinum/Thermodesulforhabdus* cluster was supported with values of 100 (data not shown). The partial sequences of strain M40/2 CIV-3.2 was inserted into the tree by applying parsimony criteria without allowing for changes in the overall tree topology. Scale bar represents 10 substitutions per 100 nucleotides.

Table 2. Similarity values (in %) of the 16S rRNA sequences of strain MT-96, strain M40/2 CIV2.3, strain M40/2 CIV3.2, and other members of the *delta* subdivision of the Proteobacteria.

Organism	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19	20
1. *Desulfobacter postgatei*	-																			
2. *"Desulfobacterium niacini"*	88.8	-																		
3. *Desulfosarcina variabilis*	87.0	88.3	-																	
4. *Desulforhabdus amnigenus*	82.5	81.0	84.9	-																
5. *Syntrophobacter wolinii*	82.3	81.5	83.9	92.8	-															
6. *Desulfacinum infernum*	82.7	81.7	85.2	88.6	88.6	-														
7. *Thermodesulforhabdus norvegicus*	79.7	80.1	81.5	87.3	87.9	87.8	-													
8. *Desulfomonile tiedjei*	83.9	84.0	86.6	87.0	86.3	85.2	83.6	-												
9. *Syntrophus buswellii*	84.4	83.9	87.3	84.2	82.9	84.4	81.7	87.5	-											
10. *Desulfuromonas acetoxidans*	85.2	84.1	86.0	85.1	84.0	85.2	82.1	87.7	86.6	-										
11. *Desulforhopalus vacuolatus*	84.2	83.7	83.7	83.9	82.4	82.3	81.0	83.5	84.2	84.9	-									
12. *Desulfovibrio desulfuricans*	82.2	81.8	82.3	81.5	80.8	81.4	80.9	81.7	81.4	83.4	81.2	-								
13. *Desulfohalobium retbaense*	80.4	79.7	81.3	82.3	81.8	82.1	80.4	81.0	82.3	82.6	81.1	85.4	-							
14. *strain TD3*	80.6	80.3	82.0	83.1	82.4	83.1	83.8	82.2	82.5	82.6	82.7	84.4	86.8	-						
15. *Desulfovibrio gabonensis*	80.7	80.4	82.7	82.2	81.4	81.8	82.2	81.4	82.2	82.9	81.2	86.9	85.8	86.0	-					
16. *Desulfovibrio halophilus*	80.9	80.5	82.8	81.2	80.0	81.7	79.1	82.1	82.0	83.2	82.3	86.6	85.6	84.7	87.9	-				
17. Strain MT-96	82.6	81.8	84.9	88.9	88.7	95.3	87.5	85.9	84.2	85.5	82.0	80.0	81.7	83.3	81.7	80.7	-			
18. OPB33	80.9	79.9	82.6	86.0	86.5	86.9	92.1	83.7	82.1	82.3	81.3	81.8	80.7	83.3	81.9	80.2	86.8	-		
19. Strain M40/2 CIV2.3	82.7	81.2	84.9	87.9	87.7	95.0	87.0	85.9	84.5	85.5	81.9	80.2	81.7	83.0	81.1	80.5	98.2	86.0	-	
20. Strain M40/2 CIV3.2	82.4	81.1	84.3	88.7	89.0	89.5	94.9	86.1	84.0	85.3	82.3	82.1	82.8	86.0	84.0	81.5	90.6	93.1	90.3	-

Phylogenetic analysis. The 16S rRNA gene sequence of strain MT-96 shared between 80 and 95.3% identity with the 16S rRNA gene sequences of other sulfate reducers of the δ-subdivision of the Proteobacteria (Table 2). The phylogenetic positions of strain MT-96, strain M40/2 CIV-2.3 and strain M40/2 CIV-3.2 are shown in Figure 2. All of them are members of the δ-subdivision of the Proteobacteria. Comparative phylogenetic analyses revealed that strain MT-96 represents a member of the genus *Desulfacinum*. and it was closely related to *Desulfacinum infernum*, (95.3 % similarity of their 16S rRNA), which is the only species of this genus so far (Figure 2). Based on the sequence of the 16S rRNA, strain M40/2 CIV-2.3 was closely related to MT-96 (98.2% similarity of their 16S rRNA sequence), whereas strain M40/2 CIV-3.2 clustered with *Thermodesulforhabdus norvegicus* (94.9% 16S rRNA sequence similarity).

DISCUSSION

The isolate, strain MT-96 is a thermophilic sulfate-reducing bacterium, that can use a large variety of substrates as electron donors. The ability to use acetate and propionate and to grow autotrophically with H_2 and CO_2 is shared with its closest relative *Desulfacinum infernum* (26) and several mesophilic sulfate reducers (37). The substrates are usually completely oxidized to CO_2, but increased substrate concentrations can lead to an incomplete oxidation of the organic substrates. For the utilization of fatty acids this might be a result of a CoA-transferase reaction as proposed for *Desulfobacterium autotrophicum* (28), whereas acetate and propionate excretion after growth on lactate might be caused by partial fermentation of the lactate via a randomizing succinate pathway as described for *Desulfobulbus propionicus* (31).

The 16S rRNA gene sequence analyses presented in this study demonstrated that strain MT-96 is a member of the genus *Desulfacinum* with *Desulfacinum infernum* as its closest relative. The physiological properties (Table 1) in combination with the results of the 16S rRNA gene sequence comparison demonstrated that strain MT-96 represents a novel species within the genus *Desulfacinum*. We propose the name *Desulfacinum hydrothermale* sp. nov. for strain MT-96^T.

Ecological relevance. By its growth characteristics, i.e. its temperature optimum and its salt tolerance, strain MT-96 seemed to be well adapted to the environment from which it was isolated. However, the pH range determined for the isolate does not cover the pH measured at the sampling site. A possible explanation could be a toxic effect of H_2S at low pH within the batch cultures, which would not occur in the natural environment, because of interaction with ferrous iron or by dilution due to exchange with the overlying water. However, strain MT-96 could also grow in microniches with different geochemical conditions (11). The fractionation of sulfur isotopes during dissimilatory sulfate-reduction obtained in batch cultures was within the

range found for mesophilic sulfate-reducers and reflected the in situ sulfur isotopic composition of the sediments (3).

The finding of members of the genus *Desulfacinum* represented by the strains MT-96 and M40/2 CIV-2.3 and of the genus *Thermodesulforhabdus* represented by strain M40/2 CIV-3.2 at both vent sites indicates an important role for the degradation of organic compounds by thermophilic sulfate-reduction at hydrothermal vents. The presence of thermophilic sulfate reduction has also been shown for a deep-sea vent site in Guaymas Basin (15, 16). In addition, it seems plausible to assume that at least strain MT-96 contributes to primary production at the vent site by growing with H_2 and CO_2, because both substances are constituents of the hydrothermal fluid (6). Our data further support the idea of Nilsen et al. (22), who proposed that species of the genera *Thermodesulforhabdus* and *Desulfacinum* are members of a deep hot biosphere (sensu [12]), that contains thermophilic and hyperthermophilic microorganisms. However, until now these two genera had not been isolated from any environment other than oil reservoirs. The present findings extend the habitat range of these genera to marine hydrothermal vents. Moreover, at least *Thermodesulforhabdus* seems also to be present in Obsidian Pool, a freshwater hot spring in Yellowstone National Park (14). The sequence OPB33 is closely affiliated with *Thermodesulforhabdus norvegicus* (Fig. 1) and it was the second most abundant sequence in the clone library. This suggests that these populations are playing an important role in this environment. While this argues for the existence for a common subterranean biosphere, the presence of *Thermodesulforhabdus norvegicus* and *Desulfacinum infernum* in oil fields could also be due to an intrusion of seawater that contained cells derived form hydrothermal sources and subsequent growth in the reservoir (32). There are only few descriptions of thermophiles from deep-sea hydrothermal vents with maximal growth temperature below 80°C, although these organisms are thought to be abundant (1). Thus, it should be worthwhile to look for the presence of members of the *Desulfacinum/Thermodesulforhabdus* cluster at deep-sea vents, which are also thought to provide a window into the subsurface biosphere (8, 34).

Description of *Desulfacinum hydrothermale* sp. nov. *Desulfacinum hydrothermale* (hy.dro.ther.ma´le. Gr.n. *hydor* water, Gr. n. *thermos* heat, M.L. neut. adj. *hydrothermale* pertaining to hot water, i.e. to hydrothermal vents). Cells are oval to short rods, 0.8-1.0 by 1.5-2.5 μm. Spore formation is absent. Cells are motile, but motility can rapidly cecline. Gram stain reaction of cells is negative. Strict anaerobe. Growth on H_2/CO_2, formate, acetate, propionate, butyrate, isobutyrate, valerate, isovalerate, octanoate, decanoate, dodecanoate, hexadecanoate, octadecanoate, ethanol, propanol, butanol, hexanol, lactate, pyruvate. Substrate oxidation is usually complete leading to CO_2, but at high substrate concentrations acetate can accumulate. Electron acceptors used are sulfate, sulfite and thiosulfate. Not used: nitrate and sulfur. Slow fermentative growth on pyruvate. Addition of at least 15 g/l of NaCl is necessary. Optimum NaCl concentration for growth is between 32 and 36

g/l. NaCl is tolerated up to 78 g/l. Vitamins are not required for growth. Temperature requirements: T_{min}, 37 °C; T_{opt}, 60 °C; T_{max}, 64 °C. The pH range for growth is 6 to 7.5; pH optimum at 7.0. The G + C content of the DNA is 59.5 mol%. The Genbank accession number for the 16S rRNA gene sequence is AF170417. The strain MT-96 is deposited at the Deutsche Sammlung von Mikroorganismen und Zellkulturen GmbH under number 13146, which is the type strain of the species.

Emended description of the genus *Desulfacinum* (26). The sentence about motility should be changed into: Oval cells which may be motile or nonmotile.

ACKNOWLEDGMENTS

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8

Fractionation of sulfur isotopes during dissimilatory reduction of sulfate by a thermophilic Gram-negative bacterium at 60°C

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ABSTRACT

Sulfur isotope ($^{34}S/^{32}S$) fractionation during reduction of dissolved sulfate was investigated with a growing batch culture of a thermophilic, Gram-negative, sulfate-reducing bacterium (strain MT-96) at 60°C. The completely oxidizing strain was isolated from geothermally heated sediments of a shallow-water hydrothermal vent in the Mediterranean Sea. The hydrogen sulfide produced in the experiments was enriched in ^{32}S by approximately 19‰ compared to sulfate, which indicates that stable isotope discrimination by this thermophile is within the range found previously for mesophilic sulfate-reducing bacteria and only slightly higher than observed for the thermophilic Gram-positive *Desulfotomaculum nigrificans*.

INTRODUCTION

Microbial dissimilatory sulfate reduction is an important process in the biogeochemical sulfur cycle of natural sediments (17, 28, 30) and associated with a fractionation of the stable sulfur isotopes (see, e.g., reference 18). Due to the lower bond-strength of ^{32}S-O compared to ^{34}S-O in the sulfate molecule, it is preferentially broken by sulfate-reducing bacteria, and the reduction process leads to the formation of hydrogen sulfide depleted in ^{34}S relative to dissolved sulfate. Depletions of up to 46‰ have been observed in studies with pure cultures of mesophilic sulfate-reducing bacteria (18). Using resting-cell and chemostat experiments it has been found that the degree of isotope discrimination by *Desulfovibrio desulfuricans* is inversely related to the specific sulfate reduction rate (9, 15, 18, 19). In resting-cell experiments the sulfate reduction rate was controlled by the changing temperature between 0 and 45°C, and the results were consistent with those obtained under constant temperature in a chemostat (9). Additionally, McCready (22) and Chambers et al. (10) carried out experiments at between 38° and 55°C with strains of the genus *Desulfotomaculum,* including *D. nigrificans,* but did not find a significant influence of temperature on isotope partitioning. The thermophilic strain *Desulfotomaculum nigrificans* belongs to the group of low G+C Gram-positive bacteria. Stable sulfur isotope fractionations by Gram-negative, thermophilic sulfate-reducing bacteria at temperatures exceeding 55°C, however, have not been investigated experimentally.

In the present study, we report for the first time on sulfur isotope discrimination during reduction of sulfate by a thermophilic Gram-negative bacterium at 60°C. The partitioning of sulfur isotopes during this reaction is similar to previous experimental results observed for mesophilic sulfate-reducers and the thermophilic Gram-positive *D. nigrificans*, indicating that similar metabolic processes are responsible for the fractionation of sulfur isotopes in the various types of sulfate-reducers.

MATERIALS AND METHODS

Strain MT-96 was isolated from geothermally heated sediments of a shallow-water hydrothermal vent in the Mediterranean Sea near Milos Island (27), and was cultivated at 60°C in an artificial seawater medium (31) with lactate (10 mM) and sulfate (20 mM). Based on the analysis of the nearly complete sequence of the 16S-rRNA gene, strain MT-96 belongs to the δ-subdivision of the *Proteobacteria.* The closest relative is *Desulfacinum infernum* strain BaG1; the sequences a of the 16S rRNA genes have a similarity of 95.8 % (26). *Desulfacinum infernum* is a thermophilic sulfate-reducing bacterium that was isolated from a petroleum reservoir and grows optimally at 60°C (25). Similar to *D. infernum* strain BaG1, strain MT-96 forms oval cells and is capable of growing with acetate. It grows between 37 and 64 °C

(optimum 60°C). The cells have a length of 2-3 μm and a width of 1-1.3 μm (26). Glass bottles (110 ml) with medium (95 ml) were inoculated with cells of freshly grown cultures that had been harvested by centrifugation and washed with 50 mM phosphate buffer (pH 7) under inert gas. Initial cell numbers were approximately 1.8 x 10^5 cells cm^3. The bottles were sealed with black butyl rubber stoppers. Batch experiments were terminated as described below after distinct time intervals (between 1 and 14 days) to achieve different proportions of substrate consumption. During the course of the experiments the pH values (solutions cooled quickly to room temperature) rose from 7.2 to, for instance, 7.8 (at a corresponding concentration of 2.5 mM H_2S).

Before opening, the bottles were quickly cooled to room temperature (ice bath) and aliquots for sulfate, pH, hydrogen sulfide and cell number analyses were subsampled from the homogenized solution (ultrasonic bath; 3 min.) through the butyl rubber stopper with syringes previously flushed with inert gas. After that, the bottles were opened and the residual solution was immediately mixed with 20% Zn acetate solution to precipitate dissolved sulfide (essentially H_2S and HS^-) quantitatively as ZnS. ZnS was converted into H_2S (1) which was trapped quantitatively as Ag_2S in a 0.3 M $AgNO_3$ solution. From a filtered aliquot (0.2 μm; polynitrate filter) of the Zn acetate-fixed solution, sulfate was precipitated as $BaSO_4$ for stable isotope determination. Dissolved sulfide concentrations were determined gravimetrically (Ag_2S) and photometrically after precipitation as CuS (12) or ZnS (11). Sulfate was determined by ion chromatography. Samples for the determination of total cell counts were fixed in 4% (w/v) borax-buffered formaldehyde and stored at 4°C in the dark. Aggregates of bacteria were disaggregated using a sonic probe with a tapered microtip (Sonopuls HD 200, Bandelin, Berlin, Germany), and, when necessary, samples were diluted with a filter-sterilized 3.5 % (wt/vol.) NaCl solution and at least 2 ml were filtered through black Nuclepore polycarbonate filters (0.2 μm pore size, Costar). Cells were stained in 0.01 % (w/v) acridine-orange and were counted with a Zeiss Axiolab epifluorescence microscope.

$BaSO_4$ and Ag_2S precipitates were washed, dried and analysed for isotopic composition by combustion isotope-ratio-monitoring mass spectrometry (C-irmMS) using a Carlo Erba EA 1108 elemental analyzer which was connected to a Finnigan MAT 252 triple collector gas mass spectrometer via a Finnigan Conflo II split interface (4). International silver sulfide standards IAEA-S-1 and IAEA-S-2 were used to calibrate the mass spectrometer. $\delta^{34}S$ values of +20.6‰ and -32.3‰ were obtained for the international standards NBS-127 ($BaSO_4$) and IAEA-S-3 (Ag_2S), respectively (3). Replicate measurements agreed within ±0.2‰. The isotopic composition is given in the δ-notation relative to the V-CDT standard according to:

$$d^{34}S\ [‰] = \{(^{34}S/^{32}S_{sample})/(^{34}S/^{32}S_{V\text{-}CDT}) - 1\} \cdot 1000.$$

It should be noted, that in the experiments conducted in the present study, no influence of isotope exchange reactions between dissolved sulfate and sulfide on the results is expected, since the rate of isotope exchange at temperatures below 100°C and circum-neutral pH is extremely slow (22). Under isotope-exchange equilibrium conditions at 60°C, sulfide would be enriched in ^{32}S by about 59‰ compared to coexisting sulfate (22).

RESULTS AND DISCUSSION

During the incubation with lactate as the electron donor and with sulfate as the electron acceptor, the Gram-negative thermophilic strain MT-96 reduced up to more than 8 mM sulfate, corresponding to about 42% of the initial concentration (Fig. 1). At the same time, the cell numbers increased, and the isotopic composition of the residual sulfate rose from its initial com-

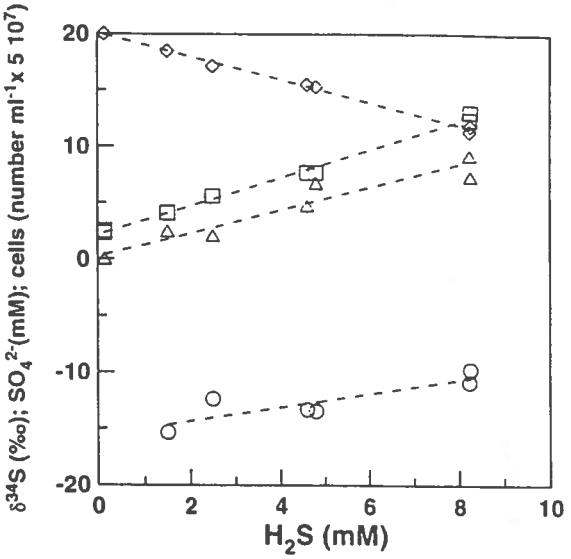

Figure 1. Sulfate concentrations (◊), cell numbers (Δ), and $\delta^{34}S$ values of sulfate (□) and hydrogen sulfide (○) during sulfate reduction (increasing sulfide concentrations, x-axis) by growing batch cultures of the thermophilic, Gram-negative, sulfate-reducing strain MT-96 at 60°C. Data for hydrogen sulfide were corrected for the initial concentration and isotopic composition.

position continuously up to $\delta^{34}S$ values of +14‰ (Fig. 1). Dissolved sulfide produced during sulfate reduction had significantly less ^{34}S than did dissolved sulfate (Fig. 1), and the isotopic composition increased as the reaction progressed, as expected for a reaction in a system limited in sulfate (e.g., 16, 21).

The enrichment factors in per mill (e = {a - 1} 1000; a: fractionation factor) derived from the $\delta^{34}S$ values of sulfate and hydrogen sulfide were evaluated using Rayleigh equations for a closed system according to Mariotti et al. (21):

$$\delta^{34}S(SO_4^{2-}) = \delta^{34}S(SO_4^{2-0}) + e \ln f \quad (1)$$

$$\delta^{34}S(H_2S) = \delta^{34}S(SO_4^{2-0}) - (f \ln f) / (1 - f) \quad (2)$$

In these equations, f denotes the fraction of unreacted sulfate and $\delta^{34}S(SO_4^{2-0})$ the initial isotopic composition of dissolved sulfate (+2.4 ± 0.1‰ vs. V-CDT; n = 2).

Evaluation of the sulfur isotopic composition of dissolved sulfate of the individual experiments according to equation (1) ranged between -18.8 and -23.7‰ (average -20.7 ± 1.7‰). Additionally, the sulfur isotope data of residual sulfate varied linearly with the logarithm of the residual sulfate fraction f (Fig. 2), as expected from equation (1). A linear regression of the sulfate data using equation (1), yields an e value of -19.2‰ (r^2 = 0.994; n = 8)), and the calculated isotopic composition for the initial sulfate ($\delta^{34}S$ = +2.6‰) is consistent with the measured value ($\delta^{34}S$ = +2.4‰). A combined evaluation according to equations (1) and (2) of all isotope data measured for substrate and products (Fig. 2) yields an e value of -18.1‰ (r^2 = 0.996; n = 14).

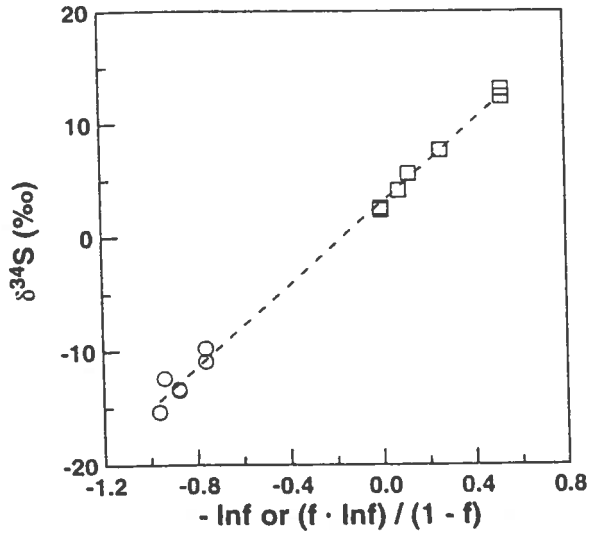

Figure 2. Sulfur isotope fractionation in substrate (□; sulfate) and product (○; hydrogen sulfide) during sulfate reduction by the thermophilic sulfate reducer strain MT-96.

The results obtained in the present study at 60°C with the thermophilic sulfate reducer MT-96 are within the range observed previously in experiments at lower temperatures with the thermophilic *D. nigrificans* and with different mesophilic sulfate reducing bacteria that use lactate as the electron donor (Fig. 3). An evaluation of 194 separate experiments from the literature yields an

average value of e = -14.3 ± 7.1‰ without a significant effect of reaction temperature (0 - 55°C; Fig. 3). Using a different electron acceptor at 55 °C, Chambers et al. (10) have obtained a maximum fractionation of -15‰ with *D. nigrificans*. The strong variability in isotope discrimination observed for mesophilic sulfate reducers (Fig. 3) can be related to the cell-specific sulfate reduction rate which seems to be the main factor determining the degree of sulfur isotope

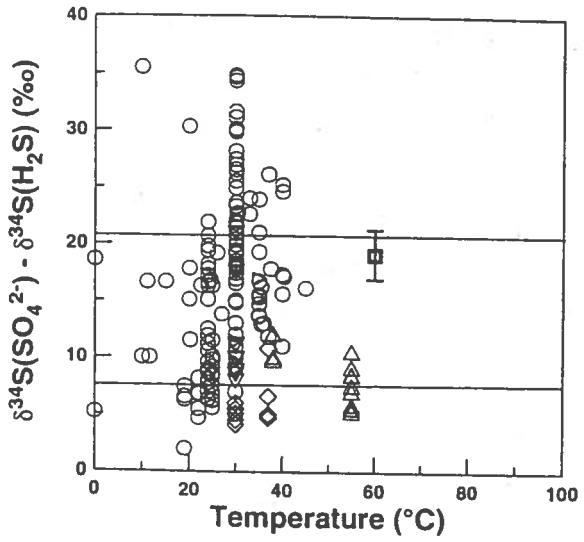

Figure 3. Experimental sulfur isotope fractionation in experiments using lactate as the electron donor with the mesophilic *Desulfovibrio desulfuricans* (○; ref. 9, 10, 15, 19, 22 and 29), Desulfovibrio salexigens (>; ref. 10 and 22), *Desulfovibrio vulgaris* (∇; ref. 22), *Desulfotomaculum orientis* (◊; ref. 22), and the thermophilic Gram-positive *Desufotomaculum nigrificans* (Δ; ref. 22) as compared to the results from the present study with the thermophilic Gram-neagtive strain MT-96 (□). Lines indicate range previously observed for isotope fractionation in experiments with mesophilic bacteria and with Gram-positive, thermophilic, sulfate-reducing bacteria using lactate (average ± SD).

fractionation (9, 15, 18, 19). Sulfur isotope fractionation in relation to an estimate for the specific sulfate reduction rate (sSRR) obtained in our experiments with the thermophilic sulfate-reducing bacterium MT-96 [log sSRR (μmol cell^{-1} h^{-1}) = -9.6 ± 0.5] is in agreement with previous measurements with the mesophilic *Desulfovibio desulfuricans* (18, 9). Therefore, the processes responsible for the fractionation of sulfur isotopes in mesophilic and in thermophilic sulfate-reducing bacteria seem to be similar and to be associated with comparable sulfur isotope effects. Additionally, there seems to be no significant difference in sulfur isotope fractionation between Gram-negative and Gram-positive sulfate reducers, which is in accordance with the experimental findings of McCready (22) and Chambers et al. (10). Stable isotope fractionation during dissimilatory sulfate reduction is caused by a superimposition of different reaction steps in the cell: After the uptake into the cell, sulfate is activated by ATP sulfurylase (20). Both steps are believed to be associated with insignificant or small sulfur isotope effects (24). Two reductive steps in the cytoplasm, the reduction of adenosine-5'-phosphosulfate to sulfite and the

subsequent reduction of sulfite to hydrogen sulfide are associated with breaking of S-O bonds. This probably causes most of the observed isotope effects, leading to a maximum fractionation of -46‰ during bacterial dissimilatory sulfate reduction (18, 24). During the reaction of iron and dissolved sulfide to form solid iron sulfides, no significant isotope fractionation occurs (5). Therefore, the isotopic composition of hydrogen sulfide produced during bacterial metabolism in natural environments should be reflected by the isotopic signature of sedimentary sulfides. However, natural sulfides have been found to isotopically much more enriched in ^{32}S than it can be explained by sulfate reduction alone (6, 8). Based on recent experimental studies, the reoxidation of hydrogen sulfide to intermediate sulfur species (e.g., elemental sulfur, sulfite, thiosulfate) followed by their bacterial disproportionation to hydrogen sulfide and sulfate (7, 13, 14) is likely responsible for this additional isotope discrimination. Field data on the sulfur isotope partitioning between sulfate and reduced sulfur species at hydrothermal vents from Milos (2), however, are close to the results observed in the experiments of the present study.

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