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Kultivierungsabhängige Analyse mikrobieller Gemeinschaften in Sapropelen des östlichen Mittelmeeres

(Cultivation-based analysis of microbial communities in Eastern Mediterranean sapropels)

Von der Fakultät für Mathematik und Naturwissenschaften
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Jacqueline Süß

geboren am 28.11.1972 in Cottbus

Gutachter: Prof. Dr. Heribert Cypionka

Zweitgutachter: Prof. Dr. Meinhard Simon

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Für meine Eltern und Großeltern

Erklärung

Die Ergebnisse dieser Arbeit sind bei Fachzeitschriften publiziert (Kapitel 2.1, 2.3), eingereicht (Kapitel 2.4) bzw. werden in Kürze eingereicht (Kapitel 2.2). Mein Beitrag an der Erstellung der Manuskripte wird im Folgenden erläutert.

Publikationen

J. Süß, B. Engelen, H. Cypionka & H. Sass. Quantitative analysis of bacterial communities from Mediterranean sapropels based on cultivation-dependent methods. 2004. FEMS Microbiology Ecology 51, 109-121.

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Tagungsbeiträge

J. Süß, B. Engelen, H. Cypionka & H. Sass. Successful cultivation and characterisation of Mediterranean sapropel bacteria. VAAM-Jahrestagung Berlin, Sediment Wilhelmshaven (2003) **Poster**

J. Süß, B. Engelen, H. Cypionka & H. Sass. Microbiological and molecular biological analysis of Mediterranean sapropel bacteria. Joint Colloquium of IODP and ICDP Bremen (2004) **Poster**

J. Süß, B. Engelen, H. Cypionka & H. Sass. Surprising detection of *Agrobacterium* in eastern Mediterranean sapropel layers and its quantitative analysis by cultivation and molecular biological methods. VAAM-Jahrestagung Braunschweig (2004) **Poster**

J. Süß, H. Sass, H. Cypionka & B. Engelen. Widespread distribution of *Rhizobium radiobacter* in Mediterranean sediments Joint Symposium of ISSM and ISEB, Jackson Hole, Wyoming, USA (2005) **Vortrag**

M. Seidel, **J. Süß**, K. Herrmann, H. Sass & J. Rullkötter. Molecular analysis of intact phospholipids of bacteria from Mediterranean sapropels. 22. International meeting on organic Geochemistry Sevilla, Spanien (2005) **Poster**

J. Süß, H. Sass, H. Cypionka & B. Engelen. Distribution of *Rhizobium radiobacter* in sediments of the Eastern Mediterranean. 2. Tagung der DGHM und VAAM Göttingen (2005) **Poster**

K. Ziegelmüller, **J. Süß**, H. Sass, H. Cypionka & B. Engelen. Use of molecular methods for optimizing cultivation strategies. 2. Tagung der DGHM und VAAM Göttingen (2005) **Poster**

J. Süß, K. Herrmann, M. Seidel, B. Engelen, H. Cypionka, H. Sass. Diversity of subsurface *Photobacterium* sp. as a response to specific *in situ* conditions? VAAM-Jahrestagung Jena (2006) **Poster**

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Zusammenfassung

In Sedimenten des östlichen Mittelmeeres treten in regelmäßigen Abständen schwärzlich-grüne Schichten mit einem hohen Gehalt an organischem Material auf. Diese so genannten Sapropele weisen deutlich erhöhte Zellzahlen und mikrobielle Aktivitäten auf und werden von bisher unkultivierten Vertretern der *Chloroflexi* und der marinen *Crenarchaea* dominiert. Da Kultivierungsversuche bisher erfolglos waren, sind die physiologischen Kapazitäten und Anpassungen indigener Mikroorganismen an die Bedingungen in Sapropellagen unbekannt.

In dieser Arbeit wurden verbesserte Kulturmedien und neue Anreicherungstechniken eingesetzt, um relevante Vertreter der Sapropelgemeinschaften zu kultivieren. Dadurch konnte die Kultivierungseffizienz im Vergleich zu vorangegangenen Studien um mehrere Größenordnungen gesteigert (bis zu 3,3 %) und eine einzigartige Stammsammlung etabliert werden (27 Phylotypen aus 6 bakteriellen Gruppen). Die molekularbiologische Analyse ausgewählter Anreicherungen zeigte, dass noch wesentlich mehr Phylotypen zum Wachstum stimuliert, jedoch nicht in Reinkultur gebracht werden konnten. Dazu gehörten unter anderem Vertreter der *Chloroflexi*.

Erstaunlicherweise wurden aus allen beprobten Sedimentschichten nahe Verwandte des Bodenbakteriums *Rhizobium radiobacter* isoliert. Um die ökologische Rolle dieser Mikroorganismen aufzuklären, wurde zunächst ihre Abundanz *in situ* bestimmt. Bei dieser Analyse wurde erstmals die numerische Dominanz eines kultivierten Vertreters der Tiefen Biosphäre am Standort nachgewiesen. Es zeigte sich, dass *R. radiobacter* in den Mittelmeersedimenten weit verbreitet ist und einen erheblichen Anteil der Bakterien-gemeinschaft in den Sapropelen einnimmt (bis zu 5 %). Die Detektion dieser Mikroorganismen in der Tiefen Biosphäre wirft grundsätzliche Fragen hinsichtlich der Interaktion zwischen Oberflächen- und Untergrundhabitaten auf.

Vertreter der Gattung *Photobacterium* bildeten einen weiteren großen Teil der Kultursammlung. Auch diese Mikroorganismen wurden bisher nicht als typische Vertreter der Tiefen Biosphäre angesehen. Physiologische und genetische Analysen dieser eng verwandten Isolate zeigten, dass sie zu zwei Populationen gehören, die sich grundsätzlich in ihrer Verteilung in den Sedimenten und ihrer Diversität unterscheiden. Die Ergebnisse der vorliegenden Arbeit machen deutlich, dass Sapropele von diversen mikrobiellen Gemeinschaften besiedelt werden, deren Vertreter auf unterschiedliche Weise an die Bedingungen in diesen Schichten angepasst sind.

Summary

Eastern Mediterranean sediments are characterised by the periodical occurrence of dark green, organic rich layers. These so called sapropels exhibit significantly elevated cell numbers and activities and are dominated by so far uncultured members of the *Chloroflexi* and the marine *Crenarchaea*. However, cultivation attempts have met with low success. Therefore, physiological capacities and adaptations of indigenous sapropel microbes to their environment are only poorly understood.

In the present study improved culture media and new cultivation techniques were applied to cultivate relevant members of sapropel communities. Thereby cultivation efficiency could be increased by several orders of magnitude (up to 3.3 %) and a unique culture collection was established (27 phylotypes of six bacterial phyla). Molecular analysis of selected initial enrichments revealed that much more sapropel bacteria, including members of the *Chloroflexi*, were stimulated to grow but were not brought into pure culture.

Phylogenetic analysis of pure cultures revealed that 30 % of the strain collection was represented by close relatives of the soil bacterium *Rhizobium radiobacter*. In order to unravel the ecological role of these microbes first their *in situ* abundance was determined. By this analysis the numerical dominance of a cultivated deep biosphere bacterium in the environment was demonstrated for the first time. It was shown that *R. radiobacter* is widespread in Mediterranean sediments and contributes substantially to the bacterial communities in sapropels (up to 5 %). The detection of these microbes in the deep biosphere raises fundamental questions concerning interactions between surface and subsurface environments.

Members of the genus *Photobacterium*, which were previously not recognized as typical deep biosphere microorganisms, also formed a large fraction of the culture collection. Investigating the physiology and genetics of these closely related isolates revealed that they belong to two distinct populations that differ significantly in microdiversity and spatial distribution.

The results of this work demonstrate that sapropels harbour diverse microbial communities. Their members exhibit different strategies to survive in this challenging environment.

Abkürzungsverzeichnis

AQDS	9,10-Anthrachinone-2,6-5 Disulfonat
ATP	Adenosintriphosphat
cAMP	zyklisches Adenosinmonophosphat
DGGE	*Denaturing Gradient Gel Electrophoresis*
DNA	*Desoxy Ribonucleic Acid*
ERIC	*Enterobacterial Repetitive Intergenic Consensus*
HPLC	*High Performance Liquid Chromatography*
mbsf	metres below seafloor
MPN	*Most Probable Number*
nifH	Gen der eisenhaltigen Untereinheit der Nitrogenase
OTU	*Operational Taxonomic Unit*
PCR	*Polymerase Chain Reaction*
PSU	*Practical Salinity Unit*
qPCR	*quantitative PCR*
TOC	*Total Organic Carbon*
RNA	*Ribonucleic acid*
rrn	Gen der ribosomalen RNA
v/v	*volume per volume* (Volumenprozent)
w/v	*weight per volume* (Gewichtsprozent)

1. Einleitung

1.1 Kultivierungsabhängige Analyse mikrobieller Gemeinschaften

Einleitende Betrachtung. Aufgrund ihrer vielfältigen metabolischen Fähigkeiten, ihrer ubiquitären Verbreitung und ihrer großen Gesamtbiomasse sind Prokaryoten von essentieller Bedeutung für biogeochemische Prozesse auf der Erde (Whitman et al. 1998). Um die ökologische Rolle der Mikroorganismen an einem bestimmten Standort verstehen zu können, müssen (i) die Zusammensetzung der mikrobiellen Gemeinschaft, (ii) die Abundanz der Mikroorganismen und (iii) deren Aktivität *in situ* untersucht werden. Lange Zeit waren die dafür zur Verfügung stehenden Methoden auf die Kultivierung und Charakterisierung von Reinkulturen beschränkt. Da meist nur ein geringer Anteil der in einem Habitat lebenden Prokaryoten kultiviert werden kann (Amann et al. 1995), wurden nicht nur die Biomasse und die Diversität, sondern auch die Ausdehnung der mikrobiellen Biosphäre lange Zeit unterschätzt.

Die Entwicklung molekularer Methoden in den 70er und 80er Jahren kam einem Quantensprung gleich. Die Verwendung ribosomaler Gene als phylogenetische Marker für die Rekonstruktion von Verwandtschaftsbeziehungen (Woese & Fox 1977) revolutionierte die bis dahin bestehende Einteilung lebender Organismen in Prokaryoten, Protisten, Pilze, Pflanzen und Tiere (Whittaker 1959). Der universelle Baum des Lebens umfasst heute die drei Domänen *Bacteria, Archaea* und *Eucarya* (Woese et al. 1990). Molekularbiologische Techniken ermöglichten die Analyse mikrobieller Gemeinschaften unabhängig von deren Kultivierung (Olsen et al. 1986) und enthüllten die erstaunliche Diversität der Prokaryoten (Giovannoni et al. 1990, Ward et al. 1990). Während bis vor 20 Jahren 12 phylogenetische Großgruppen innerhalb der *Bacteria* und zwei innerhalb der *Archaea* bekannt waren, umfassen diese Domänen mittlerweile über 50 verschiedene Divisionen, von denen viele ausschließlich Sequenzen bisher nicht kultivierter Organismen enthalten (Hugenholtz et al. 1998, Keller & Zengler 2004). Der tatsächlich vorhandenen Biodiversität, die auf bis zu 10^9 Taxa geschätzt wird, stehen etwa 6000 beschriebene Arten gegenüber (Fry 2000, Keller & Zengler 2004).

Molekulare Methoden ermöglichten außerdem die Quantifizierung spezifischer phylogenetischer Gruppen mit fluoreszenzmarkierten RNA- oder DNA-Sonden. Die Fluoreszenz *in situ* Hybridisierung (FISH; Amann et al. 1990) und die quantitative PCR (Lie & Petropoulos 1998) sind heute neben der Fluoreszenzmikroskopie und kultivierungs-

abhängigen Methoden, gängige Techniken zur Quantifizierung von Mikroorganismen in Umweltproben. Man schätzt die Anzahl aller Prokaryoten auf der Erde heute auf 4-6 x 10^{30} Zellen, die über 60 % der gesamten Biomasse der Erde enthalten (Whitman et al. 1998). Es gibt kaum einen Lebensraum, der nicht von Prokaryoten besiedelt wird (McInerney James et al. 2002).

Auch für die Analyse mikrobieller Aktivitäten *in situ* stehen heute exzellente Techniken zur Verfügung. Radioaktiv markierte Substrate und Mikrosensoren ermöglichen die Messung von Stoffflüssen und die Berechnung von Umsatzraten (Jørgensen 1978, Minz et al. 1999). Mit fluoreszenzmarkierten Substratanaloga kann die Aktivität zellspezifischer Exoenzyme bestimmt werden (Hoppe 1983) und molekulare Methoden ermöglichen die Detektion und Quantifizierung von Schlüsselgenen standortrelevanter Prozesse (Neretin et al. 2003, Inagaki et al. 2004, Schippers & Neretin 2006). Mit Hilfe des *Stable Isotope Probings* (Boschker et al. 1998) oder der Kombination von Mikroautoradiografie und FISH (Lee et al. 1999) können Mikroorganismen, die am Standort aktiv bzw. für spezifische Stoffwechselwege verantwortlich sind, identifiziert werden. Obwohl diese Methoden zum grundlegenden Verständnis mikrobieller Prozesse beitragen, können spezifische Standort-anpassungen und das gesamte Spektrum an physiologischen Kapazitäten eines Mikroorganismus jedoch nur an Reinkulturen untersucht werden (Fry 2000). Die Kultivierung relevanter Vertreter mikrobieller Gemeinschaften ist nach wie vor eine der größten Herausforderungen für Mikrobiologen.

Verbesserung von Kultivierungstechniken. Obwohl nachgewiesen wurde, dass der größte Teil der an einem Standort lebenden Mikroorganismen metabolisch aktiv ist (Fry 1990, Karner & Fuhrman 1997) wachsen meist weniger als 1 % der mikroskopisch detektierbaren Zellen unter Laborbedingungen an (Amann et al. 1995). Für die „Unkultivierbarkeit“ von Mikroorganismen gibt es zwei Gründe (i) entweder die Zellen befinden sich in einem Zustand, in dem sie sich nicht vermehren können oder (ii) die natürlichen Lebensbedingungen des Organismus können im Labor nicht hinreichend simuliert werden.

Faktoren wie Zellschäden (Nyström 1998), die Induktion temperenter Phagen (Wommack & Colwell 2000) oder der Übergang der Zellen in den *viable but non culturable* Zustand, in dem sie aktiv, aber nicht teilungsfähig sind (Roszak & Colwell 1987), verhindern das Wachstum im Labor, sind jedoch wenig beeinflussbar. Andere Probleme, wie z.B. der Tod der Zellen infolge unnatürlich hoher Nährstoffkonzentrationen in Kulturmedien (Postgate & Hunter 1963) können durchaus gelöst werden. Durch den Einsatz extrem geringer

Substratmengen konnten deutlich höhere Kultivierungseffizienzen erzielt (Bussmann et al. 2001, Eilers et al. 2001, Connon & Giovannoni 2002) und Vertreter ubiquitärer, jedoch selten kultivierter Phyla z.B. der SAR 11-Gruppe der *γ-Proteobacteria* isoliert werden (Rappé et al. 2002). Auch der Einsatz natürlicher Medien ohne künstlich zugesetzte Substrate, wie z.B. sterilisiertes See- bzw. Meerwasser oder Sedimentextrakte, war in vergangenen Studien sehr erfolgreich (Schut et al. 1993, Vester & Ingvorsen 1998, Selje et al. 2005). Kaeberlein und Lewi (2002) simulierten die natürlichen Standortbedingungen in Durchflusskammern und konnten auf diese Art und Weise Vertreter der *Cytophaga-Flexibacter-Bacterioides*-Gruppe isolieren.

Der Kultivierungserfolg wird außerdem erheblich durch die Zusammensetzung synthetischer Medien beeinflusst. Der Einsatz vieler verschiedener Substrate oder Variationen in den Kultivierungsbedingungen, z.B. Veränderungen des CO_2-Partialdrucks, erwiesen sich in der Vergangenheit als sinnvoll. Auf diese Weise konnten viele neue Arten innerhalb der *Proteobacteria*, *Actinobacteria*, *Acidobacteria*, *Chloroflexi* und *Verrumicrobia* aus Bodenproben isoliert werden (Janssen et al. 2002, Sait et al. 2002, Stevenson et al. 2004, Davis et al. 2005).

Um die Vermehrung abundanter, aber langsam wachsender Arten ohne Konkurrenzdruck zu ermöglichen, hat sich die Vereinzelung der Zielorganismen durch starkes Verdünnen von Umweltproben bewährt (Schut et al. 1993, Connon & Giovannoni 2002, Rappé et al. 2002, Rappé & Giovannoni 2003). Dabei besteht jedoch die Gefahr, dass Mikroorganismen aufgrund fehlender Interaktionen, wie Syntrophie oder Zellkommunikation, nicht anwachsen (Kaiser & Losick 1993). Zengler und Mitarbeiter (2000) separierten Mikroorganismen deshalb in Nano-Droplets, die den Austausch von Metaboliten und Signalmolekülen erlaubten. Andere Möglichkeiten sind Co-Kulturen (Kaeberlein et al. 2002) oder die Zugabe von spezifischen Signalstoffen wie cAMP oder Homoserinlakton (Bruns et al. 2002).

In den meisten Studien wurden die einzelnen Strategien miteinander kombiniert und durch lange Inkubationszeiten, sensitive Methoden zum Nachweis mikrobiellen Wachstums (Fluoreszenzmikroskopie, Durchflusszytometrie) und molekulare Methoden zur gezielten Detektion spezifischer Gruppen ergänzt (Mitsui et al. 1997, Connon & Giovannoni 2002, Janssen et al. 2002, Zengler et al. 2002, Stevenson et al. 2004).

Die Rolle kultivierter Mikroorganismen am Standort. Die häufig beobachtete Diskrepanz zwischen den Ergebnissen molekularbiologischer und kultivierungsabhängiger Analysen legt

die Vermutung nahe, dass die meisten kultivierten Arten nur von untergeordneter Bedeutung für die Stoffumsätze in ihrer natürlichen Umgebung sind (Felske et al. 1999, Burns et al. 2004, Lysnes et al. 2004). Deshalb ist die Bestimmung der Abundanz kultivierter Mikroorganismen am Standort genauso essentiell wie physiologische, biochemische und genetische Analysen, die helfen ihre ökologische Rolle aufzuklären.

Dazu bieten sich prinzipiell verschiedene molekulare Techniken an. Eine Variante sind Hybridisierungsverfahren. Dazu werden ausgehend von der 16S rRNA-Genssequenz der Isolate spezifische rRNA- komplementäre Sonden konstruiert, die zur Detektion von Zellen mittels FISH (Amann et al., 1990) oder zur Quantifizierung von rRNA-Konzentrationen durch rRNA-*Slot-Blots* (Stahl et al. 1988) eingesetzt werden. Die *Catalyzed Reporter Deposition Fluoreszenz in situ Hybridisation* (CARD-FISH, Pernthaler et al. 2002) ist eine Weiterentwicklung der FISH-Methode, die die Detektion von Zellen mit geringem rRNA-Gehalt ermöglicht.

Eine Alternative zu diesen Hybridisierungstechniken ist die quantitative PCR. Diese Technik, ursprünglich für die medizinische Diagnostik entwickelt, fand in den letzten Jahren in zunehmendem Maße Anwendung in der mikrobiellen Ökologie (Walker 2002, Valasek & Repeta 2005). Der Vorteil dieser Methode ist ihre extrem hohe Sensitivität. Theoretisch kann mit Hilfe PCR-basierter Methoden eine einzelne Genkopie nachgewiesen werden (van Kuppeveld et al. 1992). Limitierend wirkt dagegen die oft geringe Effizienz der DNA-Extraktion aus Umweltproben (Head et al. 1998). Um dieses Problem zu umgehen wird meist der relative Anteil spezifischer Gene an der Gesamtheit aller in einer Probe vorliegenden 16S rRNA-Gene bzw. an der Gesamt-DNA bestimmt (Bach et al. 2002, Stubner 2002).

Das Prinzip der quantitativen PCR. Bei der quantitativen PCR wird die Amplifikation eines spezifischen DNA- oder RNA-Fragments in „Echtzeit“ verfolgt, weshalb diese Methode auch *real-time* PCR genannt wird. Dazu können interkalierende Substanzen (Wittwer et al. 1997) oder TaqMan-Sonden (Holland et al. 1991) verwendet werden. Interkalierende Substanzen (z.B. SybrGreenI) lagern sich in doppelsträngige DNA ein und senden bei UV-Anregung 1000-fach höhere Fluoreszenzsignale aus als frei in der Lösung vorliegende Moleküle (Abb. 1A). Bei TaqMan-Sonden handelt es sich um kurze DNA-Sonden, die spezifisch an das zu amplifizierende DNA-Fragment binden. Diese Sonden tragen an ihrem 5´-Ende einen fluoreszenzmarkierten *reporter* und an ihrem 3´-Ende einen *quencher*, der das Fluoreszenzsignal des *reporters* abdämpft. Durch die Exonukleaseaktivität der Taq-

Polymerase werden diese beiden Moleküle voneinander getrennt und ein Fluoreszenzsignal emittiert (Abb. 1B). Bei beiden Varianten steigt die Fluoreszenz im PCR-Ansatz proportional zur Menge an gebildetem Produkt an (siehe Abb. 2 Kapitel 2.3).

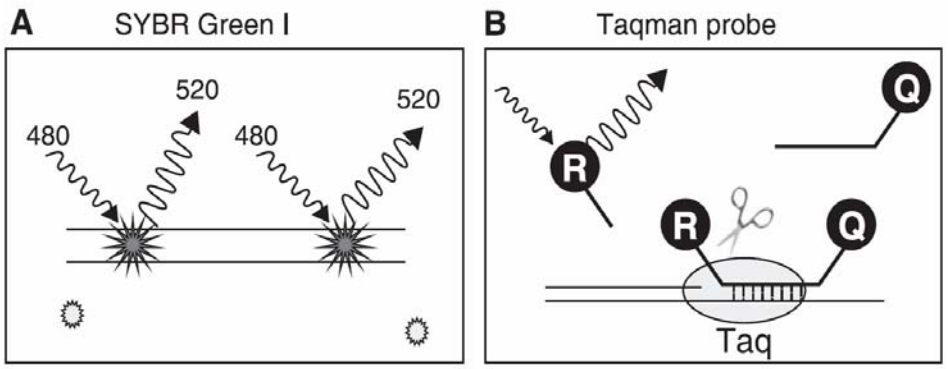

Abb. 1. Detektionssysteme für die quantitative PCR. Die Akkumulation spezifischer Genfragmente während der Amplifikation kann direkt durch den Einsatz interkalierender Substanzen (SybrGreenI) oder indirekt mittels fluoreszenzmarkierter TaqMan-Sonden verfolgt werden. (Aus: Valasek & Repeta, 2005)

Die Quantifizierung basiert auf der Kinetik der PCR-Reaktion, wobei der so genannte C_T-Wert (*treshold cycle*) die wichtigste Kenngröße ist. Der C_T-Wert ist als der PCR-Zyklus definiert, bei dem die Fluoreszenz erstmals signifikant die Hintergrundfluoreszenz übersteigt. Die Amplifikation befindet sich hier in der exponentiellen Phase, in der ihre Effizienz am größten ist und noch keine reaktionsbedingten Limitationen auftreten (*Primer*-Mangel, nachlassende Enzymaktivität, Endprodukthemmung). Durch den Vergleich mit einer Standardkurve kann die Menge der ursprünglich in dem PCR-Ansatz vorhandenen Menge an DNA- bzw. RNA-Fragmenten berechnet werden.

1.2 Das Untersuchungsgebiet: Sapropele des östlichen Mittelmeeres

Das moderne Mittelmeer. Das Mittelmeer ist ein typisches Randmeer. Es wird vom europäischen und afrikanischen Kontinent vollständig eingeschlossen und ist nur über eine schmale Verbindung, die Straße von Gibraltar, mit den großen Ozeanen, in diesem Falle dem Atlantik, verbunden. Seine Lage an der geografisch-tektonischen Kollosionszone zwischen zwei Kontinententalplatten bedingt die komplizierte, durch Schwellen gegliederte Basimetrie des Mittelmeeres. Die Sizilien-Malta-Schwelle trennt das Mittelmeer in einen westlichen und einen östlichen Teil. Letzterer wird vom Mittelmeerischen Rücken nochmals in zwei Bereiche untergliedert, das Ionische und das Levantinische Becken. Während die Schwellen nur wenige

hundert Meter tief sind, findet man in den Becken zwischen den einzelnen Schwellen ausgedehnte Tiefseebereiche mit über 4000 m Wassertiefe (Vanney & Gennesseaux 1985).

Im modernen Mittelmeer übersteigt die Verdunstungsrate den Fluss- und Niederschlagseintrag. Diese negative Wasserbilanz wird durch den Zustrom aus dem Atlantik ausgeglichen, wodurch sich ein anti-ästuarines Zirkulationsmuster ausbildet, das den Export von Nährstoffen aus dem östlichen Mittelmeer zur Folge hat. Nährstoffarmes Oberflächenwasser strömt aus dem Atlantik in das westliche Mittelmeer ein. Auf dem Weg in das östliche Becken nehmen Salinität und Dichte des Wassers durch Verdunstung zu, die Oberflächenwasser sinken ab und fließen als Intermediärwasser in das westliche Mittelmeer zurück. Augrund dieses Zirkulationsmusters ist das östliche Mittelmeer ein extrem oligotrophes Habitat mit geringer Primärproduktion, niedrigen Sedimentationsraten und oxischen Verhältnissen in den Tiefseebereichen.

In jüngster Zeit rückten zwei Besonderheiten des Mittelmeeres in den Mittelpunkt mikrobiologischer Untersuchungen: (1) hypersaline anoxische Becken, die durch freiliegende Evaporite (Salzstöcke) im Gebiet des Mittelmeerischen Rückens ausgebildet wurden (Sass et al. 2001, van der Wielen et al. 2005) und (2) die zyklisch in den Sedimenten auftretenden Sapropellagen (Cragg et al. 1998, Coolen & Overmann 2000, Coolen et al. 2002).

Sapropele. Die pliozänen bis holozänen Sapropele (griechisch: Faulschlamm) wurden als diskrete Sedimentlagen mit einer Mächtigkeit von mindestens einem Zentimeter und einem Gehalt an organischem Kohlenstoff (TOC) von mindestens 2 % definiert (Kidd et al. 1978). Der TOC-Gehalt der Sapropele kann jedoch über 30 % des Trockengewichts betragen (Passier et al. 1999). Das organische Material der Sapropele ist bis zu 5,3 Millionen Jahre alt (Emeis et al. 1996) und besteht hauptsächlich aus dunkelbraunem, amorphem, hochrefraktärem Kerogen (Aksu et al. 1999) und ist größtenteils marinen Ursprungs (Emeis et al. 1996, Bouloubassi et al. 1999). Sapropele treten zeitgleich im gesamten Bereich des östlichen Mittelmeeres auf und sind auch vereinzelt im westlichen Mittelmeer zu finden (Stanley 1978).

Hypothesen zur Entstehung von Sapropelen. Die Zyklizität der Mittelmeersedimente ist eng an regelmäßige Variationen orbitaler Erdparameter (Milankovitch Zyklen, Abb. 2) und damit verbundener klimatischer und ozeanografischer Veränderungen gekoppelt (Rohling 1994, Rossignol-Strick et al. 1998, Emeis et al. 2000).

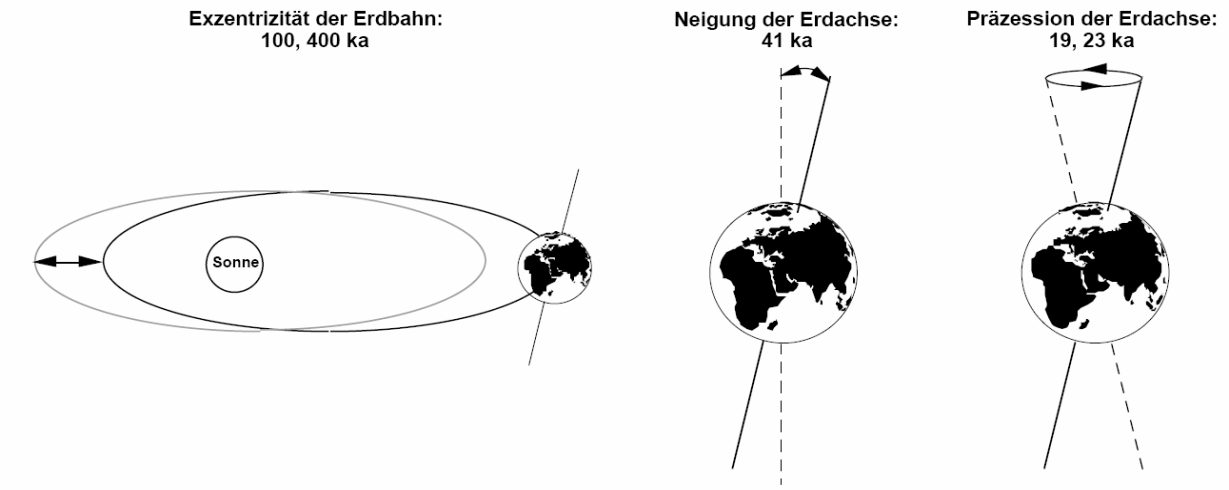

Abb. 2. Zyklische Variationen der orbitalen Parameter (Milankovitch-Zyklen). Variationen in der Exzentrizität der Erdbahn beeinflussen den Kontrast zwischen den Strahlungsintensitäten bei sonnennächster (Perihel) und sonnenfernster Position (Aphel). Schwankungen in der Neigung der Erdachse wirken sich hauptsächlich auf die Unterschiede zwischen Sommer- und Wintereinstrahlung in hohen Breiten aus während Änderungen in der Präzession der Erdachse sich in niedrigen Breiten bemerkbar machen. Die Kombination dieser Variationen führt etwa alle 21 ka zu einer erhöhten Sonneneinstrahlung auf der Nordhalbkugel. 1 ka = 1000 Jahre

Periodische Schwankungen der Exzentrizität, der Neigung und der Präzession der Erdachse (Abb. 2) führen etwa alle 21.000 Jahre zu einer erhöhten Sonneneinstrahlung auf der nördlichen Hemisphäre (Insolationsmaxima). Zu diesen Zeiten werden Sapropele gebildet. Unter den derzeit herrschenden Bedingungen (Insolationsminima) werden dagegen carbonathaltige, organisch-arme Zwischenschichten abgelagert (Fischer & Bottjer 1991).

Nach wie vor werden die Paläoumweltbedingungen, die zur Bildung von Sapropelen führen, kontrovers diskutiert. Die bislang plausibelste Erklärung ist ein vermehrter Süßwassereinstrom, der durch humides Klima während der Insolationsmaxima hervorgerufen wird. Mögliche Eintragsquellen sind der Nil (Rossignol-Strick 1983, 1985), die Flüsse der nördlich an das Mittelmeer angrenzenden Gebiete (Cramp et al. 1988, Rohling & Hilgen 1991) und Schmelzwasser aus dem Schwarzen Meer (Ollausson 1961). In Folge des Süßwassereinstroms könnte es zu einer Anhebung der Dichtesprungschicht bis in die photische Zone (Rohling & Gieskes 1989, Rohling 1994) oder zur Umkehr des derzeitigen Zirkulationsmusters gekommen sein (Sarmiento et al. 1988). Letzteres hätte das Recycling von Nährstoffen im östlichen Mittelmeer und somit eine erhöhte Primärproduktion zur Folge (Sutherland et al. 1984, Krom et al. 1992). Im Gegensatz dazu postulieren Sachs und Repeta (1999) ausgehend von Stickstoffisotopenmessungen fossiler Chlorophylle, oligotrophe Verhältnisse zu Zeiten der Sapropelbildung, unterstützen jedoch die Annahme einer geschichteten Wassersäule. Umgekehrte Strömungsverhältnisse oder die Schichtung der Wassersäule hätten in jedem Fall die Ausbildung anoxischer Tiefenwasser zur Folge, was

letztendlich zur Konservierung des organischen Materials in den Sedimenten führte. Die Detektion von Biomarkern grüner Schwefelbakterien in den Sapropelen deuten darauf hin, dass nicht nur in den Tiefseebereichen, sondern bis in die photische Zone hinein euxinische (sulfidische) Verhältnisse geherrscht haben müssen (Passier et al. 1999, Menzel et al. 2002)

Viele Sapropele weisen Laminierungen (Negri et al. 2003, Capozzi et al. 2006) und/oder Unterbrechungen auf (Myers & Rohling 2000, Meyers & Arnaboldi 2005). Zudem existieren zu den Sapropelen mit den höchsten TOC-Gehalten des östlichen Beckens keine Gegenstücke im westlichen Mittelmeer (Bouloubassi et al. 1999). Demnach könnten regionale Faktoren einen ebenso großen Einfluss auf die Bildung der Sapropele gehabt haben, wie globale klimatische Veränderungen.

1.3 Sapropele als *Hot Spots* der marinen Tiefen Biosphäre

Die Entdeckung der marinen Tiefen Biosphäre. Obwohl marine Sedimente das größte Kohlenstoffreservoir der Erde darstellen (Hedges & Keil 1995), bestand lange Zeit die Ansicht, dass diese wegen ihrer lebensfeindlichen Bedingungen (geringe Substratverfügbarkeit, hoher Druck, abnehmende Porosität) nur in den oberen Metern besiedelt sind (Parkes et al. 2000). Morita und Zobell (1955) erklärten eine Tiefe von 7,47 Metern als untere Grenze der marinen Biosphäre, da aus darunter liegenden Schichten keine Mikroorganismen mehr angereichert werden konnten. Obwohl Änderungen in der Zusammensetzung des Porenwassers, Gasproduktion, der Abbau organischen Materials und Änderungen im Verhältnis stabiler Isotope auf mikrobielle Aktivitäten in großen Tiefen hinwiesen, gab es bis vor etwa 20 Jahren keine mikrobiologischen Untersuchungen, die dies hätten belegen können (Sinclair & Ghiorse 1989). Im Jahr 1986 konnten auf einer Ausfahrt des *Ocean Drilling Programms* in den Pazifik (ODP Leg 112) erstmals ungestörte, unkontaminierte Sedimentkerne aus mehr als 700 m Sedimenttiefe gewonnen werden. Gesamtzellzahlbestimmungen (Parkes et al. 1990), PCR-basierte DNA-Analysen (Rochelle et al. 1994), die Isolierung von Bakterien mit spezifischen Anpassungen an Tiefseehabitate (Bale et al. 1997, Barnes et al. 1998) und Aktivitätsmessungen (Cragg et al. 1992) zeigten die Anwesenheit lebender Mikroorganismen und bewiesen somit die Existenz der marinen Tiefen Biosphäre. Heute geht man davon aus, dass die ozeanische Kruste bis in mehrere Kilometer Tiefe besiedelt ist (Parkes et al. 2000, Cowen et al. 2003, Huber et al. 2006) und mehr als 50 % der gesamten mikrobiellen Biomasse der Erde beherbergt (Whitman et al. 1998). Deshalb wird

eine große Bedeutung der marinen Tiefe Biosphäre für globale Stoffkreisläufe und die fortschreitende Diagenese von Sedimenten postuliert (Parkes et al. 1994, Sass et al. 2003, Webster et al. 2003).

Die Stimulation mikrobieller Gemeinschaften in tiefen marinen Sedimenten. Prinzipiell hängt die mikrobielle Aktivität in marinen Sedimenten von dem in der photischen Zone durch Primärproduktion gebildeten organischen Kohlenstoff ab (Abb. 3). Da weniger als 0,5 % des dort gebildeten organischen Materials überhaupt in die Tiefseebereiche der Ozeane gelangt (Hedges 1992), zeichnet sich die Tiefe Biosphäre im marinen ebenso wie im terrestrischen Bereich durch extrem geringe Stoff- und Energieflüsse aus (Chapelle & Lovley 1990). Die Stoffwechselaktivitäten indigener Mikroorganismen sind dementsprechend niedriger als an der Oberfläche (Lovley & Chapelle 1995).

Da Sauerstoff nur wenige Millimeter tief ins Sediment eindringt (Revsbech et al. 1980), wird organisches Material in tieferen Schichten hauptsächlich durch anaerobe Atmung und Gärung abgebaut. Die Verwertung alternativer Elektronenakzeptoren folgt dabei ihrem abnehmenden Redoxpotenzial (Jørgensen 1989), wodurch sich eine typische Abfolge metabolischer Prozesse ergibt (Elektronenturm, Abb. 3, Mitte). Mit zunehmender Tiefe werden nacheinander Nitrat, Mangan (IV), Eisen (III) und Sulfat reduziert. In den darunter liegenden Schichten schließt sich bei abnehmenden Sulfatkonzentrationen die energetisch ungünstigere Methanogenese an. D´Hondt und Mitarbeiter (2004) zeigten, dass diese Prozesse in tiefen Sedimenten in umgekehrter Folge ablaufen können (inverser Elektronenturm, Abb. 3, unten). Ursachen dafür sind Sulfateinträge aus unterirdischen Solen, die Freisetzung von Eisen und Mangan aus eingelagerten Mineralien oder Sauerstoff- und Nitrat-Einträge aus Wasserleitern, die die Basalte unterhalb der Sedimente durchströmen.

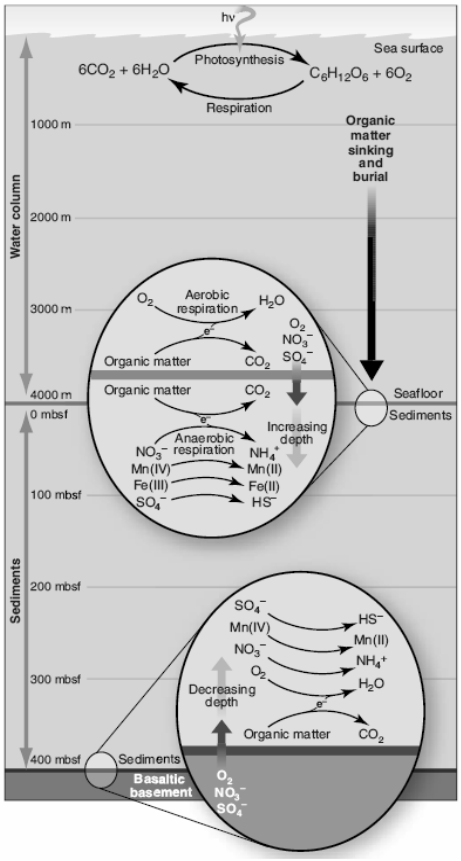

Abb. 3. Kohlenstoffremineralisierung in der Wassersäule, an der Sedimentoberfläche und an der Sediment-Basalt-Übergangszone. Aufwärtsdiffusion von Sauerstoff, Nitrat und Sulfat aus dem Basalt (Aus: DeLong 2005).

Trotz der generell geringen Aktivitäten gibt es auch in sehr tiefen Bereichen Zonen, in denen mikrobielle Prozesse stimuliert werden (*Hot Spots*). Dazu gehören geochemische Grenzschichten (Methan-Sulfat-Grenzschicht, Parkes et al. 2005) oder diskrete Sedimentlagen, die sich lithologisch von den umgebenden Schichten unterscheiden wie Diatomeen- (Parkes et al. 2005) oder Ascheschichten (Inagaki et al. 2003). In diesen Zonen wurden erhöhte Zellzahlen und Aktivitäten detektiert, die meist mit einem deutlichen *Populationsshift* einhergingen. Auch Sedimente mit eingelagerten Methanhydraten sind durch erhöhte mikrobielle Aktivitäten gekennzeichnet (Cragg et al. 1995a, Cragg et al. 1995b). In und unterhalb dieser Hydrate wurden Methanogeneseraten gemessen, die um mehrere Größenordnungen höher lagen, als in oberflächennahen Sedimenten. Außerdem gibt es Hinweise, dass mikrobielle Gemeinschaften in sehr tiefen Schichten infolge ansteigender Temperaturen durch thermogen gebildete Substrate (Acetat, Methan, Wasserstoff) stimuliert werden (Wellsbury et al. 1997, Parkes et al. 2000).

Mikrobielle Aktivität in Sapropelen. Auch Sapropele unterscheiden sich durch ihren hohen TOC-Gehalt von anderen Standorten der marinen Tiefen Biosphäre. Während die Zellzahlen von Prokaryoten in organisch armen Sedimenten mit der Tiefe um mehrere Größenordnungen abnehmen (Parkes et al. 1994, D'Hondt et al. 2004, Schippers et al. 2005), triff dies auf Sapropele nicht zu. Cragg et al. (1998) zeigten, dass die Zellzahlen in Sapropelen mit der Tiefe bzw. dem Alter der entsprechenden Lagen sogar zunehmen. Die vergleichsweise hohen Zellzahlen der Sapropele korrelieren mit einem erhöhten Anteil an sich teilenden bzw. geteilten Zellen (bis zu 10 %, Cragg et al. 1998, Coolen et al. 2000), was auf aktiv wachsende Populationen hindeutet (Getliff et al. 1992). Erhöhte ATP-Gehalte (Overmann et al. 2001) und physiologische Aktivitäten unterstützen diese Annahme (Coolen et al. 2002). Demnach stimuliert das organische Material der Sapropele seit mehreren Millionen Jahren das Wachstum indigener Mikroorganismen und unterliegt im Gegenzug einem fortwährenden mikrobiellen Abbau. Da sogar 5,3 Millionen Jahre alte Sapropele bis in die heutige Zeit erhalten geblieben sind, geschieht dies offensichtlich extrem langsam.

Vorangegangene Studien lieferten erste Ergebnisse zu den mikrobiellen Prozessen in diesen ungewöhnlichen Sedimentschichten. Die dissimilatorische Sulfatreduktion, einer der dominierenden anaeroben Prozesse in organisch reichhaltigen Sedimenten (Jørgensen 1982), scheint in Sapropelen trotz hoher Sulfatkonzentrationen von untergeordneter Bedeutung zu sein (Böttcher et al. 1998, Passier et al. 1999). Die in und unterhalb der Sapropele abgelagerten Pyrite (FeS_2) zeigen jedoch, dass Sulfatreduktion während und auch noch nach

ihrer Ablagerung eine große Rolle gespielt haben muss (Passier et al. 1999). Coolen und Mitarbeiter (2000, 2002) fokussierten ihre Untersuchungen auf die Analyse initialer Schritte der anaeroben Atmungskette, d. h. die Hydrolyse von Biopolymeren durch zellspezifische Exoenzyme und den Abbau von Monomeren. Aufgrund erhöhter β-Glukosidaseaktivitäten und Abbauraten ^{14}C-markierter Glukose in den Sapropelen schlossen sie, dass Glukose ein Substrat für indigene Mikroorganismen sein könnte. Dies wurde durch vergleichsweise hohe Glukoseumsatzraten unter *in situ* Bedingungen bestätigt. Die Annahme, dass Sapropele noch labile organische Substrate enthalten, würde sich mit Untersuchungen von Moodley et al. (2005) decken. Anhand von Laborexperimenten postulierten sie, dass der Abbau des organischen Materials während der Bildung der Sapropele durch extrem hohe Sulfidkonzentrationen unterbunden wurde und reaktive Substanzen erhalten geblieben sind. In jedem Fall lieferten die Untersuchungen von Coolen und Mitarbeitern (2002) Hinweise darauf, dass primäre und sekundäre Gärer eine wichtige Rolle beim Abbau des organischen Materials der Sapropele spielen könnten.

Dominanz selten kultivierter Phyla in Sapropelen. Obwohl eine Vielfalt an metabolischen Stoffwechselaktivitäten in tiefen marinen Sedimenten nachgewiesen wurde, weiß man bisher nur wenig über die Organismen, die für diese Prozesse verantwortlich sind. Wie die meisten bisher untersuchten Standorte der marinen Tiefen Biosphäre (Rochelle et al. 1994, Li et al. 1999, Kormas et al. 2003, Webster et al. 2004) werden die mikrobiellen Gemeinschaften der Sapropele von Organismen dominiert, deren physiologische Eigenschaften weitgehend unbekannt sind. Mittels PCR-DGGE und anschließender Sequenzierung dominanter DGGE-Banden wurden mit einer Ausnahme (*Geobacter sulfurreducens*) ausschließlich Vertreter der *Chloroflexus*-Gruppe und der *Marine Group* I *Crenarchaea* detektiert (Coolen et al., 2002). Quantitative Analysen zeigten, dass beide Gruppen zusammen einen erheblichen Anteil der Sapropelgemeinschaften bilden (bis zu 84 %; Anteil spezifischer DNA an Gesamt-DNA). *Archaea* wurden nur in Sapropelen, jedoch nicht in den Zwischenschichten detektiert, und nahmen im Durchschnitt 16 % ein, während der Anteil der *Chloroflexi* bis zu 70 % betrug.

Obwohl Vertreter beider Phyla weit verbreitet sind, gibt es bisher nur einige wenige Isolate. *Dehalococcoides ethenogenes*, der nächste kultivierte Verwandte der in den Sapropelen und anderen Bereichen der Tiefen Biosphäre detektierten *Chloroflexi* (Chandler et al. 1998, Parkes et al. 2005, Inagaki et al. 2006) wurde aus verunreinigtem Grundwasser isoliert (Maymo-Gatell et al. 1997). Dieser Organismus ist auf den Abbau halogenierter Kohlenwasserstoffe spezialisiert. Diese Fähigkeit wurde jedoch sehr wahrscheinlich durch

horizontalen Gentransfer erworben (Regeard et al. 2005). Bei dem einzigen kultivierten Vertreter der *Marine Group* I *Crenarchaea*, *Nitrosopumilus maritimus*, handelt es sich um einen autorophen Ammoniumoxidierer (Könneke et al. 2005). Ob die in den Sapropelen lebenden Vertreter dieser Phyla ähnliche Fähigkeiten besitzen wie ihre nächsten kultivierten Verwandten, ist unklar.

Meteorausfahrt M51/3. Kultivierungsversuche hinsichtlich indigener Mikroorganismen waren bisher wenig erfolgreich. Mit traditionellen Ansätzen konnten lediglich aus einer einzigen Sapropelprobe Mikroorganismen angereichert werden, wobei die Kultivierungseffizienz bei 0,000024 % lag (Coolen & Overmann 2000). Die physiologischen Kapazitäten und Anpassungen indigener Mikroorganismen an die Bedingungen in den Sapropelen sind deshalb vollkommen unbekannt.

Im November 2001 fand eine weitere Forschungsausfahrt ins östliche Mittelmeer statt. Während dieser Ausfahrt wurden an mehreren Standorten (#562, #567, #575; Kapitel 2.1) bis zu 12 m lange Sedimentkerne gewonnen und verschiedene Sapropele und Zwischenschichten beprobt. Der Fokus der nachfolgenden Untersuchungen lag auf der Kultivierung relevanter Vertreter der mikrobiellen Gemeinschaften dieser Schichten. Um den Kultivierungserfolg zu steigern, wurden im Wesentlichen drei Strategien verfolgt:

1) Einsatz vieler verschiedener, auch schwer abbaubarer Substrate in verschiedenen Kombinationen und geringen Konzentrationen, um das Wachstum vieler verschiedene Mikroorganismen zu stimulieren
2) Einsatz von Verdünnungsreihen zur Vereinzelung abundanter, aber langsam wachsender Mikroorganismen
3) Anreicherung von Mikroorganismen in ungestörtem Sedimentmaterial.

Um ein breites Spektrum an Medienvariationen bearbeiten zu können, wurden Verdünnungsreihen in Mikrotiterplatten angesetzt. Zum Vergleich wurden Flüssigkulturen in Glasröhrchen und Gradientenflüssigkulturen beimpft. Letztere haben den Vorteil langsam ansteigender Substratkonzentrationen. Zur Umsetzung von Strategie 3) wurden Sedimentstücke in einem Substratgradienten in Agar eingebettet (Sedimentgradientenkulturen, Kapitel 2.2, Abb. 1).

1.4 Zielsetzung der Arbeit

Ziel dieser Studie war es, verschiedene Kultivierungsansätze zu evaluieren und durch die Isolierung und Analyse von Reinkulturen Einblicke in die Diversität der Sapropel-gemeinschaften und in die physiologischen Fähigkeiten und Anpassungen indigener Mikroorganismen zu erhalten.

Zur Abschätzung der Kultivierungseffizienz wurden zunächst Lebendzellzahlen bestimmt und mit den Gesamtzellzahlen in den entsprechenden Schichten verglichen. Aus den höchsten bewachsenen Verdünnungsstufen der MPN-Serien und den Anreicherungskulturen wurden Isolate gewonnen, die die Grundlage für weitere Untersuchungen bildeten. Anhand der Isolate und der molekularen Analyse ausgewählter Originalanreicherungen wurden die Kultivierungsansätze evaluiert und miteinander verglichen. Diese Untersuchungen lieferten wichtige Einblicke in das Potenzial der eingesetzten Techniken und Substratklassen und sind somit richtungweisend für zukünftige Kultivierungsansätze. Die Ergebnisse dieser Analysen sind in den Kapiteln 2.1 und 2.2 zusammengefasst.

Ein weiteres wichtiges Ziel der Arbeit war es, die ökologische Rolle häufig kultivierter Phylotypen am Standort aufzuklären. Dazu wurden zum einen Vertreter der Gattung *Rhizobium* herangezogen. Diese typischerweise in Böden lebenden Mikroorganismen wurden aus allen beprobten Sedimentschichten des Mittelmeeres und auch aus Pazifiksedimenten isoliert (D´Hondt et al. 2004) und waren deshalb besonders interessant. Der Fokus der in Kapitel 2.3 dargestellten Analysen lag auf dem Nachweis der Verbreitung und Abundanz dieser Mikroorganismen in den Mittelmeersedimenten. In dieser Studie konnte erstmals die numerische Dominanz eines kultivierten Vertreters der Tiefen Biosphäre am Standort nachgewiesen werden.

Ein weiterer großer Teil der Kultursammlung wurde von Vertretern der Gattung *Photobacterium* repräsentiert. Diese Isolate wurden für eine hochauflösende geno- und phänotypische Analyse herangezogen (Kapitel 2.4). Diversität unterhalb des Artlevels (Mikrodiversität), ist ein in Oberflächenhabitaten häufig beobachtetes Phänomen (Fuhrman & Campbell 1998, Schloter et al. 2000). Es wurde mehrfach gezeigt, dass Vertreter einer Art distinkte Subpopulationen ausbilden, die unterschiedliche ökologische Nischen besiedeln können (Jaspers & Overmann 2004). Die vorliegende Studie sollte Aufschluss darüber geben, ob Bakterienspezies auch in tiefen marinen Sedimenten einer gewissen Variabilität unterworfen sind, und ob diese von ökologischer Relevanz ist.

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2 Publikationen

2.1 Quantitative analysis of bacterial communities from Mediterranean sapropels based on cultivation-dependent methods

Jacqueline Süß, Bert Engelen, Heribert Cypionka, Henrik Sass

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Quantitative analysis of bacterial communities from Mediterranean sapropels based on cultivation-dependent methods

Jacqueline Süß, Bert Engelen, Heribert Cypionka, Henrik Sass *

Institut für Chemie und Biologie des Meeres, Universität Oldenburg, Carl-von-Ossietzky Straße 9-11, D-26111 Oldenburg, Germany

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Abstract

Microbial communities of ancient Mediterranean sapropels, buried sediment layers of high organic matter, were analyzed by most probable number (MPN) approaches. Mineral media containing different carbon sources in sub-millimolar concentrations were used. MPN numbers were elevated in sapropels and at the sediment surface, which mirrored total cell count distributions. Highest MPN counts were obtained with a mixture of different monomeric and polymeric substrates, with amino acids or with long-chain fatty acids as sole carbon sources. These values reached up to 2×10^7 cm^{-3}, representing 3.3% of the total cell count. A total of 98 pure cultures were isolated from the highest positive dilutions of the MPN series, representing the most abundant microorganisms culturable by the methods used. The strains were identified by molecular biological methods and could be grouped into 19 different phylotypes. They belonged to the α-, β-, γ-, and δ-Proteobacteria, to the Actinobacteria and the Firmicutes. However, about half of the number of isolates was closely related to the genera *Photobacterium* and *Agrobacterium*. Regarding the high cultivation success, these organisms can be assumed to be typical sapropel bacteria, representing a substantial part of the culturable indigenous microbial community.

Keywords: Deep-sea sediment; Sapropel; MPN; Culturability; Marine subsurface sediment

1. Introduction

In sediments of the eastern Mediterranean Sea, conspicuous dark sediment layers, called sapropels, occur repeatedly in the sediments. They are characterized by elevated organic carbon contents, ranging from 2 to over 30% of dry weight [1,2] and were formed periodically approximately every 20,000 years, following the Milankovic cycles [1,3,4]. It is thought that sapropels were formed in response to climate changes leading to elevated nutrient input, higher productivity and increased sedimentation rates [1,3,4]. The resulting formation of anoxic bottom water led to the enhanced preservation of organic material in the sediments. After restoration of oxic conditions in the bottom water, the sapropels were buried under low organic carbon calcareous sediments [5].

It has been estimated that subsurface sediments contain more than 90% of the microbial biomass on earth [6,7]. However, these habitats exhibit only very low microbial activities [8,9] due to their depletion in easily degradable organic material. Sapropels, on the other hand are elevated in organic carbon, which despite its age and kerogenic state, still provides an energy source for indigenous microorganisms [10,11]. Recent studies showed that sapropels have elevated numbers of

* Corresponding author. Present address: School of Earth, Ocean and Planetary Sciences, Cardiff University, Park Place, Main Building, Cardiff CF1O 3YT, Wales, UK. Tel.: +44 29 208 76001; fax: +44 29 2087 4329.

E-mail address: henrik@earth.cf.ac.uk (H. Sass).

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microorganisms compared to intervening low organic carbon layers, and that these can even reach those found at the sediment surface [10,11]. Even in almost 220,000-year-old sapropel layers active microbial populations were detected by the use of fluorescent substrate analogues and ^{14}C-labelled substrates [11]. Molecular analyses of the indigenous microbial communities indicated the dominance of sequences related to the Green non sulfur bacteria (GNSB) and to Crenarchaeota [11], both of them so far without cultured counterparts.

Although sapropels contain active microbial communities, these appear to have low viability since cultivation approaches employing standard media, yielded no more than 1500 cells g^{-1} sediment [12]. Nevertheless, to investigate and understand the ecology of the sapropel microbiota and their physiological adaptations, it is necessary to obtain typical sapropel microbes in culture for study. In order to enrich a broad diversity of microorganisms, in particular GNSB, whose metabolism is so far unknown, we used a range of different media for cultivation. Besides media considered selective for terminal oxidizers such as sulfate-reducing bacteria, substrate combinations were applied, which should allow a broad variety of different microorganisms to grow. Since it is likely that environmental microorganisms are exposed to a 'substrate shock' after transfer into substrate-rich medium lowering their viability [13,14], all substrates were provided in sub-millimolar concentrations. Additionally, we used a 'natural' sapropel extract medium, since it was reported previously that sediment extract media achieved higher MPN counts than synthetic media [15].

2. Material and methods

2.1. Sampling

Sediment samples were obtained during cruise M51/3 of the R/V *Meteor* in November 2001 from three stations (562, 567, 575, Table 1) in the Eastern Mediterranean Sea. The temperature at the sediment surface was around 14 °C. Surface sediment was taken by multicorer, whilst for deeper sediment (up to 12 m) a gravity corer was used. The recovery speed of the sampling devices ranged from 0.3 to 0.5 m s^{-1}. Aseptic sampling of sediment was performed as described by Coolen et al. [11, supplementary material]. From the different sediment layers, 1 cm^3 was taken by use of cut-off sterile plastic syringes, and was transferred into 9 ml sterile anoxic artificial seawater. Until processing, these sediment slurries were kept under a nitrogen atmosphere in the dark at 4 °C, although part of each slurry was fixed immediately with glutaraldehyde (final concentration 2%) for determination of total cell counts. These samples were stored at 4 °C in the dark.

2.2. Determination of total cell counts

Fixed sediment slurry (5–7 μl) was added to an equal volume of Tween 80 (0.5%, sterile-filtered) and 50 μl of particle-free sterile PBS buffer (130 mM NaCl, 5 mM NaH_2PO_4, 5 mM Na_2HPO_4, pH 7.2) in a sterile 1.5 ml Eppendorf reaction tube. After short ultrasonic treatment (5 × 5 s), the solution was filtered through a white polycarbonate membrane (0.1 μm pore size, 25 mm diameter, Anodisc 25, Whatman, Maidstone, UK). Bacterial cells were stained by the method described by Jaspers et al. [16] using a staining solution consisting of 70 μl DAPI (4′,6-diamidino-2-phenylindole, Sigma, 10 μg ml^{-1}), 1 ml of fixing solution (paraformaldehyde final conc. 4%, Triton 100 final conc. 0.1% in PBS buffer) and 930 μl ddH_2O. For comparison, samples were also stained with acridine orange as described by Coolen and Overmann [17].

The air-dried filter membranes were mounted in DABCO (25 mg Diazabicyclo-octan, 1 ml PBS, 9 ml glycerol) under a coverslip. Counting was performed using an epifluorescence microscope (Zeiss Axiolab, Oberkochen, Germany) equipped with filter sets for DAPI (BP365, FT395, LP397) and acridine orange (BP459-490, FT510 and LP515). For each layer, three replicates were counted, each up to at least 200 cells.

2.3. Growth media

An artificial seawater medium was used for preparing sediment slurries, MPN series and isolation of pure cultures. This medium contained (in g l^{-1}): NaCl (24.3), $MgCl_2 \cdot 6H_2O$ (10), $CaCl_2 \cdot H_2O$ (1.5), KC1 (0.66), Na_2SO_4 (4), KBr (0.1), H_3BO_3 (0.025), $SrCl_2 \cdot 6H_2O$ (0.04), NH_4Cl (0.021), KH_2PO_4 (0.0054), NaF (0.003). The medium was supplemented with 1 ml l^{-1} trace element solution SL10 [18] and 0.2 ml l^{-1} of a selenite and tungstate solution [19]. After autoclaving, the medium was cooled under N_2/CO_2 (80/20, v/v). To the cold medium, 10 ml of a solution of 10 vitamins [20] and 30 ml l^{-1} of a 1 M $NaHCO_3$ solution were added from sterile stocks. Finally, the medium was reduced by addition of Na_2S and acid $FeCl_2$ solutions to final concentrations of 1.5 and 0.5 mmol l^{-1}, respectively. The pH of reduced medium was set to 7.2–7.4 with sterile HC1 or Na_2CO_3.

For oxic MPN counts and isolation of aerobic microorganisms, a slightly modified medium was used. Instead of bicarbonate and CO_2 the medium was buffered with Hepes (2.4 g l^{-1}) and the pH was adjusted to 7.2–7.4 with NaOH before autoclaving. After autoclaving, the oxic medium was cooled under air and then supplemented with vitamins and sodium bicarbonate (final concentration 0.2 g l^{-1}).

A diluted yeast extract–peptone medium was used for maintenance of aerobic pure cultures and for tests of aerobic growth. It consisted of the HEPES-buffered oxic

Table 1
Sampling stations 562, 567 and 575 of *Meteor* cruise leg 51/3 in the Eastern Mediterranean Sea

Station	Position	Depth (m)	Sampled sediment layers	Depth of sediment layer (mbsf)
562	32°46.42′N	1391	Z5	2.71–3.70
			S6	3.70–4.00
	19°11.55′E		Z6	4.00–4.16
			S7	4.16–4.38
567	34°48.79′N	2153	Surf.	<0.05
			S1	0.17–0.35
	27°17.13′E		Z1	0.35–2.50
			S5	3.75–4.60
575	34°31.39′N	2330	Surf.	<0.05
			S3	1.31–1.35
	31°46.40′E		S4	1.96–2.00
			Z5	2.71–3.45

Surf, surface layer; S, sapropel layer; Z, carbon-lean intermediate layer.

seawater described above, amended with yeast extract (0.03 $g l^{-1}$), peptone (0.06 $g l^{-1}$), sodium lactate (5 $mmol l^{-1}$), glucose (1 $mmol l^{-1}$), sodium thiosulfate (1 $mmol l^{-1}$), vitamins and sodium bicarbonate (0.2 $g l^{-1}$).

2.4. Substrates used for MPN series

Six different substrate combinations were used as electron and carbon sources for MPN series: (1) AS (amino acids), containing 20 different L-amino acids (final concentration 0.1 $mmol l^{-1}$ each), (2) ALC (*n*-alcohols), containing methanol, ethanol, *n*-propanol and *n*-butanol (final concentration 0.1 $mmol l^{-1}$, each), (3) LFA (long chain fatty acids), containing saturated straight chain fatty acids with 14 to 18 carbon atoms (final concentration 0.1 $mmol l^{-1}$, each), (4) ARO (aromatic compounds), containing salicylate, *p*-OH benzoate and the methoxylated aromatic compounds vanillate, coumarate, ferulate, sinapinate and syringate (final concentration 0.1 $mmol l^{-1}$, each), (5) MKS (defined substrate mixture), containing a broad range of different carbon compounds, in particular the amino acids and alcohols as listed above, but also the short chain fatty acids formate, acetate, propionate, butyrate, valerate and caproate, and additionally glycerol, glucose, lactate, fumarate, malate, succinate (0.1 $mmol^{-1}$ final concentration, each). The MKS medium was additionally supplemented with the polysaccharides laminarin, xylan and chitin (0.1 $g l^{-1}$ each). (6) SED (sediment extract medium). Equal amounts of freeze–dried sapropel material and oxic mineral medium were thoroughly mixed and boiled for at least 2 h. After coarse particles had settled, the turbid supernatant was filtered to remove particles and was finally autoclaved. After autoclaving, the sapropel extract medium was cooled under air. The cooled medium was supplemented with vitamins and bicarbonate (0.2 $g l^{-1}$) as described above. For the preparation of sapropel extract medium, only a limited amount of sapropel material was available. Therefore, for the last isolation steps sediments from a tidal flat from the North Sea were used. In order to guarantee that older sediments with a low content of easily degradable organic matter were used, material was dug out from a depth of approximately 50 cm. Additionally, MPN series without added substrates were inoculated as a blank.

2.5. Preparation and incubation of MPN series

Viable counts were determined by MPN series that were inoculated on shipboard using the sediment slurry described above. All equipment necessary for preparation of the MPN series was placed into a polyethylene chamber (AtmosBag, 280 l, Aldrich, Milwaukee, WI), which was then flushed with nitrogen gas, evacuated and filled with N_2 again. The procedure was repeated up to five times to remove atmospheric oxygen. The MPN series were incubated in polypropylene 96-deep-well plates (Beckman, Fullerton, CA). Every well contained 450 μl medium, to which 50 μl inoculum were added. After inoculation the plates were covered-with sterile lids (CAPMAT, Beckman, Fullerton, CA), sealing each well separately. Each plate contained four different MPN series with three replicates and six 10-fold dilutions. Furthermore, in each plate four uninoculated dilution series were prepared as control.

The inoculated and sealed MPN plates were put into gas-tight plastic bags equipped with a gas generating and catalyst system for anoxic conditions (Anaerocult C mini, Merck, Germany). Incubation proceeded normally for 12 weeks at 20 °C, but long-chain fatty acid medium was incubated for 6 months.

2.6. Analysis of MPN series

The MPN plates were analyzed for microbial growth by epifluorescence microscopy for higher sensitivity. Cell densities in positive cultures were generally not very

high, and many of the microorganisms found were attached to particles, e.g. FeS, hence detection by phase contrast microscopy proved difficult.

The gas-tight plastic bags containing the MPN plates were carefully transferred into an anaerobic hood. The lid was cut into small pieces and carefully lifted using sterile tweezers. From each well, 10 μl of culture was transferred into a cavity of a diagnostica slide. To each sample, 2 μl of a solution of SybrGreen II (1:100, Molecular Probes, Leiden, Netherlands) was added and mixed. The stained samples were incubated for at least 1 h in the dark prior to examination. For epifluorescence microscopy, an UV excitation filter set for SybrGreen II (BP450-490, FT510 and LP515) was used. The MPN counts were calculated as described by de Man [21] and corrected for the values obtained in substrate-free MPN series.

2.7. Isolation of pure cultures

For the isolation of pure cultures, the highest positive dilutions were used. All subculturing and isolation procedures were performed with the same media as in the MPN series. Isolation of strains from the oxic MPN series was done on agar plates. For the isolation of anoxic cultures, the deep-agar dilution method was used. Purity of cultures was checked by microscopy and by denaturing gradient gel electrophoresis (DGGE) [22].

2.8. Molecular biological screening of pure cultures by signature PCR

In order to affiliate the pure cultures with major bacterial groups, a signature PCR (SIG-PCR) following the protocol of Uphoff et al. [23] was carried out for all isolates. The applied primer mixture contained the universal primers for Eubacteria (BAC8f and UNI1493r) and the group specific primers for α-, β- and γ-Proteobacteria (αδP688r, βεP774r and γβP1403r), Actinobacteria (HGC1153r), *Cytophaga-Flavobacterium-Bacteroides* phylum (CFB947r) and for marine phototrophic α-Proteobacteria belonging to the *Roseobacter* and *Erythrobacter* clusters (αER247r). The PCR mixture had a final volume of 50 μl and contained: 1 U RedTaq polymerase (Sigma), the 10×polymerase buffer according to manufacturers specification, BSA (0.4 μg), dNTPs (200 $\mu mol l^{-1}$ each) and the SIG-PCR primer mix (0.1 $\mu mol l^{-1}$ each). The $MgCl_2$ concentration was adjusted to 2.1 $mmol l^{-1}$. The SIG-PCR followed a touch down protocol with decreasing annealing temperatures (59–53 °C, 0.2 °C step) as described by Uphoff et al. [23]. PCR products were analyzed by agarose gel electrophoresis. According to the applied primer combination, PCR products of the following lengths were expected: 100 bp for γ-Proteobacteria, 350 bp for Actinobacteria, 650 bp for α-Proteobacteria, 700 bp for (β-Proteobacteria, 1000 bp for the *Cytophaga Flavobacteria* phylum, and 1400 bp for Eubacteria.

2.9. Denaturing gradient gel electrophoresis (DGGE)

For DGGE analysis 630-bp long fragments of the 16S rDNA of each strain were amplified using the universal Eubacterial primer set GC357f (containing a 40-bp long GC clamp) and 907r. The PCR was carried out according to the protocol of Sass et al. [22]. DGGE was performed using an INGENYphorU-2 system (Ingeny, Leiden, The Netherlands). PCR products were loaded onto polyacrylamide gels (6% wt/vol) stored in 1×TAE (40 $mmol l^{-1}$ Tris, 20 $mmol l^{-1}$ acetate, 1 $mmol l^{-1}$ EDTA), with a denaturing gradient from 50% to 70% (100% denaturant correspond to 7 $mol l^{-1}$ urea and 40% formamide). Electrophoresis was performed at a constant voltage of 100 V and at a temperature of 60 °C for 20 h. After electrophoresis, the gels were stained for 2 h with 1× SybrGold in 1×TAE (Molecular Probes, Leiden, The Netherlands), washed for 20 min in distilled water, and documented digitally (BioDocAnalyze, Biometra, Göttingen, Germany).

2.10. Amplification and sequencing of the 16S rDNA

The almost complete 16S rRNA gene was amplified using the Eubacteria-specific primer set 8f and 1492r (10 $pmol l^{-1}$ each). A step down PCR was performed. Initial denaturation was done at 96 °C for 4 min; followed by 10 cycles with denaturation at 94 °C for 30 sec, annealing at 59 °C for 45 sec and extension at 72 °C for 1 min., and another 20 cycles with the same denaturation and extension parameters, but with annealing for 45 s at 54 °C. A terminal post-extension was done for 10 min at 72 °C. After amplification, PCR products were purified using the QIAquick PCR Purification Kit (Qiagen GmbH, Hilden, Germany). The DNA concentration of the purified products was quantified fluorometrically using the PicoGreen® dsDNA Quantitation Kit (Molecular Probes, Leiden, Netherlands) according to the manufacturers protocol. For sequence analysis, the DNA content of the template was set to 4 $ng \mu l^{-1}$.

The DNA sequencing reactions were performed using the DYEnamic Direct Cycle Sequencing Kit (Amersham Biosciences Europe GmbH, Freiburg, Germany) following the manufacturers instructions with a modified protocol for the PCR mixture, containing 1 μl 7deaza dNTPs, 2 μl IRD-labelled primer (0.5 $pmol \mu l^{-1}$) and 2 μl template. The 16S rDNA sequences were determined by cycle sequencing with the LiCor DNA Sequencing System 4000 (MWG Biotech, Ebersberg, Germany) as previously described by Overmann and Tuschak [24].

Sequences most closely related to those obtained in the present study were retrieved from GenBank using the BLASTN tool [25]. The nucleotide sequences of rep-

resentative strains of each phylogentic group presented in this study are available at the GenBank database [24] under the Accession Nos. AJ630143 to AJ630199 and AJ748349 to AJ748351.

3. Results

3.1. Total cell counts

Total cell counts were determined at the sediment surface (Surf), in low carbon intermediate layers (Zl to Z6) and in the organic-rich sapropels (S1 to S7) at the three stations studied (Table 2). In order to get reliable counts, sediment slurries had to be treated by ultrasound and by the addition of detergents for cell detachment. This treatment turned out to be necessary since the major part of the cells appeared to be stuck to sediment particles, and therefore could not be reliably distinguished from the background fluorescence of the sapropel material. After the treatment described above, most cells were detached from particles and could be counted easier.

Total cell counts after DAPI-staining were in the range of 2×10^{8}–1×10^{9} cells cm^{-3}. The acridine orange direct counts revealed almost identical results (data not shown). These values are up to tenfold higher than in previous reports [10,11], most likely due to the modified staining procedure. Cell counts showed only a limited decrease with sediment depth. In sapropels, cell numbers were elevated compared to intermediate layers (Table 2).

3.2. Viable counts and cultivation efficiency

MPN counts were generally high and reached maximum values of 2×10^{7} cells cm^{-3} in the surface layer (stations 567 and 575) and in the sapropel layers S1 and S5 at station 567 (Table 2). In general, MPN counts decreased with depth but were elevated in sapropels compared to the intermediate sediment layers. However, with some substrate combinations MPN counts remained more or less constant or even increased with depth. This was in particular the case for aromatic compounds, that produced MPN counts orders of magnitude lower than those with other substrates at sediment surface. In the deeper layers, however, they were as high as or even exceeded MPN counts with the other substrate combinations.

Surprisingly, MPN counts obtained after oxic and anoxic incubation were similar, indicating the presence of high numbers of facultatively anaerobic microorganisms. Sediment extract medium yielded lower MPN counts than the mineral media used (Table 2). In substrate-free controls, counts were below the detection limit.

From total cell numbers and MPN counts cultivation efficiencies were calculated. The highest values reached 3.3% and were obtained at the surface, in sapropel 1, but also in sapropel 5. The substrates with the highest cultivation efficiencies were the substrate mixture MKS, amino acids but also long chain fatty acids. Considering the 95% confidence levels given in Table 2 the highest cultivation success was in the range of 0.8–15% of the total cell count.

3.3. Isolation of pure cultures

To identify the most abundant microorganisms growing in the various media, pure cultures were isolated from the highest positive MPN dilutions. In total, 144 subcultures were inoculated. However, some of these failed to grow after repeated transfers or did not form visible colonies. In particular, no pure cultures were obtained with aromatic compounds and long chain fatty acids as substrates. Finally, 98 pure cultures were obtained (Table 3). Most of these (71 strains) were isolated under anoxic conditions, compared to 27 aerobic strains. Screening for aerobic growth revealed, that only six out of the 71 anaerobic isolates were strict anaerobes, having no visible colonies developed within two months under oxic conditions.

Generally, growth under anoxic conditions was very slow. In most cases, the bacteria in deep-agar dilutions needed at least six, but sometimes up to 12 weeks to form small visible colonies. Furthermore, anaerobically grown colonies were not very large in size. However, the size of the colonies was depending on the growth substrate as well as on the phylogeny of each strain. Especially, on substrates which are difficult to ferment like alcohols, and also on sapropel extract medium, colonies were rarely larger than 0.2 mm in diameter. Whilst on amino acids or the defined substrate mixture MKS, colonies could reach up to 4 mm in diameter.

3.4. Molecular biological screening and phylogenetic affiliation of pure cultures

A two-step screening was applied to avoid the expensive and time-consuming procedure of 16S rDNA analysis of all isolates. In the first step, a signature PCR (SIG-PCR) with a mixture of six different primer pairs was used to roughly affiliate the isolates with the most common phylogenetic groups (Fig. 1). With this approach 42% of the isolates belonged to the γ-Proteobacteria, 21% to the α-Proteobacteria and 17% to the Actinobacteria. Twelve isolates yielded none, or only the 1400-bp long PCR product for Eubacteria. No SIG-PCR products indicative for β-Proteobacteria or members of the *Cytophaga-Flavobacterium-Bacteroides* phylum were obtained. The strictly anaerobic isolates were not screened by SIG-PCR.

Table 2
Total cell counts and MPN counts [$\times cm^{-3}$ sediment] in the investigated sediment layers at stations 562, 567 and 575

Station	Layer	Total cell counts	Oxic MPN series		Anoxic MPN series					
			Sediment extract	Mixed substrates	*n*-Alcohols	Amino acids	Fatty acids (C14–C18)	Aromatic compounds	Mixed substrates
562	Z5	$3.7 \times 10^8 \pm 3.3 \times 10^7$						2,200 (800–4800)	5.6×10^4 ($2.2–15 \times 10^4$)
	S6	$3.6 \times 10^8 \pm 1.4 \times 10^8$						80 (20–420)	800 (200–4200)
	Z6	$2.3 \times 10^8 \pm 1.0 \times 10^7$						300 (120–1000)	2.2×10^5 ($0.8–6.8 \times 10^5$)
	S7	$4.2 \times 10^8 \pm 9.4 \times 10^7$						4.0×10^5 ($1.4–12 \times 10^5$)	1.0×10^4 ($0.2–4.2 \times 10^4$)
567	Surf	$1.0 \times 10^9 \pm 2.2 \times 10^8$	4.0×10^6 ($1.4–12 \times 10^6$)	1.0×10^7 ($0.2–24 \times 10^7$)	4.0×10^5 ($1.4–12 \times 10^5$)	1.4×10^6 ($0.4–5.6 \times 10^6$)	2.2×10^7 ($0.8–6.8 \times 10^7$)	400 (140–1200)	5.6×10^5 ($2.2–15 \times 10^5$)
	S1	$8.2 \times 10^8 \pm 1.5 \times 10^8$	4.0×10^5 ($1.4–12 \times 10^5$)	2.2×10^7 ($0.8–6.8 \times 10^7$)	5.6×10^5 ($2.2–15 \times 10^5$)	5.6×10^5 ($2.2–15 \times 10^5$)	2.2×10^5 ($0.8–6.8 \times 10^5$)	5,600 (2000–14,000)	5.6×10^5 ($2.2–15 \times 10^5$)
	Z1	$3.7 \times 10^8 \pm 4.5 \times 10^7$	n.d.	5.6×10^5 ($2.2–15 \times 10^5$)	220 (80–480)	3.0×10^5 ($1.2–8.2 \times 10^5$)	4.2×10^4 ($1.6–12 \times 10^4$)	60 (20–340)	5.6×10^5 ($2.2–15 \times 10^5$)
	S5	$6.6 \times 10^8 \pm 1.1 \times 10^8$	n.d.	5.6×10^5 ($2.2–15 \times 10^5$)	4.0×10^4 ($1.4–12 \times 10^4$)	2.2×10^7 ($0.8 –6.8 \times 10^7$)	1.4×10^4 ($0.4–5.6 \times 10^4$)	220 (80–6800)	4.0×10^5 ($1.4–12 \times 10^5$)
575	Surf	$7.5 \times 10^8 \pm 1.2 \times 10^8$		4.0×10^5 ($1.4–12 \times 10^5$)	4.0×10^5 ($1.4–12 \times 10^5$)	2.2×10^7 ($0.8–6.8 \times 10^7$)	4000 (1400–12,000)	4000 (1400–12,000)	
	S3	$8.0 \times 10^8 \pm 1.6 \times 10^8$		2.2×10^4 ($0.8–6.8 \times 10^4$)	4.0×10^5 ($1.4–12 \times 10^5$)	5.6×10^5 ($2.2–15 \times 10^5$)	400 (140–1200)	4.0×10^5 ($1.4–12 \times 10^5$)	
	S4	$5.4 \times 10^8 \pm 5.2 \times 10^7$		2200 (800–6800)	2.2×10^5 ($0.8–4.8 \times 10^5$)	2.2×10^4 ($0.8–6.8 \times 10^4$)	2200 (800–6800)	2.2×10^5 ($0.8–6.8 \times 10^5$)	
	Z5	$5.9 \times 10^8 \pm 1.5 \times 10^8$		<60	4000 (1400–12,000)	80 (20–420)	400 (140–1200)	4.0×10^5 ($1.4–12 \times 10^5$)	

95% confidence levels of MPN counts are given in brackets.
Surf., surface layer; S, sapropel layer and Z, carbon lean intermediate layer and n.d., not determined.

Table 3
Phylogenetic affiliation of the closest relatives of isolates from Mediterranean sapropels and intermediate layers

Closest relative in GenBank	Similarity (%)	Origin of closest relative	Origin of isolates			Number of isolates		Substrate	
			562	567	575	Oxic	Anoxic	Oxic	Anoxic
α-Proteobacteria									
Agrobacterium sp. JS71 (AY174112)	98	Contaminated soil	Z6, S7	Surf, S1, Z1,S5	Surf, S4	1	16	SED	MKS, Alk
Roseobacter sp. PIC-68 (AJ534238)	95	Northern Atlanic Ocean sediment		Surf		1		SED	
Erythrobacter citreus^T (AJ294340)	98	Western Mediterranean Sea	Z6				2		MKS
Paracoccus marcusii^T (Y12703)	99	Adriatic Sea		S1			1		Alk
β-Proteobacteria									
Janthinobacterium sp. An8 (AJ551147)	96	Deep-sea sediment		S5			1		AS
γ-Proteobacteria									
Photobacterium profundum^T (AB003191)	95–98	Deep-sea sediment		Surf, S1, Z1, S5	S4	10	23	MKS	MKS, AS
Marinobacterium jannaschii^T (AB006765)	95	Western Mediterranean Sea		Surf	S3		2		MKS, Alk
Vibrio splendidus^T (AJ515229)	97	Marine fish			Surf, S3, S4		5		Alk, AS
Vibrio halioticoli IAM 14598 (AB000392)	96	Marine snail		S5		1		MKS	
δ-Proteobacteria									
Desulfofrigus fragile^T (AF099065)	99	Arctic marine sediments			S1		4		AS
Actinobacteria									
Brachybacterium rhamnosum^T (AJ415376)	99	Corn steep liquor		Surf, S5	Surf	4		MKS	
Brachybacterium sp. V589 (AF324202)	97–98	Lake Vostoc (Antarctica)	S6	Surf, Z1			5		MKS, SED
Micrococcus luteus^T (AB023371)	97–99	Unknown		Surf, S1	Surf, S3	5	1	SED, MKS	AS
Micrococcus sp. V4.MO.20 (AJ244665)	97–99	Deep-sea sediment, Mediterranean Sea		Z1,S5		2		SED	
Firmicutes									
Bacillus licheniformis^T (AF276309)	99	Medieval wall painting		Surf		1		MKS	
Bacillus barbaricus^T (AJ422145)	98–99	Medieval wall painting			Surf, S3	2		MKS	
Bacillus jeotgali^T (AF221061)	99	Sea food		S1,Z1, S5			8		MKS, AS, SED
Sporosarcina macmurdoensis^T (AJ514408)	96	Cyanobacterial mat, Antartica		Z1			1		AS
Uncultured clone CD13F11 (AF441880)	96	Black band disease of corals			Surf, S1		2		AS, MKS

Surf, surface layer; S, sapropel layer; Z, carbon lean intermediate layer; SED, sediment extract medium; MKS, substrate mixture; AS, amino acids and ALC, *n*-alcohols.
Bacterial cultures or sequences most closely related to the isolates were retrieved from the GenBank database. Also shown are data concerning the origin of our isolates with regard to sampled sediment layer of sampling stations 562, 567 and 575, incubation conditions and applied substrates as well as number of existing isolates from oxic and anoxic enrichments.

After screening the isolates via SIG-PCR, denaturing gradient gel electrophoresis (DGGE) was applied to subdivide the isolates based on sequence differences of the 16S rDNA. In total, the DGGE analysis produced nineteen different melting types: five in the γ-Proteobacteria, four in each of the Actinobacteria, the α-Proteobacteria and the strains that produced only the eubacterial band in the SIG-PCR screening,

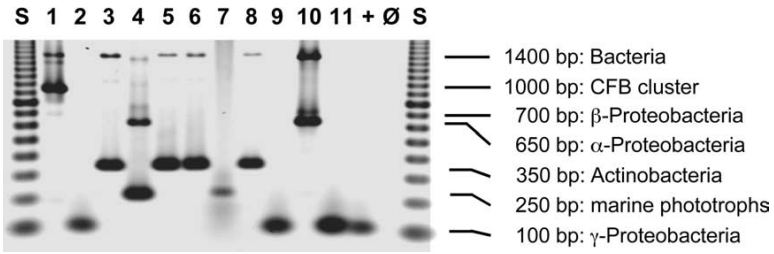

Fig. 1. Example for the separation of Signature PCR products. Negative image of an ethidium bromide-stained 1.5% agarose gel. On the right site the expected fragment lengths for each taxonomical group are given. The lanes were as follows: (1)–(4) DSMZ strains used as standards; (1) *Cytophaga-Flavobacterium-Bacteroides* phylum: *Muricauda ruestringensis*^{T} (DSM 13258), (2) γ-Proteobacteria: *Pseudalteromonas atlantica*^{T} (DSM 6839), (3) Actinobacteria: *Arthrobacter nicotinovorans*^{T} (DSM 420), (4) marine phototrophic α-Proteobacteria: *Roseobacter gallaeciensis*^{T} (DSM 12440); (5)–(11) strains from Mediterranean sapropels and intermediate layers isolated during the present work; (+) *E. coli* was used as positive control, (ø) water as negative control and (S) a 100-bp ladder as standard.

and two in the strictly anaerobic strains. Two to twelve representatives of each melting type were identified by partial sequencing of the 16S rDNA (400–850 bp long fragments). The grouping by molecular methods was supported by similarities in the phenotypical appearances of colonies and cells. With only one single exception, strains belonging to a single melting type shared sequence similarities of at least 98%.

The BLAST analysis of the target group revealed that the majority of the γ-Proteobacterial strains (33) was related to *Photobacterium profundum*^{T} (Table 3). Within this group 16S rDNA sequence differences of up to 4% were found. Closest relatives of the other γ-Proteobacteria were *Marinobacterium jannaschii*^{T}, *Vibrio splendidus*^{T}, and *Vibrio halioticoli* IAM 14598. One strain affiliated with the γ-Proteobacteria by SIG-PCR, turned out to be a β-Proteobacterium with *Janthinobacterium* sp. An8 as closest relative. The second-largest group of isolates (21 strains) belonged to the α-Proteobacteria. Of these, seventeen were closely related to *Agrobacterium* sp. JS71. The other four were affiliated with *Erythrobacter citreus*^{T}, *Paracoccus marcusii*^{T}, and *Roseobacter* sp. PIC-68. The strains identified as Actinobacteria (17 strains) were related to the genera *Brachybacterium* (nine strains) and *Micrococcus* (eight strains). All strains that could not be classified after signature PCR turned out to be Firmicutes. Primers specific for Firmicutes were not in the SIG-PCR primer set. Firmicutes isolated under anoxic conditions were affiliated with *Bacillus jeotgali*^{T} and *Sporosarcina macmurdoensis*^{T}, while those isolated as aerobes were related to *Bacillus licheniformis*^{T} and *Bacillus barbaricus*^{T}. Of the six strictly anaerobic strains directly analysed by partial 16S rDNA sequence analysis, four were closely affiliated with *Desulfofrigus fragile*^{T} within the δ-Proteobacteria and two were identified as Firmicutes, most closely related to an uncultured member of the Clostridiales.

4. Discussion

4.1. Cultivation efficiency

In the present study the first quantitative cultivation-based data on ancient sapropels from the Eastern Mediterranean Sea are published and remarkable cultivation efficiencies of more than 3% of the total cell numbers were obtained, in considering the age of these sapropels (8000–195,000 years old). Considering the large confidence levels of MPN results (Table 2, in the range of a 1/4 of to 5-times the MPN count), the MPN counts are probably in a range between 0.8% and 15% of the total cell count. These values are clearly higher than those typically found in marine sediments (up to 0.1% of the total cell count, Amann et al. [26]), and those obtained during a previous investigation on sapropels (0.01% or less [Sass et al., unpublished]).

Up to now, there are only very few cultivation-based quantitative studies on the microbial communities in marine subsurface sediments [2,27–29]. Although in a few cases enrichment of up to 30% of the total cell counts was reported (Table 4), in most cases the cultivation efficiencies were far below 1%. However, in the present study high MPN counts were achieved in almost all sediment layers investigated. The media applied here seem, therefore, to support not only a relatively high cultivation success but also consistently elevated results. This might be due to the use of media with low substrate concentrations compared to standard media. Low nutrient media have recently been shown to increase the number and diversity of cultured bacteria from pelagic habitats [30–33]. Microorganisms enriched and isolated using low substrate concentrations should typically be oligocarbophilic or oligotrophic bacteria [34,35], which are assumed to represent the majority of the environmental microorganisms. In nutrient-rich media these generally slow growing organisms can easily be outcompeted by copiotrophic bacteria [34,35], and are likely to be irreversibly damaged after exposure to

Table 4
Total and viable cell counts in various deep-sea sediments

Deep-sea sediment	Total cell count	Viable cell count	% Cultivated
Japan Sea, ODP Leg 128 [8]			
Surface	1×10^9 cm^{-3}	1×10^5 cm^{-3}	0.0001
0.8 mbsf	1×10^8 cm^{-3}	7.9×10^5 cm^{-3}	0.79
500 mbsf	7.8×10^6 cm^{-3}	1.6×10^4 cm^{-3}	0.21
Blake Ridge, ODP Leg 164 [27]			
Surface	2.5×10^9 cm^{-3}	2.8×10^6 cm^{-3}	0.11
1 mbsf	4.4×10^7 cm^{-3}	1×10^6 cm^{-3}	2.27
365 mbsf	3.2×10^6 cm^{-3}	1.2×10^5 cm^{-3}	3.75
Woodlark Basin, ODP Leg 180 [28]			
Surface	3.3×10^8 ml^{-1}	9.4×10^4 ml^{-1}	0.028
10 mbsf	1.6×10^7 ml^{-1}	1×10^2 ml^{-1}	0.001
366 mbsf	4.2×10^6 ml^{-1}	1.3×10^6 ml^{-1}	30.9
Cascadia Margin, ODP Leg 146 [29]			
Surface	3.7×10^8 cm^{-3}	6.3×10^4 cm^{-3}	0.017
10 mbsf	1×10^7 cm^{-3}	8.4×10^3 cm^{-3}	0.084
200 mbsf	1×10^7 cm^{-3}	1.6×10^4 cm^{-3}	0.16
Mediterranean Sediment [this study]			
Surface	7.5×10^8 cm^{-3}	2.2×10^7 cm^{-3}	2.9
Sapropel S1, 0.25 mbsf	8.3×10^8 cm^{-3}	2.2×10^7 cm^{-3}	2.7
Sapropel S5, 4 mbsf	6.6×10^8 cm^{-3}	2.2×10^7 cm^{-3}	3.3

high substrate concentrations (substrate shock [13,14]). Besides the application of low substrate concentrations, a second strategy to prevent the isolation of copiotrophic bacteria was the use of highly diluted samples as inoculum or of dilution series cultures. In this respect the use of MPN series and the isolation from the highest positive dilutions might be advantageous. Microorganisms growing in the highest positive dilutions represent theoretically the most abundant microbes that can be cultured in the medium used.

The use of "natural media" was reported to yield high MPN counts in recent studies [15]. It was supposed that this medium contained the electron donors present also in the natural environment. This is debatable, since the heating and autoclaving most likely will lead to the release of low molecular weight organic material [36]. This would not be available for the microorganisms in situ. However, in our case the applied sapropel extract medium was outmatched by the artificial but defined seawater medium (Table 2).

Another improvement in the media was the use of FeS as reducing agent for anoxic media. FeS is common in marine sediments and was present in the sapropel layers [37]. Therefore, indigenous microbes should be adapted to it. FeS is less reactive than other reducing agents, like dithionite or titanium chloride and should, therefore, be less aggressive towards cells. Furthermore, the concentration of free hydrogen sulfide was decreased. The presence of FeS particles seemed also to support growth of several strains, as these preferentially grew attached to particles.

The cultivation success in this study appeared to be independent from the type of substrate provided, since relatively high MPN counts (10^5–10^7 cells cm^3) were obtained with nearly all substrate combinations, particularly at the sediment surface. Even the use of aromatic compounds seemed to be useful to stimulate the growth of microbes from very old sediment layers. Obviously, microbes in the old sediments are accustomed to deal with these compounds that are recalcitrant under anoxic conditions. In most cases the number of microorganisms stimulated to grow on the substrate mixture MKS was as high as on the single substrates or even higher. This result was to be expected, since MKS represents a combination of the single substrates used. Why the MPN numbers with MKS from sapropel S5 (#567) are lower than those with amino acids alone can only be speculated.

Despite the small volume of the inocula, the deep-well plates turned out to be well suited for MPN analyses. Because of their small size and because all steps can be carried out with multichannel pipettes, they allow a large number of incubations at small volume and relatively quickly. A possible negative effect of the small volume of inocula on cultivation success is unlikely. Zhang et al. [38] did not find any correlation between viable counts from subsurface sediments and sample size.

It the present study total cell counts were higher than in other studies on Mediterranean sapropel-bearing sediments [10,11]. A possible explanation is the treatment of fixed samples with ultrasound and the addition of detergents. Both procedures were addressed to the

detachment of cells from sediment particles and it is possible that these were therefore easier to detect. It is unlikely that the use of DAPI caused the elevated total counts, for example by unspecific staining of sediment particles, because acridine orange counts produced almost the same total cell numbers.

4.2. Diversity of isolated microorganisms

In the present study the MPN series were not only used for quantitative investigations, but also to isolate abundant microorganisms. The application of media containing different carbon sources was aimed at the enrichment of a broad variety of different sapropel microorganisms. Because the MPN counts reached a relatively high percentage of the total cell count, pure cultures obtained from the highest positive MPN dilutions represent the most abundant culturable members of the microbial community. With 98 different strains isolated, most of them under anoxic conditions, it is by far one of the most extensive strain collections obtained from marine subsurface habitats so far. A few reports described extensive aerobic culture collections that were obtained from surface sediments [39,40] or marine chemoclines [22], but not subsurface marine sediments. Despite the age of most of our sediments, the diversity (indicated as Shannon–Weaver index [41], data not shown) was in the same range as described for cultures from surface freshwater and marine sediments [39,40] or chemoclines [22]. A rarefaction analysis indicated that almost all bacteria from the sediments that were capable of growth in our media were present in the culture collection (Fig. 2). However, Green non sulfur bacteria (GNSB), which were identified as dominant bacterial group in sapropels [11] and subsurface sediments from the Nankai Trough [42], were not present in the culture collection. So far, there is no counterpart of this phylogenetic group in culture and their metabolism and physiology can only be speculated. However, as long as the needs of these organisms are unknown, it is hard to design specific cultivation medium. On the other hand, it could also be that they are strict barophiles and are, therefore, either unable to grow at atmospheric pressure or that they were damaged by decompression during sampling.

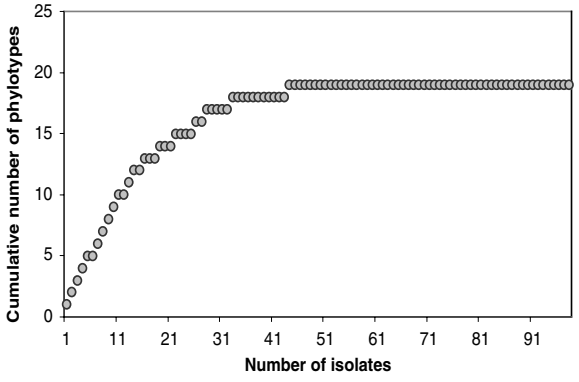

Fig. 2. Diversity of phylotypes in the culture collection. After randomizing, the 19 detected phylotypes were plotted in a cumulative manner against the total number of isolated strains (98).

4.3. Predominance of facultative anaerobes within culture collection

One remarkable finding was the predominance of aerobes among our isolates. This finding was supported by the high MPN counts obtained after oxic incubation. Even most of the strains isolated under anoxic conditions turned out to be only facultative anaerobes. Nonetheless, these bacteria were growing without oxygen and it has to be assumed that most of these microorganisms are anaerobically active in situ. Conversely, the Eastern Mediterranean Sea is a highly oligotrophic environment with a low productivity and little sedimentation. As a consequence, the input of organic matter into the sediment is low and only little microbial activities can be detected. At some sites oxygen penetrated down to a depth of 210 mm [12] and nitrate and oxidized manganese or iron species were present along the whole surface sediment penetrating the upper layers of the youngest sapropel S1 [43]. These oxidized but oxygen-free conditions would be favourable for facultative anaerobes. Only a minority of the isolates were spore formers, and hence might have been dormant in the sediment.

The low number of sulfate-reducing bacteria in the culture collection is surprising. In the sapropels and intermediate layers sulfate is still present and it should be expected that sulfate reduction is the dominant terminal oxidation process. Sulfate reduction was shown to occur in sapropels [37], although rates were relatively low and no sulfate decrease within sapropels could be detected. However, despite their importance in the biogeochemical cycles, in most sediments sulfate reducers are not the numerically dominating group of microorganisms. Even in sediments with high sulfate reduction, sulfate-reducing bacteria account for less than 12% of the total cell count [44]. On the other hand it could be possible that some fermenting microorganisms within the sapropels are using the kerogenic organic material as an electron sink as it was described for humic acids [45,46]. In this case they would not rely on the cooperation with terminal oxidizers.

4.4. Phylogenetic affiliation of isolates

Members of 19 different operational taxonomic units (OTU) were isolated. Most of these are known to be typical marine bacteria frequently found in the deep sea or in marine sediments. Some of them have also been detected in subsurface habitats, e.g. *Micrococcus* sp., *Rho-*

dococcus sp. or *Pseudomonas* sp. [47–49]. During a previous study on sapropels some of the OTUs found in the present study were also detected (e.g. *Photobacterium* sp., *Vibrio* sp., *Erythrobacter* sp. [Sass et al., unpublished]). In previous studies on sapropels almost exclusively Green non-sulfur bacteria were detected by culture-independent methods [11]. However, in newer investigations also several other sequences were retrieved [Overmann et al., unpublished], some of them related to *Sphingomonas* sp. and *Marinobacter* sp., genera present in our culture collection.

Strikingly, *Agrobacterium*-related organisms were repeatedly isolated from the different sediment layers and formed a dominant group within the culture collection. This finding seems surprising since *Agrobacterium* sp. are typical soil bacteria, some of them known to be even plant pathogens. Nevertheless, *Agrobacterium*-related 16S rDNA fragments were retrieved from sapropels by PCR and DGGE [Overmann et al., unpublished]. Commonly, only 16S rDNA sequences that constitute more than 1% of all template molecules in a natural bacterial community are detectable by the PCR-DGGE approach [50]. Therefore, these organisms seem to be quite common in the sapropels and intermediate layers. In addition, organisms with *Agrobacterium*-related 16S rDNA fragments have been isolated from the terrestrial deep subsurface [47,49]. Despite their high similarity on basis of 16S rDNA the *Agrobacterium*-like isolates from the Mediterranean Sea and the 'normal' *Agrobacterium* sp. do not necessarily have the same metabolism. The 16S rDNA gene is normally exposed to a limited environmental selection [51] and it might be possible that it can remain almost unchanged over a long period while the rest of the genome is probably undergoing adaptive changes. How these organisms thrive in their natural habitat and what metabolism they possess is a task for future research.

4.5. Phylogenetic screening of the culture collection by SIG-PCR and DGGE

At present time, analysis of the 16S rRNA gene sequence is the principle approach for the phylogenetic classification of microorganisms. However, the sequence analysis of a large number of isolates is time-consuming and expensive. For that reason, a pre-screening step was used to sort the isolates into different operational taxonomical units (OTU). Of each of these OTUs, two or a few representatives were analyzed, avoiding an unnecessary large sequencing effort. SIG-PCR was chosen for the sorting strains into major bacterial phyla and the correspondence of bands in DGGE, together with the phenotypical appearance of cells and colonies, was used for grouping the strains into the different OTUs. In general, this procedure worked well despite the fact that no primers for δ-Proteobacteria and Firmicutes were available. This is due to the fact that SIG-PCR was invented for pelagic systems [23] but for future approaches the system can be adapted. The fact that strains related to the β-Proteobacteria were first assigned to the γ-Proteobacteria was caused by γ-Proteobacteria specific primer also fits to some β-Proteobacteria [23].

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2.2 Development and evaluation of new techniques for the cultivation of sapropel bacteria

Jacqueline Süß, Katja Ziegelmüller, Andrea Schlingloff, Heribert Cypionka, Henrik Sass, Bert Engelen

In Bearbeitung

Development and evaluation of new techniques for the cultivation of sapropel bacteria

Jacqueline Süß, Katja Ziegelmüller, Andrea Schlingloff, Henrik Sass[1], Heribert Cypionka, and Bert Engelen[*]

Institut für Chemie und Biologie des Meeres, Carl von Ossietzky Universität Oldenburg, Carl von Ossietzky Straße 9-11, D-26111 Oldenburg, Germany

[1] Present address: School of Earth, Ocean and Planetary Science, Cardiff University, Main Building, Park Place, Cardiff CF10 3YE, Wales UK

Running title

New techniques for the cultivation of sapropel bacteria

Keywords

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[*]Corresponding author. Mailing address: Institut für Chemie und Biologie des Meeres, Paläomikrobiologie, Universität Oldenburg, Postfach 2503, D-26111 Oldenburg, Phone: +49-441-798-5376, FAX: +49-441-798-3583, email: engelen@icbm.de

Summary

In the present study different, partly newly developed cultivation techniques (liquid cultures, liquid gradient cultures, sediment gradient cultures) were applied to samples of up to 200,000 years old Mediterranean sapropels. As growth substrates, amino acids (AS), fatty acids (FS), tricarboxylic acids (TCA), *n*-alcohols (ALC) and aromatic compounds (ARO) were provided. Besides strain purification, the cultivation potential of the different approaches was evaluated by PCR-DGGE analysis of initial enrichments. In total, 23 phylotypes were enriched or isolated affiliating with the α-, β-, γ- and δ-*Proteobacteria*, *Actinobacteria*, *Firmicutes* and the deep-biosphere related subdivision of the *Chloroflexi*. With nearly all applied substrate combinations high numbers of phylotypes were obtained (AS/TCA/FS: 14; ALC/FS: 11; ARO: 9). Sediment gradient cultures offered a high potential for the cultivation of various different (17) and even typical sapropel bacteria (e.g. *Chloroflexi*). The application of serially diluted sapropel material in liquid cultures and liquid gradient cultures, in turn, led to the repeated cultivation of the same species. Regarding the discrepancy between the phylotypes detected in the initial enrichments and those present in the strain collection, cultivation-based studies appear to be mainly hampered by selective isolation procedures. Our study demonstrates that the recovery of (subsurface) microbes can be significantly improved by cultivation techniques that simulate environmental settings and provides useful guidance for future cultivation attempts.

1. Introduction

Since the first investigations on deep-sea sediments about 50 years ago [1] it became obvious that microbial life is widespread in the sub-seafloor. Several studies have demonstrated the presence of active microbial populations in sediments as deep as 800 mbsf [2, 3] and as old as 16 million years [4]. Molecular analyses have revealed a previously unknown and diverse microbial community within this deep biosphere [5-8]. However, a comprehensive understanding of the physiology of these microbes and of the biogeochemical processes in which they engage will undoubtedly require their cultivation. Up to now, only a few new species have been isolated from enrichments of deep sediments [9-12] and Most probable number (MPN)-counts have been very low in most cases [13-15]. Hence, the development of appropriate techniques that allow to grow these microbes in laboratory culture is one of the major tasks in subsurface microbiology.

In a recent study the cultivation efficiency in samples of Mediterranean subsurface sediments could be increased from less than 0.001 % to up to 3.3 % by the application of various different substrates in submillimolar concentrations [16]. The subsequent isolation procedure led to a large culture collection comprising 19 different phylotypes within the α-, β-, γ- and δ-*Proteobacteria*, the *Actinobacteria* and the *Firmicutes*. Similar improvements were successfully applied during studies on deeply buried sediments in the equatorial Pacific Ocean and the continental margin of Peru [10] as well as in the Nankai Trough [12]. Mediterranean sediments differ from these latter sites in that they contain a series of dark, organic-rich layers called sapropels. Despite its age the organic matter of these unusual layers still promotes the growth of indigenous microbes resulting in higher prokaryotic numbers and activities than found in adjacent carbon-lean oozes [13, 17, 18]. Contrary to the composition of our strain collection the majority of prokaryotes detected in sapropels by molecular techniques affiliated with the *Chloroflexus*-subdivision II (Coolen et al. 2002). Members of this lineage are ubiquitous and presumably abundant in subsurface environments [5, 8, 19] but up to now resist cultivation.

To further improve the recovery of these and other typical subsurface microbes we applied newly developed cultivation techniques to samples of Mediterranean sapropels. Amongst others, we established liquid cultures embedded in a substrate gradient (liquid gradient culture) in which the microbes are exposed to slowly increasing nutrient concentrations. As a further improvement we used undisturbed sapropel material as an inoculum (sediment gradient cultures). In these enrichments the microbes were grown within

their habitual surroundings what was recently shown to be very successful [20, 21]. As substrates a variety of different, in part unconventional carbon sources like aromatic compounds were provided. To gain further insights into the cultivation potential of the different approaches, PCR-DGGE was applied in a systematic analysis of 40 initial enrichments.

2. Material and Methods

2.1. Sampling

Up to 12 m long sediment cores were obtained from three sites (#562, #567, and #575) in the eastern Mediterranean Sea during cruise M51/3 of R/V Meteor in November 2001 using a gravity corer [16]. Aseptic sampling of sapropels was performed as described by Coolen et al. [13]. From the different sapropel layers, 1 cm^3 was taken by use of cut-off sterile plastic syringes, and transferred into 9 ml sterile anoxic artificial seawater [16]. The sediment slurries were kept under a nitrogen atmosphere at 4°C in the dark until further processing.

2.2. Preparation of enrichment cultures

All enrichment cultures were inoculated directly on board the ship. As basal medium anoxic artificial seawater was used [16]. Liquid cultures were set up in sterile glass tubes containing 10 ml of the basal medium amended with the respective substrates or using 10 ml of a sediment extract medium [16]. Liquid gradient cultures were prepared in glass tubes as follows: The substrates were placed at the bottom of a glass tube, and 1 ml of 4 % agar was added. After cooling and solidifying, this substrate reservoir was overlain with 6 ml of anoxic substrate-free basal medium mixed with 3 ml of 4 % agar. This agar-solidified overlay served as a spacer between the substrate reservoir and the liquid culture and was in turn overlain with 5 ml substrate-free basal medium. All liquid cultures were inoculated with ten-fold dilutions of sapropel material (final dilutions of 10^{-2} to 10^{-4}). Sediment gradient cultures

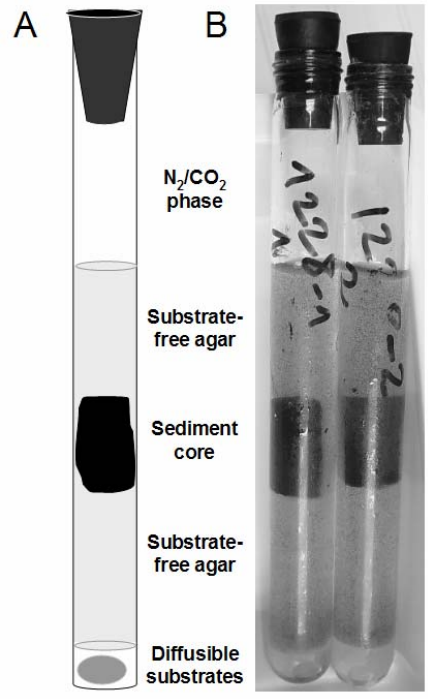

Fig. 1. Enrichment of microbes in undisturbed sediment samples. Scheme (A) and two examples (B) of sediment gradient cultures.

were prepared as liquid gradient cultures (Fig. 1). Instead of a liquid phase on the top of the spacer a 1 cm^3 sediment sample was embedded in a mixture of 5 ml basal medium and 2 ml of 4 % agar as described by Köpke et al. [21]. The head space of all cultures was flushed with nitrogen for at least 2 min and the tubes were sealed with a butyl rubber stopper. All cultures were incubated at 20°C in the dark for at least three months. Potential growth was determined by epifluorescence microscopy.

2.3. Substrates for enrichment cultures

The following substrates were used in different combinations (each at a final concentration of 0.1 mM) AS: a mixture of 20 different L-amino acids, TCA: a mixture of the organic acids containing DL-malate, fumarate, succinate and DL-lactate, FS: a mixture of short chain fatty acids containing formate, acetate, propionate, butyrate, valerate and capronate, ALC: a mixture of *n*-alcohols containing methanol, ethanol, *n*-propanol and *n*-butanol, ARO: a mixture of salicylate, *p*-OH benzoate and the methoxylated aromatic compounds vanillate, coumarate, ferulate, sinapinate and syringate. As alternative electron acceptors Fe: containing $Fe(OH)_3$ (final concentration, 0.5 mM), Mn: manganese oxides (final concentration 2 mM) and AQDS: 9,10-anthra-quinone-2,6-disulfonate (final concentration 0.5 mM) were used.

2.4. Isolation and phylogenetic analysis of pure cultures

All subculturing and isolation procedures were performed in analogous media using deep agar dilution series**.** Subculturing and isolation from gradient cultures were also done in gradient tubes; the agar-solidified top layer with the sediment was replaced by liquid culture or deep agar dilution series, respectively. The purity of cultures was tested by microscopy. Pure cultures were screened by a two-step molecular procedure comprising Signature-PCR [22] and DGGE. For the phylogenetic analysis the almost complete 16S rRNA gene of the isolates was amplified and sequenced as described previously [16].

2.5. DNA extraction from initial enrichments

Nucleic acids from sediment samples and sediment gradient cultures were extracted using the FastDNA® SPIN Kit for Soil according to the manufacturers protocol (Qbiogene Heidelberg, Germany). In order to saturate DNA binding sites of sediment compounds, 0.4 mg of poly-adenylic acid (PolyA) and 1.3 mg of sodium pyrophosphate (NaPP) were added to the lysis mixture [23, 24]. Spin and matrix binding times were extended to yield a high amount of

DNA recovery in 100 µl elution volume according to Webster et al. [25]. DNA extraction from liquid cultures was performed with a combination of bead-beating and enzymatic digestion with lysozyme followed by a phenol/chloroform treatment [26]. For this purpose, 1 ml of each culture was concentrated on Nucleopore polycarbonate filter (Whatman plc, Brentford, UK), transferred to a reaction tube containing 0.5 g of nealed zirconium beads (0.1 mm in diameter, BioSpec Products, Inc., Bartlesville, OK) and incubated with 400 µl extraction buffer (100 mM Tris-HCl, 50 mM EDTA, pH 8), 0.4 mg PolyA, 1.3 mg NaPP and 40 µg lysozyme on ice for 10 min. After addition of 20 µl SDS (20 % v/v, pH 7.2) and incubation for 40 min on ice, cell lysis was performed by beat-beating. After centrifugation the supernatant was removed and purified as described by Süß et al. [26]. The remaining precipitate was incubated with 200 µl extraction buffer, 50 µl SDS and 0.1 mg Proteinase K (Boehringer Mannheim GmbH, Penzberg, Germany) at 64°C for 1 h and centrifuged. In addition, the supernatant was also subjected to the purification procedure. After purification nucleic acids were precipitated, washed (EtOH, 70 %), resuspended in TE-buffer (10 mM Tris, 1 mM EDTA, pH 8) and stored at -20°C until further processing. As a contamination control during all extractions, a blind extraction without sediment or culture was included.

2.6. PCR-DGGE analysis

From each sample 630-bp long fragments of the 16S rRNA genes were amplified using the bacterial primer set GC357f (containing a 40-bp long GC clamp) and 907r [27] and separated in a denaturing gradient from 50 % to 70 % as described previously [16]. After electrophoresis the gels were stained for 2 h with 1x SybrGold in 1xTAE (Molecular Probes, Leiden, The Netherlands), washed for 20 min in distilled water, and documented digitally (BioDocAnalyze, Biometra, Göttingen, Germany). Distinct DNA bands were excised, dissolved in 40 µl PCR water (Ampuwa® Aqua dest, Fresenius, Bad Homburg, Germany) and kept at 4°C until further processing.

DGGE bands were reamplified using the universal primer set 357f/907r [27] according to Wilms et al. [28]. For sequence analysis the DNA content of the purified PCR product was set to 0.5 $ng \cdot \mu l^{-1}$. DNA sequencing reactions were performed using the DYEnamic Direct Cycle Sequencing Kit (Amersham Biosciences Europe GmbH, Freiburg, Germany) following the manufacturers instructions with a modified protocol for the PCR mixture, containing 1 µl 7deaza dNTPs, 2 µl IRD-labelled primer (0.5 $pmol \cdot \mu l^{-1}$) and 2 µl template. The 16S rDNA sequences were determined by cycle sequencing with the LiCor DNA Sequencing System 4000 (MWG Biotech, Ebersberg, Germany) as described by Overmann and Tuschak [29].

Sequences most closely related to those obtained in the present study were retrieved from GenBank using the BLASTN tool [30].

3. Results

3.1. Diversity of pure cultures

In the present study liquid cultures, liquid gradient cultures and sediment gradient cultures were applied to samples of Mediterranean sapropels. Microscopic inspection revealed the presence of various different, partly unusually shaped morphotypes in almost all initial enrichments. The subsequent isolation procedure led to a total of 74 strains. Based on partial sequences of the 16S rRNA genes the pure cultures were grouped into 15 phylotypes within the *Proteobacteria*, the *Actinobacteria* and the *Firmicutes* (Table 1). *Actinobacteria* (55 %) and α-*Proteobacteria* (34 %) were the most abundant bacterial groups in the strain collection whilst *Firmicutes* represented only a minor fraction (9 %). The most frequently isolated types showed highest sequence similarities to *Brachybacterium* sp. (97 %) and *Rhizobium radiobacter* (98 %) and accounted for 27 and 20 isolates, respectively (Table 1, Fig. 2).

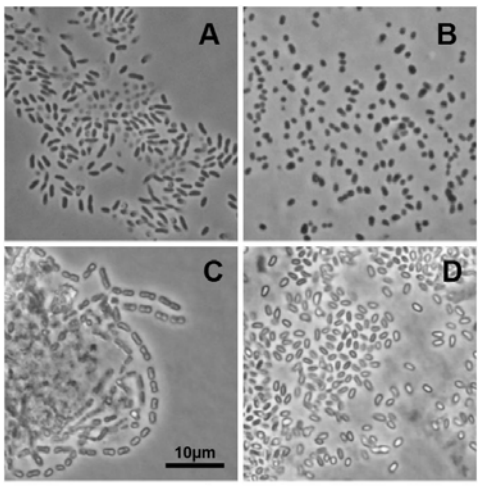

Fig. 2. Phase-contrast photomicrographs of sapropel strains. A: *Rhizobium* sp. J117, B: *Brachybacterium* sp. J80, C: *Fulvimarina* sp. J180, C: *Paenibacillus* sp. J36.

These microbes have been previously isolated from sapropels by using an improved MPN technique [16] and most likely represent abundant members of the original communities [31]. This applies also for *Erythrobacter* sp., *Micrococcus* sp., *Micrococcus luteus* and *Bacillus jeotgali*. Although γ-*Proteobacteria* were frequently isolated from the MPN-series only one

member of this bacterial group was obtained in the present study. However, the improved cultivation techniques led to the isolation of nine phylotypes previously not recognized in sapropels. Two of these were only distantly related to known bacterial species and showed highest similarities to environmental clones obtained from deep-sea sediments (uncultured hydrocarbon seep bacterium BPC060 and uncultured actinomycete BD4-12). The others affiliated either with the genera *Fulvimarina*, *Sphingomonas* and *Pseudomonas* within the *Proteobacteria* or with the Gram-positives *Citricoccus*, *Rhodococcus* or *Paenibacillus* (Table 1, Fig. 2). In addition, a new *Bacillus* species was discovered that showed highest similarity to the methanol-utilising *Bacillus methanolicus* (98 %).

Most of these newly detected phylotypes were obtained from cultures inoculated with undisturbed sediment material (Table 1). In general, the isolation out of these enrichments yielded the highest number of phylotypes (11). The use of liquid cultures and liquid gradient cultures, in turn, led to the repeated isolation of the same species (*B. paraconglumeratum* and *R. radiobacter*) resulting in a significant lower number of pure cultures (5 and 6 phylotypes, respectively). Nevertheless, three new phylotypes were obtained with these techniques (Table 1).

3.2. Molecular analysis of enrichment cultures

Original sediment samples, initial enrichments, and selected MPN series were screened by PCR-DGGE. The different bacterial types within the samples were identified by excision and sequencing of dominant DGGE bands and compared to pure cultures. In almost all enrichment cultures obtained by the different cultivation approaches at least three but sometimes as many as seven distinct bands were detected as a result of stimulated growth of certain bacterial species. Most enrichment cultures were unique in their phylogenetic composition and showed little overlap with enrichments achieved by other methods (Fig. 3). No differences were observed between liquid cultures in glass tubes and microtiter plates (Fig. 3).

Confirming the results of the isolation procedure, the highest diversity of microbes was detected in sediment gradient cultures (Table 1). In the dilution series established for liquid and liquid gradient cultures a clear shift towards dominant cultivable members of the sapropel communities was observed (Fig. 4). In most cases the highest dilution steps (10^{-4}) contained only a single bacterial species indicating that this technique facilitated the isolation of pure cultures.

In total, 21 bands were sequenced representing 12 different genotypes. Most of them affiliated with the α-, β- and γ- *Proteobacteria,* the *Actinobacteria* and the *Firmicutes* (Table 1). One sequence revealed a rather unclear phylogenetic affiliation but was assigned to the δ-*Proteobacteria* due to its high similarity (97 %) to an environmental sequence obtained from intertidal subsurface sediments [19]. In addition, a member of the as-yet-uncultured *Chloroflexus*-group was detected in a sediment gradient culture. It showed highest similarity (97 %) to a DGGE band obtained from sapropel S1 during a previous study [17] suggesting that this technique offers a high potential for the cultivation of typical sapropel microbes.

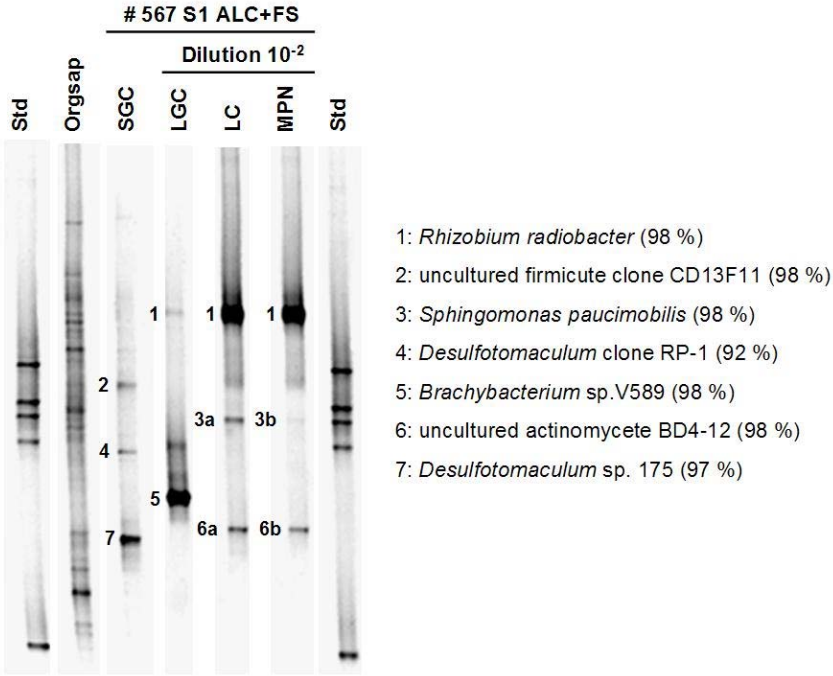

Fig. 3. DGGE profiles of the original community of sapropel S1 of site #567 (Orgsap) and initial enrichment cultures amended with *n*-alcohols (ALC) and fatty acids (FS). Negative image of SybrGold stained polyacrylamid gel. Std: standard, SGC: sediment gradient culture, LGC: liquid gradient culture, LC: liquid culture, MPN*:* Most Probable Number-series (amended only with *n*-alcohols).

3.3. Isolation efficiency

The direct comparison of the sequences derived from the DGGE bands with those of the isolates obtained from the same enrichments revealed that only few of the molecularly detected phylotypes were gained as pure cultures. Two of these DGGE bands matched the dominant phylotypes of the culture collection (*B. paraconglumeratum* and *R. radiobacter*) and another corresponded to the newly isolated actinomycete (clone BD4-12). Additionally, a *Sphingomonas* species was detected in liquid cultures although this organism was isolated from a sediment gradient culture (Table 1) and another DGGE-band corresponded to a strain

obtained from MPN-series (Firmicutes clone CD13F11, [16]). In all cases the sequences received from the DGGE bands and the respective isolates showed a similarity of nearly 100 %. However, none of the other isolated strains was retrieved in the enrichment cultures by PCR-DGGE. This might be partly due to the fact that several bands could not be sequenced. In particular those obtained from sediment gradient cultures gave ambiguous sequences, probably due to the presence of several phylotypes in a single band. Our results indicate that subculturing during the isolation procedure led to a population shift from the types dominating the primary enrichment to less-dominant but presumably faster growing types.

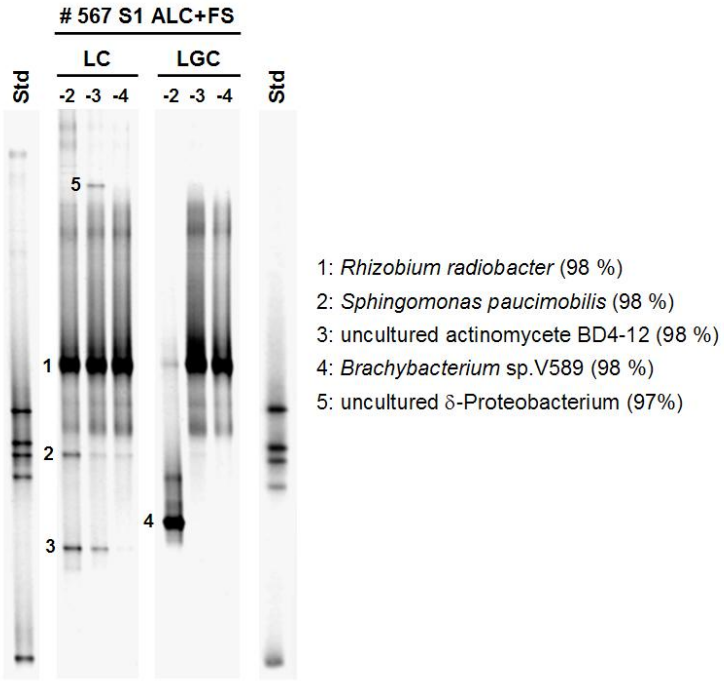

Fig. 4. DGGE profiles of dilution series of liquid cultures (LC) and liquid gradient cultures (LGC) inoculated with diluted material (10^{-2}-10^{-4}) of sapropel S1 of site #567. As growth substrates *n*-alcohols (ALC) and fatty acids (FS) were provided. Negative image of ethidium stained polyacrylamid gel. Std: standard

3.4. Comparison of the applied substrate combinations

In order to stimulate growth of a broad diversity of microbes various combinations of organic acids, *n*-alcohols and aromatic compounds were provided in the different enrichment approaches. Additionally, in liquid cultures alternative electron acceptors and a sediment extract medium were applied. PCR-DGGE analysis of selected cultures revealed that with each substrate combination a unique set of microbes was enriched (data not shown). The use of organic acids led to the highest number of phylotypes in total (14), but also to the highest

number of previously unrecognized sapropel microbes (Table 1), and the highest isolation efficiency (about 70 %). However, the mixture of *n*-alcohols in combination with fatty acids and also the mixture of aromatic compounds stimulated the growth of several different sapropel bacteria (11 and 9 phylotypes, respectively), some of which were obtained exclusively with these substrates (Table 1). The sediment extract medium, in turn, yielded only one phylotype (*Brachybacterium* sp., data not shown) and even the application of Mn, Fe and AQDS as alternative electron acceptors did not significantly increase the diversity of the culture collection (Table 1). Since these cultures were not included in the PCR-DGGE screening their actual cultivation potential remains unknown.

4. Discussion

In the present study a large number of subsurface bacteria could be either enriched or isolated from up to 200,000 years old sapropel layers. The newly developed sediment gradient technique turned out to be appropriate not only for the cultivation of various, as-yet-unrecognized sapropel bacteria but also for the enrichment of typical members of sapropel communities such as *Chloroflexi*.

4.1. Cultivation of typical subsurface microbes

Whatever cultivation attempt is performed one is confronted with the question whether the obtained organisms represent relevant members of the original communities. As already mentioned, several phylotypes of our culture collection have been isolated from highest positive dilutions of MPN-series during our recent investigation on sapropels [16] and can be considered as numerically important members of the sapropel communities. Furthermore, many of our isolates have been recognised in other subseafloor habitats by cultivation-based methods such as *Rhizobium* sp., *Bacillus* sp., *Paenibacillus* sp. and *Micrococcus* spp. [10], *Pseudomonas* sp. [12] and *Rhodococcus* sp. [32]. Others matched environmental sequences obtained from marine subsurface sediments or affiliated with terrestrial subsurface isolates (Table 1). This was also true for almost all phylotypes detected in the initial enrichments. Many of them showed highest similarity to organisms detected in deep subsurface groundwater (Table 1) or to DGGE-bands recovered from 4 m deep tidal flat sediments that are dominated by deep biosphere-related phylotypes [19].

Table 1. Diversity of cultivated sapropel bacteria obtained with different cultivation techniques and growth substrates.

Closest relative in GenBank	No. of Isolates	Cultivation technique			Substrate combination				Special properties/origin of closest relative and/or presence in other subsurface habitats[1]
		LC	GLC	SGC	TCA+AS/FS	ALK/FS	ARO	TCA/AS+Fe +Mn+AQDS	
α-Proteobacteria									
Rhizobium radiobacter (98%)*	20	i/m	i/m	i/m	i/m	i/m	i/m	i	Detected in deep subsurface sediments (a)
Erythrobacter sp. IC114 (99%)*	1			i	i				Carbazol degradation
Fulvimarina sp. K416 (98%)	3	i						i	Isolated from deep-sea invertebrates
Sphingomonas paucimobilis (98%)	2	m	m	i	i/m				PAH degradation, detected in sub seafloor habitat (b)
β-Proteobacteria									
Delftia tsuhuratensis AD9 (95%)	-		m				m		Aniline degradation
γ-Proteobacteria									
Pseudomonas sp. BU (98%)	1			i	i				Humic acid utilization, detected in deep subsurface (c)
Stenotrophomonas sp. Ellin162 (98%)	-	m	m	m	m	m	m		Detected in subsurface sediment (d)
δ-Proteobacteria									
Uncultured δ-*Proteobacterium* DGGE band IIB1 (97%)	-	m				m			Subsurface sequence
Actinobacteria									
Brachybacterium sp. V589 (98%)*	27	i	i	i/m	i	i/m	i	i	Isolate from deep subsurface accretion ice of Lake Vostoc
Micrococcus luteus (98%)*	2	i		i	i				Detected in deep subsurface sediments (a)
Micrococcus sp. P_wp 0222 (98%)*	6		i	i	i	i	i		PAH degradation, deep-sea sediment isolate
Citricoccus sp. 2216.25.22 (98%)	2		i		i	i			Isolated from coastal subseaflor sediments
Rhodococcus sp. LB1 (98%)	1			i		i			Toluene degradation, detected in subseafloor sediments (e)
Uncultured actinomycete BD4-12 (98%)	2	i/m	i/m		m	i/m	m	i	Deep-sea sediment clone
Firmicutes									
Paenibacillus glucanolyticus (98%)	3			i			i		Detected in deep subsurface sediments (a, e)
Bacillus jeotgali (99%)*	3			i	i				Detected in the marine/terrestrial deep biosphere (a, f)
Bacillus methanolicus (98%)	1		i				i		Methanol utilization
Uncultured hydrocarbon seep bacterium BPC060 (99%)	1			i	i				Deep-sea sediment clone
Uncultured firmicute clone CD13F11 (96%)*	-			m		m	m		Detected in deep subsurface groundwater (h)
Uncultured low G+C Gram-positive clone SRB6c14 (99%)	-			m	m				Petroleum degradation, marine sediment clone
Desulfotomaculum clone RP-1 (92%)	-			m		m			Syntrophic propionate-degradation with metahogens (i)
Desulfotomaculum sp. 175 (97%)	-			m		m			Detected in deep subsurface groundwater (h)
Chloroflexi									
Uncultured bacterium Seq2 (S1) (97%)	-			m	m				Sapropel sequence
OTU total / isolated (only with this technique)	**23/ 15**	**8 / 5 (1)**	**9 / 6 (3)**	**17 / 11 (6)**	**14 / 10 (5)**	**11 / 6 (3)**	**9 / 5 (2)**	**4 / 4 (1)**	

LC: liquid culture, LGC: liquid gradient culture, SGC: sediment gradient culture, AS: amino acids, TCA: intermediates of the citrate cycle, FS: fatty acids, ALK: *n*-alcohols, ARO: aromatic compounds, i: isolated, m: detected by PCR-DGGE, * isolated from sapropels by MPN-technique. [1] according to closest matches in GenBank and/or referees (a) D´Hondt et al. [10], (b) Huber et al. [49], (c) Toffin et al. [12], (d) Wilms et al. [19], (e) Inagaki et al. [32], (f) Morgan et al. unpublished, (h) Shimizu unpublished, (i) Imachi et al. [50].

If the bacterial species obtained in the present study represent typical subsurface microbes they should be adapted to the harsh conditions in these habitats. Subsurface environments are usually characterised by a poor availability and quality of organic matter. Sapropels, for example, consist mainly of amorphous highly recalcitrant kerogen [33]. In fact, many phylotypes present in our culture collection or in the initial enrichments are closely related to organisms that possess the capacity to utilise hardly degradable substances such as polycyclic aromatic hydrocarbons and humic acids or were shown to be involved in the mineralization of similar compounds in marine sediments (Table 1). Further support comes from the cultivation success achieved with aromatic compounds indicating that even the most frequently isolated bacteria such as *Rhizobium* sp. and *Brachybacterium* sp. seem to be accomplished with the degradation of recalcitrant substrates.

4.2. Influence of substrate gradients and medium variations on the diversity of cultivable sapropel bacteria

Enrichment cultures have a long tradition in microbiology [34] but it is well known that classical techniques often underestimate the bacterial diversity within natural environments [35]. In the present study, the traditional approach was improved by the use of substrate gradients. The slowly increasing concentration of the respective carbon sources should have favoured the growth of oligotrophic types and likely helped to prevent a “substrate shock” to which microbes are exposed in traditional enrichments [36]. Indeed, a direct comparison of the initial cultures suggested that liquid gradient cultures contained phylotypes differing from those in cultures without a substrate gradient (Fig. 3). However, the diversity of pure cultures obtained with both techniques was rather similar.

The medium variations used in this study turned out to be appropriate for the cultivation of sapropel microbes. Several species, in particular the most abundant phylotypes of the culture collection, grew with nearly all substrates provided, indicating the ability to utilize a broad spectrum of nutrients. However, each substrate combination was more or less selective for a unique set of microbes indicating that no single medium is suitable to recover the entire diversity of cultivable microbes from a certain sample. The high diversity of phylotypes enriched or isolated with mixtures of organic acids is consistent with high viable counts achieved with similar growth substrates during our recent investigation on sapropels [16] and supports the assumption that fermenters are important constituents of sapropel communities [17]. Even the *Chloroflexi* were detected in a sediment gradient culture amended with organic acids, namely intermediates of the citrate cycle (TCA). Only recently members

of this phylum could be enriched from intertidal sediments using the same medium (Gittel, personal communication) providing evidence for TCA as useful substrates for the cultivation of sediment-dwelling *Chloroflexi*.

4.3. Dilution series vs. undisturbed sediment material

Even more than by the medium variations or the use of substrate gradients the success of our cultivation attempt was determined by the inoculum used. By diluting a sample one should favour the growth of abundant and/or slow growing microbes that are usually outcompeted by copeotrophic bacteria in traditional enrichment cultures [37, 38]. This was only partly observed in our study. In most cases the highest dilution steps contained the most frequently isolated types (*Brachybacterium* sp. and *Rhizobium* sp.) that showed superior growth with almost all provided substrates. Therefore, it can be assumed that other abundant species were overgrown in the enrichments. This could also explain the lack of γ-*Proteobacteria* in the initial cultures and the strain collection though members of this group were frequently isolated from MPN-series [16]. Actually, Pinhassi and Berman [39] observed that γ-*Proteobacteria* are selectively outcompeted by other bacterial groups (e.g. α-*Proteobacteria*) in the presence of low nutrient concentrations. Probably, higher dilutions would have been more advantageous. On the other hand, several types have been already thinned down in the dilutions applied in this study (e.g. the uncultured deep sea-actinomycete BD4-12, Fig. 4). Despite a relatively low abundance, these microbes could play a significant ecological role in the environment [40].

However, the molecular screening and the remarkable diversity of pure cultures obtained from sediment gradient cultures indicated that the use of undisturbed sapropel material offered a much higher cultivation potential than the application of dilution series. Rare faction analysis suggested that more phylotypes could have been obtained by the use of more medium variations (date not shown). An even higher cultivation success was achieved by the application of this technique during a previous study on intertidal sediments [21] probably due to the investigation of two sites from dynamic coastal systems that differed significantly in their microbial assemblages.

One major advantage of this technique was likely to be the preserved sediment texture itself. For several microbial species the presence of surfaces might be essential for growth as it was shown for *Desulfonema* sp. [41]. Takai and coworkers [42] for example isolated novel members of ε-*Proteobacteria* from a deep-sea hydrothermal vent by means of a newly designed “in situ colonization system” in which the organisms grew on synthetic pumices. In

another study several new members of the *Cytophaga-Flexibacter-Bacterioides* group were isolated from intertidal marine sediments by simulating natural settings in diffusion chambers [20]. Several of these isolates did not grow on artificial media alone but formed colonies in the presence of other microorganisms. It was supposed that the observed growth synergy was based on the exchange of specific signals indicating a familiar environment. Such interactions as well as other synergistic effects such as cross feeding most likely occurred in SGC but were probably disturbed in the dilution cultures.

4.4. Monitoring the cultivation procedure by molecular methods

The PCR-DGGE technique applied in this study enabled a relatively quick and reliable screening of the initial enrichments. The discrepancy between the types detected in the initial cultures and those that were present in the culture collection suggests that not only the cultivation techniques but also the isolation procedure itself needs to be improved. One basic requirement for the isolation of pure cultures, as performed in the present study, was the ability to form colonies in deep agar. Agar, in turn, has inhibitory effects on the growth of some bacteria [43]. The use of Gellan gum or Gelrite as gelling agents might overcome such biases. It can be also imagined that several of the types present in the initial cultures were unable to form colonies [44] and were therefore lost during isolation. For such species single cell manipulations would be appropriate [45].

We assume that even more phylotypes could have been obtained into pure culture when the isolation procedure would have been guided by molecular methods as it was done during other studies [12, 46, 47]. If the phylogenetic position of a species is consistent with its culture requirements, the design of specific culture conditions could eventually lead to a more successful separation [48]. On the other hand, many microbes do not grow independently from syntrophic partners and alternative strategies such as cocultures [20] or dialysis cultures are required for their isolation. However, future cultivation attempts should be accompanied by molecular techniques. The polyphasic approach applied in this study revealed a diversity of sapropel bacteria that could neither be detected by direct molecular retrieval [17] nor by strain purification techniques alone.

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2.3 Widespread distribution and high abundance of *Rhizobium radiobacter* within Mediterranean subsurface sediments

Jacqueline Süß, Karin Schubert, Henrik Sass, Heribert Cypionka, Jörg Overmann, Bert Engelen

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Widespread distribution and high abundance of *Rhizobium radiobacter* within Mediterranean subsurface sediments

Jacqueline Süß,[1] Karin Schubert,[2] Henrik Sass,[1†] Heribert Cypionka,[1] Jörg Overmann[2] and Bert Engelen[1*]

[1]*Institut für Chemie und Biologie des Meeres, Carl von Ossietzky Universität Oldenburg, Carl von Ossietzky Straße 9-11, D-26111 Oldenburg, Germany.*

[2]*Ludwig-Maximillians-Universität München, Department Biologie I, Bereich Mikrobiologie, Maria-Ward-Straße 1a, D-80638 Munich, Germany.*

Summary

Eastern Mediterranean sediments are characterized by the occurrence of distinct, organic-rich layers, called sapropels. These harbour elevated microbial numbers in comparison with adjacent carbon-lean intermediate layers. A recently obtained culture collection from these sediments was composed of 20% of strains closely related to *Rhizobium radiobacter*, formerly classified as *Agrobacterium tumefaciens*. To prove and quantify the *in situ* abundance of *R. radiobacter*, a highly specific quantitative polymerase chain reaction (PCR) protocol was developed. To convert quantification results into cell numbers, the copy number of *rrn* operons per genome was determined. Southern hybridization showed that our isolates contained four operons. Finally, quantitative PCR was applied to 45 sediment samples obtained across the eastern Mediterranean. *Rhizobium radiobacter* was present in 38 of 45 samples indicating an almost ubiquitous distribution. In total, 25–40 000 cells per gram of sediment were detected, corresponding to 0.001–5.1% of the bacterial cells. In general, the relative and absolute abundance of *R. radiobacter* increased with depth and was higher in sapropels than in intermediate layers. This indicates that *R. radiobacter* forms an active population in up to 200 000 years old sapropels. The present study shows for the first time that a cultivated subsurface bacterium is highly abundant in this environment.

Received 2 February, 2006; accepted 6 April, 2006. *For correspondence. E-mail engelen@icbm.de; Tel. (+49) 441 798 5376; Fax (+49) 441 798 3583. †Present address: School of Earth, Ocean and Planetary Science, Cardiff University, Main Building, Park Place, Cardiff CF10 3YE, Wales, UK.

Introduction

Climate changes in the northern hemisphere are controlled astronomically by the Milankovic cycles (Hilgen, 1991; Lourens *et al*., 1996). These result in changing hydrographical circulation patterns in the eastern Mediterranean (Kidd *et al*., 1978; Rohling, 1994). An enhanced freshwater influx (Ryan, 1972; Rossignol-Strick, 1985) in combination with higher nutrient input and primary production (Calvert, 1983; Calvert *et al*., 1992; Lourens *et al*., 1996) leads to the formation of anoxic bottom waters and the preservation of organic material (Rossignol-Strick, 1985; Passier *et al*., 1999). Following the Milankovic cycles, several centimetres thick black-greenish sediment layers are deposited periodically approximately every 21 000 years under these conditions (Emeis *et al*., 2000). The so-called sapropels are characterized by a high content of organic carbon reaching up to 30% of the sediment dry weight and are interspersed between layers that are composed of grey, carbon-lean oozes (Calvert, 1983; Calvert *et al*., 1992).

Mediterranean sapropels were shown to be subsurface hotspots exhibiting elevated bacterial cell numbers, dividing cells and potential microbial activities (Cragg *et al*., 1998; Coolen *et al*., 2002). Therefore, it was supposed that the organic matter of sapropels, although highly refractory, provides carbon and energy sources for microbial growth (Coolen *et al*., 2002). In a quantitative cultivation study up to 3.3% of the indigenous microbial communities were stimulated to grow (Süß *et al*., 2004) and 175 environmental strains were obtained. These were affiliated to the α-, β-, γ- and δ-Proteobacteria, to the Actinobacteria and the Firmicutes (Süß *et al*., 2004). Phylogenetic studies showed that 20% of all isolates were closely related to *Agrobacterium tumefaciens* (16S rRNA sequence similarity of 98%). This typical soil bacterium was recently re-classified as *Rhizobium radiobacter* (Young *et al*., 2001). Bacteria belonging to this phylotype were obtained from all examined sediment layers and even from highest dilutions of Most Probable Number-series that were proven to be positive. These enrichments are supposed to contain the most abundant cultivated

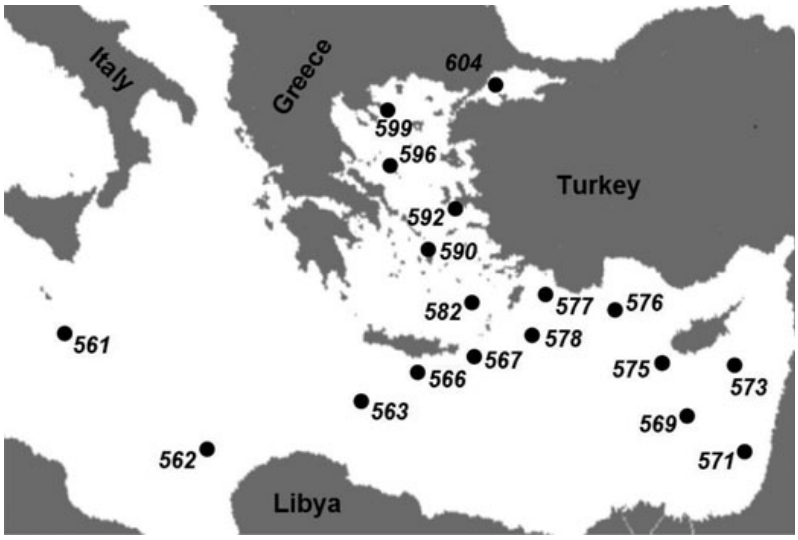

Fig. 1. Map of the eastern Mediterranean and location of sampling sites (●) of Meteor cruise Leg 51/3.

members of the original microbial communities (Rosencrantz *et al*., 1999).

One technique to prove whether an isolate shows a numerically significant *in situ* abundance is the quantitative polymerase chain reaction (qPCR). This molecular method has been successfully applied to target 16S rRNA genes or rRNAs of single species (Stubner, 2002; Panicker *et al*., 2004; Skovhus *et al*., 2004; Lloyd-Jones *et al*., 2005) as well as functional genes in different habitats (Bach *et al*., 2002; Beller *et al*., 2002; Weller and Stead, 2002; Lopez-Gutierrez *et al*., 2004). The abundance of target species in old marine sediments was determined by Schippers and colleagues (2005). In the present study, a qPCR assay for the specific and sensitive quantification of *R. radiobacter* in Mediterranean subsurface samples was established and applied in a systematic study of subfossil sediments from different sampling locations (Fig. 1, Table 1). The results indicate a widespread distribution and high abundance of *R. radiobacter* in Mediterranean sapropels.

Results

Specificity of the qPCR

Specific conditions for the quantification of *R. radiobacter*

Table 1. Study sites and sampled Mediterranean sediment layers.

Site	Latitude	Longitude	Water depth (m)	Sampled sapropels[a] and intermediate layers
561	35°47.89′N	012°59.49′E	485	Z0
562	32°46.42′N	019°11.55′E	1391	Z0, S1, S6, Z6, S7
563	33°43.30′N	023°30.06′E	1851	Z0, S1, S5, S6, S7, S8
566	34°27.98′N	025°39.96′E	1339	S1, S5
567	34°48.79′N	027°17.13′E	2153	Z0, S1, Z1, S3, Z3, S4, Z4, S5
569	33°27.22′N	032°34.78′E	1307	Z0, S1, S3, S4, S5, S6
571	32°39.29′N	034°05.78′E	1471	S1, Z1
575	34°31.39′N	031°46.40′E	2330	Z0, S1, S3, S4, S5
576	35°34.43′N	030°27.65′E	1275	Z0
577	35°54.08′N	028°30.98′E	4284	Z0
578	35°29.68′N	027°34.53′E	1367	Z0
580	35°45.19′N	027°33.18′E	723	S1
582	35°39.72′N	026°36.98′E	1495	Z0
583	35°42.59′N	026°31.52′E	1230	S1
590	37°16.42′N	026°11.54′E	580	Z0
592	37°47.66′N	026°15.80′E	1148	S1
596	38°57.29′N	024°45.20′E	883	S1
599	39°45.34′N	024°05.59′E	1085	S1
604	40°50.36′N	027°47.30′E	552	S1

a. Approximate age of sapropels: S1, 8 ka; S3, 81 ka; S4, 102 ka; S5, 124 ka; S6, 172 ka; S7, 195 ka; S8, 217 ka (Lourens *et al*., 1996). Sapropels (S1–S8) are numbered according to the system of Ryan (1972). Intermediate layers are denominated with Z0 (sediment surface) to Z4.

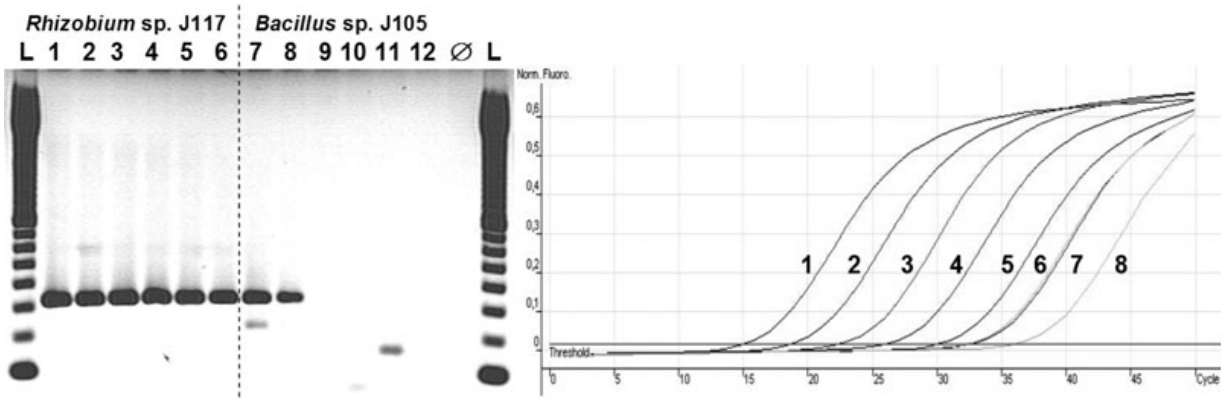

Fig. 2. Quantitative PCR of 10-fold dilution series of *Rhizobium* sp. J117 and *Bacillus* sp. J105 with primer annealing at 54°C. Left: negative image of an ethidium bromide-stained agarose gel. Right: analysis image of the corresponding PCR run. 1–6, *Rhizobium* sp. J117 10^6–10^1 targets; 7–12, *Bacillus* sp. J105 10^6–10^1 targets; Ø, negative control; L, 100 bp ladder.

target sequences in environmental samples were established by: (i) optimizing the annealing temperature for the specific primer set; (ii) excluding false positive signals by non-target strains; and (iii) determining the temperature for fluorescence read-outs to exclude the interference by primer dimers.

When the *R. radiobacter*-specific primer (Agro1r) was combined with a specific primer for α-Proteobacteria (α688f) none of the 28 tested non-target strains yielded a false positive signal at an annealing temperature of 56°C. The combination with a bacterial primer (519f) required 71°C for highest specificity and was therefore not used for further studies. However, first applications of primer pair α688f/Agro1r in qPCR showed a low PCR efficiency of 63%. This could be significantly increased to 93% by decreasing the annealing temperature to 54°C. At this lower stringency, genomic DNA of only one of the non-target strains, isolated from the same sampling site, yielded a weak signal (*Bacillus* sp. J105). Comparison of dilution series of DNA from *Rhizobium* sp. J117 and the *Bacillus* strain J105 revealed that 10^6 targets of *Bacillus* sp. J105 yielded the same CT-value as 10 targets of *R. radiobacter* (Fig. 2). As the frequencies of 16S rRNA genes of *R. radiobacter* determined in the present study were between 0.001% and 5.1% of the total bacterial cells (see below), it is highly unlikely that products resulting from unspecific amplification contributed significantly to the signal quantified.

Melting curve analyses of *R. radiobacter* amplicons revealed an increase in fluorescence above 82°C. Primer dimers were found to melt below 80°C. To circumvent the interference by primer dimers (Hein *et al.*, 2001; Stubner, 2004), fluorescence was therefore measured at 82°C. The CT-value of the no template control was always lower than the highest dilution of the positive control.

Sensitivity of R. radiobacter *quantification in sediment samples*

The quantification of *R. radiobacter* was linear down to 10 targets (Fig. 2) with a correlation coefficient of 0.998. PCR efficiencies varied between 81% and 97%. Small differences between CT-values of standards within one PCR run (0.1%) and for all runs (4%) indicated a low tube-to-tube variation and a high reproducibility.

As background DNA has been observed to dramatically decrease the sensitivity of qPCR (Skovhus *et al.*, 2004) we tested the effect of the addition of different amounts of non-target DNA. The presence of 1–10 ng of *Escherichia coli* DNA in PCR reactions did not show any effect. Analyses of different *Rhizobium* sp. J117 and *E. coli* DNA mixtures (*Rhizobium* sp. J117 DNA fraction: 0.0003–30%) gave a good accuracy over a broad range of specific target concentrations (Table 2). Similarly, qPCR was highly sensitive if 1:10 dilutions of sapropel DNA extracts were employed, whereas the application of undiluted extracts led to a significant decrease of the fluorescence signal (50%). This inhibitory effect could not be reduced by blocking with BSA concentrations of up to 2 mg ml^{-1}. Therefore, diluted environmental DNA extracts and a final

Table 2. Quantification of *R. radiobacter* in defined DNA mixtures of *E. coli* and *Rhizobium* sp. J117.

	Rhizobium sp. (%)	
No.	Theoretical	Detected
1	30	29.34 (±8.74)
2	3	2.84 (±0.64)
3	0.3	0.231 (±0.068)
4	0.03	0.035 (±0.006)
5	0.003	0.004 (±0.001)
6	0.0003	0.0008 (±0.0001)

BSA concentration of 0.2 mg ml^{-1} were used in subsequent quantifications.

Enumeration of bacteria and R. radiobacter *in Mediterranean sediments*

The abundance of bacteria in 45 sediment samples as calculated after qPCR ranged from 1.2×10^3 to 5.8×10^7 cells per gram (Table 3). The numbers were highest at the sediment surface and elevated in sapropels compared with intermediate layers. This pattern is consistent with total cell counts shown in Fig. 3 and previous investigations (Coolen and Overmann, 2000; Süß *et al.*, 2004). However, qPCR-based estimates of bacterial cell numbers were at least one order of magnitude lower than the total cell numbers (Fig. 3). This might be caused by the extraordinary high adsorption capacity of sapropels for DNA resulting in a low DNA extraction efficiency as

Table 3. Total and relative abundance of *R. radiobacter* in sediments of the eastern Mediterranean as determined by qPCR.

Sediment layer	Site	Sediment depth (mbsf)	Cells (×g^{-1} sediment)		Relative abundance of *R. radiobacter* (%)
			Bacteria	*R. radiobacter*	
Surface	561	0–0.04	5.8 (±0.6) × 10^7	3.6 (±1.7) × 10^2	0.001
	562	0–0.04	5.5 (±0.3) × 10^7	1.6 (±0.1) × 10^3	0.003
	563	0–0.04	6.9 (±1.7) × 10^6	3.2 (±1.2) × 10^2	0.005
	567	0–0.04	9.8 (±0.8) × 10^5	2.5 (±0.5) × 10^1	0.003
	569	0–0.04	1.1 (±0.2) × 10^7	2.4 (±0.9) × 10^2	0.002
	575	0–0.04	5.4 (±1.2) × 10^6	7.6 (±1.6) × 10^2	0.014
	576	0–0.04	2.2 (±0.5) × 10^7	4.5 (±0.8) × 10^2	0.002
	577	0–0.04	1.8 (±0.1) × 10^6	2.8 (±2.6) × 10^2	0.015
	578	0–0.04	2.1 (±0.5) × 10^7	2.4 (±1.5) × 10^2	0.001
	582	0–0.04	1.2 (±0.1) × 10^7	3.3 (±0.3) × 10^2	0.003
	590	0–0.04	5.8 (±0.9) × 10^7	9.7 (±3.9) × 10^2	0.002
					Average: 0.005
Sapropel					
S1	562	0.24	3.7 (±0.4) × 10^6	1.3 (±0.7) × 10^2	0.004
	563	0.23	1.8 (±0.7) × 10^6	4.4 (±2.3) × 10^1	0.002
	566	0.29	4.9 (±0.4) × 10^6	3.5 (±2.5) × 10^2	0.007
	567	0.19	4.6 (±0.2) × 10^6	–	–
	569	0.30	3.3 (±0.3) × 10^5	3.7 (±2.5) × 10^2	0.112
	571	0.55	3.2 (±0.5) × 10^6	1.4 (±0.7) × 10^4	0.594
	575	0.39	6.0 (±0.5) × 10^6	8.4 (±4.3) × 10^1	0.001
	580	1.24	3.1 (±0.5) × 10^6	1.7 (±1.1) × 10^2	0.005
	583	0.50	1.7 (±0.2) × 10^6	6.4 (±1.8) × 10^1	0.004
	592	2.22	1.6 (±0.2) × 10^6	1.4 (±0.6) × 10^2	0.009
	596	1.55	7.3 (±0.2) × 10^5	9.9 (±5.9) × 10^2	0.135
	599	1.23	9.2 (±1.6) × 10^5	7.7 (±5.1) × 10^1	0.008
	604	0.98	2.4 (±0.2) × 10^6	4.1 (±1.9) × 10^1	0.002
S3	567	2.50	4.1 (±0.4) × 10^5	2.1 (±0.8) × 10^3	0.506
	569	2.31	5.0 (±0.3) × 10^5	1.3 (±0.6) × 10^2	0.027
	575	1.33	2.0 (±0.1) × 10^6	–	–
S4	567	3.21	8.5 (±5.1) × 10^5	4.4 (±2.4) × 10^4	5.143
	569	2.95	7.3 (±0.5) × 10^4	6.1 (±5.3) × 10^2	0.836
	575	1.98	4.0 (±1.0) × 10^5	6.1 (±3.5) × 10^2	0.151
S5	563	2.26	3.0 (±0.9) × 10^6	4.1 (±3.5) × 10^2	0.014
	566	2.77	3.2 (±0.4) × 10^5	8.1 (±2.9) × 10^1	0.025
	567	4.07	1.5 (±0.3) × 10^5	6.8 (±4.4) × 10^3	4.684
	569	3.50	3.4 (±0.3) × 10^5	5.7 (±2.4) × 10^2	0.167
	575	2.63	6.5 (±0.5) × 10^5	1.9 (±1.2) × 10^2	0.029
S6	562	3.78	2.9 (±0.1) × 10^5	–	–
	563	3.28	4.5 (±2.9) × 10^6	1.2 (±0.6) × 10^3	0.027
	569	4.77	7.2 (±1.7) × 10^5	2.9 (±2.7) × 10^2	0.040
S7	562	4.28	6.8 (±2.1) × 10^3	–	–
	563	3.72	3.2 (±0.2) × 10^5	1.1 (±0.7) × 10^3	0.336
S8	563	4.07	3.6 (±0.1) × 10^5	–	–
					Average: 0.429
Intermediate layer					
Z1	567	2.35	2.8 (±0.1) × 10^4	–	–
Z1	571	2.00	7.5 (±0.9) × 10^5	7.2 (±4.8) × 10^1	0.010
Z3	567	2.88	1.2 (±0.7) × 10^3	–	–
Z4	567	3.50	6.3 (±1.8) × 10^4	1.4 (±0.7) × 10^2	0.224
Z6	562	3.93	7.1 (±0.4) × 10^4	–	–
					Average: 0.047

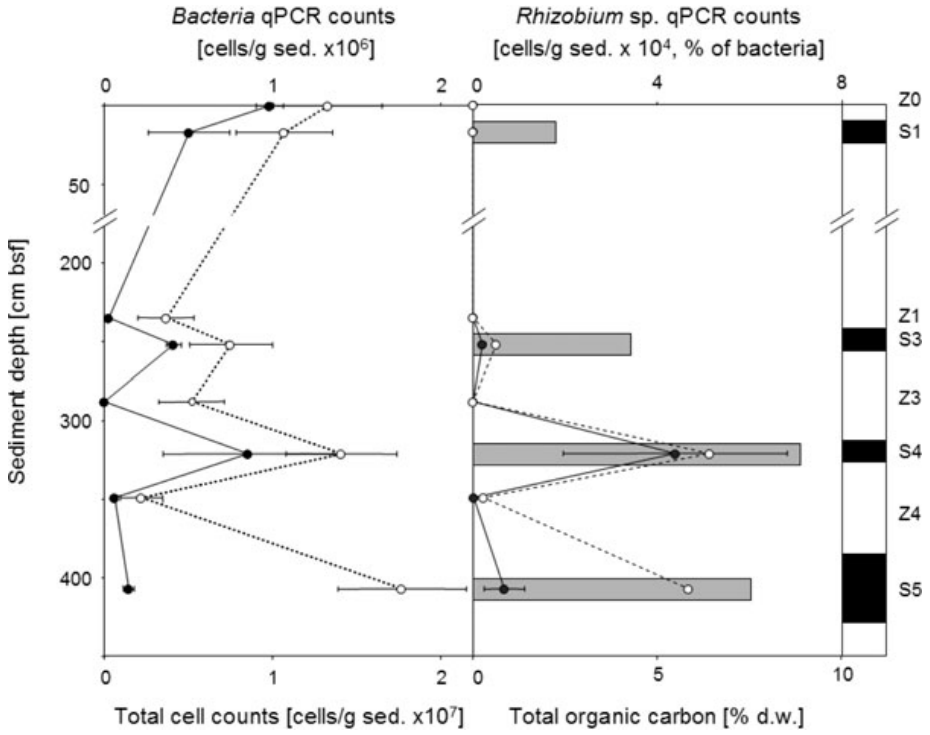

Fig. 3. Depth profiles at site 567. Left: total cell counts determined by acridine orange staining (○) and bacterial numbers estimated by qPCR (●). Right: absolute (●) and relative abundance (○) of *R. radiobacter* determined by qPCR and the content of total organic carbon (grey bars). Black bars indicate organic-rich sapropels (S1–S5), white bars carbon-lean intermediate layers (Z1–Z4).

proven by spiking experiments (Coolen and Overmann, 2000).

The enhanced sensitivity of the optimized PCR protocol permitted a detection of *R. radiobacter* in 38 of the 45 examined sediment samples (Table 3). Southern hybridization revealed that the genome of Mediterranean *Rhizobium* strains contained four *rrn* copies (Fig. 4). This factor was used for the conversion of qPCR results to cell numbers of *R. radiobacter*. The calculated values of 25–44 000 cells per gram of sediment corresponded to 0.001% and 5.14% of the qPCR bacterial numbers. The highest abundance was found in sapropels S4, S5 and S1 at site 567 and site 571 respectively. The presence of *R. radiobacter* in these layers was verified by PCR-denaturing gradient gel electrophoresis (DGGE) of 16S rRNA gene fragments and sequencing of dominant bands (data not shown). *Rhizobium radiobacter* was also isolated in pure culture from various samples in which it was not detectable by qPCR (e.g. S1, Z1 of site 567 and S3 of site 575; Süß *et al.*, 2004). It therefore appears possible that the recovery of DNA from the sediments is limited, leading to an underestimation of actual cell counts of *R. radiobacter*. However, the relative abundance of *R. radiobacter* (Table 3, Fig. 3) was generally enhanced in the sapropels (average: 0.429%) compared with intermediate (average: 0.047%) and surface layers (average: 0.005%). Calculated cell numbers had a closer correlation to the total organic carbon (TOC) content of the sediments ($r^2 = 0.678$) than

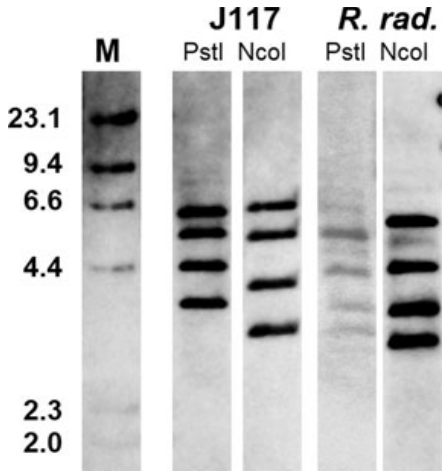

Fig. 4. Number of *rrn* operons of *Rhizobium* sp. J117 and *R. radiobacter* DSM 30147^T determined by Southern hybridization. Images of X-ray films composed of two hybridizations. M, Lambda phage size marker, molecular weights are given in kb.

the total cell counts ($r^2 = 0.538$) and bacterial cell numbers ($r^2 = 0.127$).

To test if the river Nile represents a possible source of *R. radiobacter*, we also applied the PCR protocol to sediment and water samples taken in Assuan, Egypt. Here, an abundance of 2.9×10^5 cells per gram of sediment and 500 cells per litre was determined.

Discussion

The present work demonstrates that *R. radiobacter* is widespread in Mediterranean sediments and contributes substantially to the microbial community composition even in very old sapropels. The highly specific, sensitive and accurate quantification of *R. radiobacter* in Mediterranean sediments indicates that isolates obtained from marine subsurface sediments are indigenous and abundant in the environment despite their close affiliation to surface-dwelling bacteria.

Indications for active R. radiobacter *populations in Mediterranean sediments*

Several lines of evidence indicate that *R. radiobacter* populations are active within the environment. If the cells would exist in a stage of dormancy, one would expect a decline in abundance with sediment depth and age. However, estimated numbers of *R. radiobacter* were generally elevated in deeper sediments compared with upper layers. Furthermore, isolates of *R. radiobacter* were obtained from up to 200 000 years old sapropels (S7; Süß *et al*., 2004). Consequently, dormant cells of *R. radiobacter* would have had to survive over more than 200 000 years. Survival for such a long period is so far only known for endospores or cysts, which, in turn, are not produced by this genus. Although extremely low maintenance energy requirements enable a prolonged survival of vegetative microbial cells in permafrost soils or deep subsurface sediments (Price and Sowers, 2004) this does not apply for Mediterranean subsurface microbes. Maintenance energy requirements increase with rising temperatures, and therefore must be considerable for *R. radiobacter* populations in Mediterranean sediments due to the rather high *in situ* temperatures of 13°C (Slomp *et al*., 2002).

River water as a source for Rhizobium *cells in Mediterranean sediments?*

A potential source for *R. radiobacter* in Mediterranean sediments may be a fluvial input. The river Nile, for example, had a strong impact on the eastern Mediterranean by annually occurring mud flows and particularly during times of sapropel formation via a high river discharge (Rohling, 1994). During these events, *R. radiobacter* cell numbers in the Nile water were probably higher than in recent water samples as an enhanced river run-off generally results in higher fractions of suspended fluvial material (Hellmann, 1986). However, the isolates and the terrestrial type strain exhibit the required salinity tolerance to survive the migration from the river Nile to the Mediterranean as they were able to grow in freshwater as well as in seawater medium. A prerequisite for survival under unfavourable or changing environmental conditions is a high regulatory capacity on the genome level. This might be suggested by complete genome analyses of the closest relative of our isolates (*A. tumefaciens* C58; Wood *et al*., 2001). The ability for nutrient acquisition under highly competitive conditions is indicated by a high proportion (15%) of genes encoding for transporter proteins (Wood *et al*., 2001). The combination of salt tolerance with the ability to utilize many different and even recalcitrant carbon sources, such as aromatic compounds (Stowers, 1985), may favour the proliferation of a typical soil bacterium as *R. radiobacter* within sapropels.

Implications for the in situ *metabolism of* R. radiobacter *populations*

Cells of *R. radiobacter* must be able to grow within the oxygen-depleted sediments as all isolates were obtained from anoxic enrichment cultures. Potential exoenzyme activities and glucose degradation rates point towards the presence of primary and secondary fermenters in the indigenous microbial communities in sapropels (Coolen and Overmann, 2000; Coolen *et al*., 2002). Although fermentative growth on a single carbon source was not yet proven for Mediterranean *Rhizobium* strains, fermentation might have occurred in our enrichments as they mostly contained mixtures of fermentable compounds (Süß *et al*., 2004). Another possibility is, that the kerogen material of sapropels serves as an external electron acceptor as described for humic acids (Lovley *et al*., 1996). A detailed physiological characterization of the isolates is currently in progress and will provide a deeper insight into the adaptation of *R. radiobacter* to environmental conditions.

At present, the *in situ* growth rates and the available substrates remain unknown. Potential carbon sources, such as carbohydrates, lipids, proteins and biopolymeric carbons are available in Mediterranean surface sediments, but strongly decline with depth (Luna *et al*., 2004). In sapropels, carbon compounds might be, if even present, strongly adsorbed to the kerogen matrix and hence hardly bioavailable (Coolen and Overmann, 2000). The adaptation of *R. radiobacter* to prolonged nutrient deprivation was proven by the successful application of low substrate concentrations in our media (Süß *et al*., 2004) as conventional isolation techniques did not result in any pure culture.

The re-classification of *A. tumefaciens* to *R. radiobacter* led to confusion regarding to other phenotypic properties of this species, such as nitrogen fixation or causing crown galls in plants. However, neither was uptake of elemental nitrogen described for the type strain nor the *nifH* gene was detected in our isolates (data not shown). Even though nitrogen fixation most likely played a significant role in the phototrophic zone during sapropel formation (Sachs and Repeta, 1999) geochemical profiles of the sediments indicate that nitrogen fixation might be suppressed within the sapropel layers. Nitrate penetrates the sediment column down to the top sapropel S1 and ammonium accumulates with increasing sediment depths (Slomp *et al*., 2002). These are energetically more preferable nitrogen sources for indigenous microbes than molecular nitrogen.

Is R. radiobacter *ubiquitous in deep marine sediments?*

It has been questioned whether available deep subsurface isolates represent authentic inhabitants, as they are closely related to surface-dwelling bacteria (DeLong, 2004). Recent studies identified supposedly terrestrial microbes, such as actinomycetes, as common constituents of marine microbial communities (Jensen and Fenical, 1995; Colquhoun *et al*., 1998). This might also be the case for the genus *Rhizobium*. Members of this genus have been detected in marine bacterioplankton communities (e.g. Pinhassi *et al*., 1997; 2003; Granger and Price, 1999; Kelly and Chistoserdov, 2001), in association with marine protists or metazoans (Alavi *et al*., 2001; Frias-Lopez *et al*., 2002) and in marine sediments (e.g. Cifuentes *et al*., 2000; Mills *et al*., 2004; Koepke *et al*., 2005; Stevens *et al*., 2005). Even studies on deep-sea habitats revealed the presence of *Rhizobium* species or close relatives (e.g. Dang and Lovell, 2001; Marchesi *et al*., 2001; Suzuki *et al*., 2004). Actually, in another cultivation-based study we obtained several pure cultures closely related to *R. radiobacter* from deeply buried sediments of the Pacific Ocean (D'Hondt *et al*., 2004). The last finding and our study provide strong evidences for *R. radiobacter* as a widespread microbe of marine subsurface sediments.

Experimental procedures

Sampling

Sediment samples were obtained in 2001 during the *Meteor* cruise 51/3 at several locations in the eastern Mediterranean (Fig. 1, Table 1). The sediment surface was sampled by means of a multicorer while for deeper layers a gravity corer was used (Süß *et al*., 2004). Subsamples from sapropels and intermediate layers were collected aseptically as described previously (Coolen and Overmann, 2000) and transferred into sterile Falcon tubes. Water and sediment surface samples from the river Nile were taken in 2005 in Assuan, Egypt. The sediment was recovered by using a 60 ml sterile cut-off syringe, whereas microbial cells from 300 ml of Nile water were concentrated on a 0.2 μm polycarbonate filter (Millipore, Tullagreen, Ireland). All samples were stored at –20°C until processing.

Extraction of nucleic acids

Nucleic acids of pure cultures were extracted using lysozyme (40 μg ml^{-1}) and SDS (1% w/v) treatment followed by five freeze and thaw steps (–70°C, 95°C, 3 min each). Afterwards, nucleic acids were purified with organic solvents (phenol, phenol-chloroform, chloroform, each H_2O saturated and at pH 7.5) and precipitated with a 13:1 mixture of ethanol (70% v/v) and sodium acetate (3 M, pH 7.5). The precipitate was washed (ethanol, 70% v/v), dried, re-suspended in TE buffer (10 mM Tris HCl, 1 mM EDTA, pH 8.0) and stored at –20°C.

Environmental DNA was extracted from 10 g of sediment using the MoBIO Ultra Clean Mega Prep Soil DNA Kit (Dianova, Hamburg, Germany) according to the manufacturer's specifications. To ensure cell lysis and to optimally disperse the sediment samples, an additional sonication step was performed after the chemical lysis step. The planktonic cells from the river Nile that were collected on polycarbonate filters were disrupted by bead beating and purified as described above.

DNA standards for qPCR

The 16S rRNA gene of *Rhizobium* sp. strain J117 (AJ630169) was amplified using the universal primer set 8f and 1492r (Lane, 1991) as described previously (Süß *et al*., 2004). The PCR product was purified using the QIAquick PCR Purification Kit (Qiagen GmbH, Hilden, Germany) and the DNA concentration quantified fluorometically with PicoGreen (Molecular Probes, Leiden, the Netherlands) according to the instructions of the manufacturer. The number of *Rhizobium* targets was calculated from the DNA content, the length of the amplified fragment (1484 bp) and the mean weight of a base pair (1.1×10^{-21} g). For each qPCR run a separate standard curve was generated from 10-fold dilutions of standards ranging from 10 to 10^6 target molecules.

Primers

A *R. radiobacter*-specific reverse primer (Agro1r, 5′-GTC TCC AAT GCC CAT ACC C-3′; *E. coli* positions 1000–1018) was developed using the ARB software package (Ludwig *et al*., 2004), while as forward primers a bacterial primer (519f; Lane, 1991) and a primer specific for α-Proteobacteria (α688f; Uphoff *et al*., 2001) were tested. For both primer combinations, the optimal annealing temperature was determined by gradient PCR (Eppendorf Mastercycler gradient, Hamburg, Germany). For the quantification of bacterial targets, the universal primers 519f and 907r (Muyzer *et al*., 1995) were used. All oligonucleotides were synthesized by MWG-Biotech AG (Ebersberg, Germany).

Primer specificity

Specificity of the primer sets for quantifying *R. radiobacter* was tested with the following non-target organisms obtained from the German culture collection (DSMZ): *Roseobacter gallaeciensis* (DSM 12440^T), *Ruegeria gelatinovorans* (DSM 5885^T), *Paracoccus aminophilus* (DSM 8538^T), *Burkholderia pyrrocinia* (DSM 10685^T), *Aquaspirillum delicatum* (DSM 4558^T), *Alcanivorax jadensis* (DSM 12178^T), *Alteromonas atlantica* (DSM 6839^T), *Desulfobulbus mediterraneus* (DSM 13871^T), *Pelobacter venetianus* (DSM2394^T), *Muricauda ruestringensis* (DSM 13258^T), *Streptomyces glaucescens* (DSM 40155^T), *Streptomyces violaceoruber* (DSM 40701^T), *Arthrobacter nicotinovorans* (DSM 420^T), *Bacillus marinus* (DSM 1297^T), *Methanosarcina barkeri* (DSM 800^T), *Methanobacterium bryantii* (DSM 863^T), *Methanospirillum hungatei* (DSM 864^T), *Methanococcus vannielii* (DSM 1224^T) and *Methanohalophilus portucalensis* (DSM 5703^T).

Furthermore, the following strains isolated from Mediterranean sediments (Süß *et al.*, 2004) were tested: *Marinobacterium* sp. J5 (AJ630166), *Photobacterium* sp. J4 (AJ748351), *Vibrio* sp. J8 (AJ630167), *Erythrobacter* sp. J35 (AJ630174), *Paenibacillus* sp. J36 (AJ630152), *Micrococcus* sp. J90 (AJ630182), *Brachybacterium* sp. J92 (AJ630186), *Bacillus* sp. J105 (AJ630146) and *Desulfofrigus* sp. J152 (AJ630195).

Quantification of bacteria and R. radiobacter

First studies were performed using a home-made mastermix, containing SybrGreenI, 1 U *Taq* DNA polymerase (New England Biolabs), the appropriate 10× buffer, $MgCl_2$ (2 mM), BSA (0.2 mg ml^{-1}), dNTPs (200 μM each) and the primers (10 pM each) in a final volume of 15 μl per reaction. Diluted standards and DNA extracts (10 μl each) were taken as templates. SybrGreenI was applied in different concentrations (1:5000–1:20 000) diluted in ddH_2O or TE buffer. When TE buffer was used, the $MgCl_2$ concentration of the mix was set to 2.5 mM to prevent inhibitory effects by the buffer (Karsai et al., 2002). Further quantifications were performed by use of the Finnzymes DyNAmo™ HS SYBR® Green qPCR Kit (BioCat, Heidelberg, Germany). The reaction mixture contained 12.5 μl of the double concentrated premix solution, BSA and the respective primers. Additionally, T4-Gen32-Protein (25 μg ml^{-1}; Roche Applied Science, Mannheim, Germany) was added, to stabilize single-stranded DNA during primer annealing and to avoid inhibitory effects of humic acids coextracted from the environmental samples (Tebbe and Vahjen, 1993). Quantitative PCR was performed in a RotorGene-3000 cycler (Corbett Research, Sydney, Australia). The following PCR protocol was applied for the quantification of bacteria and *R. radiobacter*: initial denaturation and activation of the hot start polymerase at 95°C for 15 min, followed by 50 cycles with denaturation at 94°C for 10 s, annealing at 54°C for 20 s, elongation at 72°C for 30 s, and fluorescence measurement at 72°C and 82°C. Subsequently a melting curve was recorded by increasing the temperature from 50°C to 99°C (1°C every 10 s). Data analysis was performed using the software package RotorGene 4.6. Specificity of PCR products was checked by melting curve analysis and agarose gel electrophoresis. Bacterial targets were measured in three different dilutions of environmental DNA extracts (1:100, 1:500 and 1:1000). Quantification of *R. radiobacter* was performed in 1:20–1:500 dilutions of environmental DNA extracts in at least fourfold parallels. Cell numbers of bacteria were estimated using an average *rrn* copy number of 3.8 (Fogel et al., 1999). For *R. radiobacter*, the *rrn* copy number was determined as described below.

Determination of the rrn *operon copy number*

In order to convert the number of detected targets to cell numbers, the rRNA gene copy number of environmental *Rhizobium* sp. strains was determined by Southern hybridization. The method was established using *R. radiobacter* (DSM 30147^T) as control strain, which contains four *rrn* copies as listed in the Ribosomal RNA Operon Copy Number Database (Klappenbach *et al.*, 2001). Genomic DNA (1–2 μg) of each strain was digested with five restriction enzymes (NcoI, PvuII, PstI, AccI and EcoRI) respectively. DNA fragments were separated on 0.75% w/v agarose gels in 0.5× TBE buffer (44.5 mM Tris base, 44.5 mM boric acid, 1 mM EDTA, pH 8.3). After depurination (0.2 N HCl; 2 × 5 min), denaturation (0.5 M NaOH, 1.5 M NaCl; 2 × 15 min) and neutralization (1 M Tris base, 1.5 M NaCl, pH 7.5; 2 × 15 min) the fragments were transferred to a positively charged nylon membrane (Boehringer Mannheim, Mannheim, Germany) by capillary blotting using 20× SSC (3 M NaCl, 0.3 M Na-citrate, pH 7.0) as a transfer solution. The DNA was then immobilized by baking for 30 min at 120°C. Specific probes for the isolates and the type strain were prepared by PCR-amplification of a 16S rRNA gene fragment using the universal primers 8f and 519r (Lane, 1991). The 470 bp fragments were separated on 1.5% w/v agarose gels, excised and purified using the QIAquick Gel Extraction Kit (Qiagen GmbH, Hilden, Germany). The probe was then labelled with digoxigenine using the DIG High-Prime DNA Labelling and Detection Starter Kit II (Roche Applied Science, Mannheim, Germany). Hybridization was carried out for 24 h at 45°C in a hybridization solution that contained 50% v/v formamide (deionized), 5× SSC, 0.1% w/v *N*-lauryl sarcosine, 0.02% w/v SDS, 2% w/v Blocking Reagent (supplied with the DIG labelling kit) and 25 ng ml^{-1} of the labelled probe. Washing and detection was performed according to the instructions supplied with the DIG labelling kit. Finally, chemiluminescence signals were visualized by exposure and development of an X-ray film.

Total cell counts

Total cell numbers were quantified directly on board the RV *Meteor* by epifluorescence microscopy employing an improved protocol for acridine orange staining. In total, 1 cm^3 of sediment was added to 9 ml of sterile synthetic seawater containing 2% v/v glutardialdehyde, and suspended by vortexing. For each sample, 5 μl aliquots were transferred into Eppendorf vials (Eppendorf, Hamburg, Germany) containing 50 μl sterile tap water and 100 μl acridine orange solution (Serva, Heidelberg, Germany). After staining for 10 min, the sediment and bacterial cells were pelleted by centrifugation and washed three times with 200 μl of particle-free water.

Finally, the pellet was evenly spread on a microscopic slide, dried on a hot plate, and covered with 5 µl of paraffin oil and a cover slip. For each sample, the occurrence of green-coloured bacterial cells in 100 separate fields was recorded. No orange-coloured cells were observed.

Determination of TOC values

The TOC content was determined as the difference between total carbon analysed by combustion in a Leco CS-444 instrument (Leco Instruments GmbH, Mönchengladbach, Germany) and inorganic carbon measured with a UIC CO_2-coulometer (UIC, Joliet, IL) according to Rütters and colleagues (2002).

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2.4 Two distinct *Photobacterium* populations thrive in ancient Mediterranean sapropels

Jacqueline Süß, Kerstin Herrmann, Michael Seidel, Heribert Cypionka, Bert Engelen, Henrik Sass

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Two distinct *Photobacterium* populations thrive in ancient Mediterranean sapropels

Jacqueline Süß, Kerstin Herrmann, Michael Seidel, Heribert Cypionka, Bert Engelen and Henrik Sass[1*]

Institut für Chemie und Biologie des Meeres, Carl von Ossietzky Universität Oldenburg, Carl von Ossietzky Straße 9-11, D-26111 Oldenburg, Germany

[1] Present address: School of Earth, Ocean and Planetary Science, Cardiff University, Main Building, Park Place, Cardiff CF10 3YE, Wales UK

Running title

Microdiversity of *Photobacterium* populations in sapropels

Keywords

Microdiversity; *Photobacterium*; metabolic properties; marine subsurface; sapropels

[*]Corresponding author. Mailing address: School of Earth, Ocean and Planetary Science, Cardiff University, Main Building, Park Place, Cardiff CF10 3YE, Wales UK, Phone: +44-29-208-76001, FAX: +44-29-2087-4329, email: henrik@earth.cf.ac.uk

Abstract

Eastern Mediterranean sediments are characterised by the periodic occurrence of conspicuous, organic-rich sapropel layers. Phylogenetic analysis of a large culture collection isolated from these sediments revealed that about one third of the isolates belonged to the genus *Photobacterium*. In the present study 22 of these strains were examined with respect to their phylogenetic and metabolic diversity. The strains belonged two distinct *Photobacterium* populations (Mediterranean cluster I and II). Strains of cluster I were isolated exclusively from organic-rich sapropel layers and were closely affiliated with *P. aplysiae* (based on their 16S rRNA gene sequence). They possessed almost identical ERIC and substrate utilization patterns, even among strains from different sampling sites or layers differing up to 100,000 years in age. Strains of cluster II originated from sapropels as well as from the surface and carbon-lean intermediate layers. They were related to *P. frigidiphilum*, but differed significantly in their fingerprint patterns and substrate spectra, even when these strains were obtained from the same sampling site and layer. Temperature range for growth (4 to 33°C), salinity tolerance (5 to 100 PSU), pH requirements (5.5-9.3) and the composition of polar membrane lipids were similar for both clusters. All strains grew readily by anaerobic respiration (nitrate, DMSO, AQDS or humic acids) or fermentation (glucose, organic acids). These results indicate that the genus *Photobacterium* comprises subsurface inhabitants with different patterns of adaptation to life in the deep biosphere.

Introduction

In the recent years the marine deep subsurface received increasing attention. Geochemical analyses and modeling [12] but also radiotracer-based activity measurements [34,35], direct microscopic or viable counts [12,41] revealed the presence of active microbial communities in up to 15 Ma old marine sediments [56]. It was also shown that microbial densities and metabolic activities in deeply buried sediments correlate with the availability of organic carbon [7,35] and/or electron acceptors [10,12,13]. Extensive molecular analyses were performed and unraveled an unexpectedly large microbial diversity [23,31], including some phylogenetic lineages appearing to be typical for deep subsurface habitats [7,55]. However, due to the low fraction of sediment microbes that have been brought into pure culture [12,51], there is a lack in knowledge of physiological adaptations of indigenous deep biosphere bacteria.

One aspect lowering the cultivation success might be the application of high substrate concentrations in standard microbiological media. Apparently, the use of media containing submillimolar substrate concentrations increased the cultivation efficiency and resulted in the subsequent isolation of a large culture collection from ancient Mediterranean sapropels [46]. These dark, periodically occurring sediment layers differ from other subsurface environments in their unusually high organic carbon contents (up to 30 % of the dry weight, [37]). Although the organic material consists mainly of highly recalcitrant kerogen, sapropels were shown to be subsurface 'hotspots' with elevated microbial numbers and increased potential microbial activities [7,8,11].

The culture collection from sapropels of the Eastern Mediterranean included 98 strains covering 19 different phylotypes. Phylogenetic analysis revealed that about a third of the strains affiliated with the soil bacterium *Rhizobium radiobacter* [46]. The occurrence of this phylotype as a member of the deep biosphere was already proven by molecular methods [47]. Another 30 % of the strains belonged to the genus *Photobacterium* [46]. This genus is one of the earliest described bacterial taxa [5] and was originally considered to be associated with marine animals [38]. Although photobacteria were found to be widespread in marine sediments (e.g. *P. profundum* and *P. frigidiphilum*; [32,43]) their prevalence in the culture collection obtained from the up to 120,000 years old sapropels is unexpected and extends their 'typical' habitat range. Since *Photobacterium* sp. are among the dominant cultured subsurface bacterial groups, analysis of their physiology might help to deepen the knowledge about metabolic adaptations of sediment microbial communities.

Several recent studies have revealed a remarkable phylogenetic [1,36,49] or physiological [3,19,29] heterogeneity at the subspecies level within single bacterial taxa. The ecological significance of this phenomenon still remains elusive [15] but it was assumed that this microdiversity is amongst others a prerequisite for longevity of bacterial populations in changing environments [2,9,42]. In the present study 22 *Photobacterium* strains originating from three different sampling sites in the Eastern Mediterranean Sea were examined with respect to their phylogenetic and metabolic diversity. The extent of the culture collection offered the opportunity to link variations in 16S rRNA genes and ERIC-PCR fingerprinting patterns to physiological differences and correlate these to spatial separation or to age and TOC-content of the sediment layers.

Material and Methods

Sample origin and isolation of the Photobacterium *strains*

All strains analyzed in this study were obtained from eastern Mediterranean sapropels and intermediate layers sampled during R/V Meteor cruises M 40/4 and M 51/3 [8,46]. Most strains were isolated from highest positive dilutions of oxic and anoxic MPN series that were supplemented with different carbon sources and electron acceptors (Table 1). Strains 67TD and 67FSB were obtained from anoxic enrichments directly inoculated with sapropel material.

Extraction of nucleic acids

Genomic DNA of the strains was extracted following a standard protocol with cell lysis by lysozyme, SDS and “freeze and thaw” cycles with subsequent purification as described by Süß et al. [47]. Purified nucleic acids were resuspended in TE buffer (10 mM Tris HCl, 1 mM EDTA, pH 8.0) and stored at -20°C.

Phylogenetic analysis

The 16S rRNA genes of the strains were amplified and sequenced as described elsewhere [46]. Sequences were compared to those available in the GenBank database using the BLASTN tool. Phylogenetic trees were constructed using the ARB software package [27]. The maximum likelihood method was used for the construction of backbone-trees considering sequences of validly described species with sequence lengths of at least 1300 bp. Sequences of recently described species [43,44,48,59] not yet available in the ARB database release were

retrieved from GenBank. Sequences <1300 bp were added afterwards by parsimony interactive using a newly constructed specific *Photobacterium* filter. To verify the stability of the Mediterranean *Photobacterium* clusters, neighbor-joining and parsimony trees were calculated as described for the maximum likelihood method.

ERIC-PCR

To investigate subspecies diversity of closely related strains genomic fingerprinting applying the primer ERIC1R and ERIC2 [53] was performed. The PCR reaction mix contained 1 U Red Taq DNA polymerase (Sigma, Munich, Germany) and the appropriate 10 x buffer, dNTP´s (200 µM each), $MgCl_2$ (2.1 mM), BSA (0.2ng·µl^{-1}), the primers ERIC1R and ERIC2 (5 pM each) and 1 to 5 µl template DNA in a final volume of 50 µl. PCR was performed in a Perkin Elmer thermocycler (Perkin Elmer Gene Amp PCR System 9600, Wellesley, MA). The following protocol was used according to Versalovic et al. [53]: 4 min denaturation at 96°C, followed by 35 cycles with denaturation for 30 sec at 94°C, annealing for 1 min at 52°C, and elongation for 8 min at 72°C. Post elongation was performed for 10 min at 72°C. Fragments were separated on 1 % (w/v) agarose gels (90 V for 3 h). Gels were stained for 20 min with ethidium bromide and documented by means of a digital imaging system and the respective software (BioDoc Analyze Biometra, Göttingen, Germany). The resulting band patterns were analyzed using the software package GelCompar II Version 2.5 (Applied Maths, St-Martens-Latem, Belgium). The densometric curves were compared using the Pearson coefficient. Dendograms were generated by the UPGMA-method (unweighted-pair group method with arithmetic averages).

Growth media

Artificial seawater was used as a basal medium. This medium contained (in g · l^{-1}): NaCl (24.3), $MgCl_2 \cdot 6H_2O$ (10), $CaCl_2 \cdot H_2O$ (1.5), KCl (0.66), Na_2SO_4 (4), KBr (0.1), H_3BO_3 (0.025), $SrCl_2 \cdot 6H_2O$ (0.04), NH_4Cl (0.021), KH_2PO_4 (0.0054), NaF (0.003). The medium was supplemented with 1 ml · l^{-1} trace element solution SL10 and 0.2 ml · l^{-1} of a selenite and tungstate solution [57]. The oxic medium was buffered with HEPES (2.4 g · l^{-1}). The pH of the medium was adjusted to 7.2 – 7.4 with NaOH prior to autoclaving. After autoclaving, the medium was cooled under air and supplemented with vitamins and sodium bicarbonate (final concentration 0.2 g · l^{-1}).

For anoxic incubations a slightly different medium was used. It contained resazurin (0.25 mg·l^{-1}) as a redox indicator. After autoclaving the anoxic medium was cooled under an

atmosphere of N_2/CO_2 (80/20, v/v). Instead of HEPES, 30 ml · l^{-1} of a 1 M sodium hydrogen carbonate solution was added as a buffer from a sterile stock. After addition of vitamins the medium was carefully reduced by addition of sterile dithionite. The pH of the reduced medium was set to 7.2 – 7.4 with sterile HCl or Na_2CO_3 if necessary.

Substrate utilization under oxic conditions

For tests on substrate utilization, 56 different carbon sources were chosen for growth tests under oxic conditions. Growth assays were prepared in polystyrene microtiter plates (Costar 3795, Corning, New York, NY). Each well was filled with 180 µl oxic basal medium. The following carbon sources were provided (final concentrations are given in brackets). (I) complex substrates: peptone (0.05 % w/v), casamino acids (0.05 % w/v), yeast extract (0.005 % w/v), (II) polysaccharides: cellulose (0.05 % w/v), starch (0.1 % w/v), chitin (0.05 % w/v), xylan (0.05 % w/v) and laminarin (0.05 % w/v), (III) mono- and disaccharides, sugar derivates: sucrose, cellobiose, maltose, trehalose, arabinose, xylose, fructose, glucose, mannose, rhammnose, mannitol, gluconate and glucosamine (each at 5 mM), (IV) organic acids: lactate and succinate (each at 10 mM), formate, acetate, malonate, fumarate, malate, 2-oxoglutarate, glycolate and pyruvate (each at 5 mM), butyrate (2.5 mM), tartrate (2 mM), citrate (2 mM), propionate (1 mM), capronate, caprylate, crotonate and valerate (each at 0.5 mM), (V) alcohols: ethanol, *n*-propanol, *n*-butanol, glycol, glycerol (each at 5 mM), methanol (2 mM) and Tween80 (0.001% w/v), (VI) L-amino acids: alanine, arginine, cysteine, glutamine, isoleucine and phenylalanine (each at 2 mM), (VII) miscellaneous compounds: betaine, benzoate, salicylate and nicotinate (each at 2 mM). Cells were harvested from exponential phase liquid cultures and washed three times. Three replicates and two substrate free controls were inoculated for each strain as well as two inoculum free controls for each substrate. The plates were incubated at 20°C for six weeks. Growth was determined by fluorometry [28] as well as by microscopy of selected wells.

Use of electron acceptors

The tests were performed in completely-filled, screw cap glass tubes with anoxic artificial seawater as basal medium. Acetate (10 mM) was used as electron donor and carbon source. NO_3^- (10 mM), $Fe(OH)_3$ (40 mM), manganese oxides (20 mM), thiosulfate (10 mM), elemental sulfur (20 mM), dimethyl sulfoxide (DSMO, 10 mM), 9,10-anthra-quinone-2,6-disulfonate (AQDS, 4 mM) and a iron-free humic acid suspension (1 mg ml^{-1}, [6]) were chosen to test anaerobic growth with alternative electron acceptors. For each strain and

electron acceptor combination three replicates were inoculated as well as an inoculum-free control and a control without electron acceptor. The assays were incubated for six weeks at 20°C in the dark. Growth was monitored microscopically. The conversion of nitrate to nitrite or ammonium was determined photometrical according to Grasshoff et al. [16]. Reduction of $Fe^{(III)}$ to $Fe^{(II)}$ was generally indicated by the formation of black precipitates. The utilization of $Mn^{(IV)}$ led to decolorisation and finally disappearance of the brown manganese oxides and the precipitation of whitish manganese carbonates. The production of sulfide due to the reduction of thiosulfate, sulfite or elemental sulfur was measured photometrical after addition of an acidic cupric solution at 436 nm in accordance to Widdel [Widdel F (1980) PhD Dissertation University Göttingen]. Reduction of AQDS to the reduced antrahydroquinone was measured photometrically at 450 nm [26].

Fermentative growth

Fermentative growth was tested in completely-filled screw cap glass tubes filled with anoxic artificial seawater supplied either with glucose (10 mM), a mixture of L-amino acids (alanine, threonine, lysin-monohydrochloride, arginine, asparagine, aspartate, leucine, isoleucine, glumatate, glutamine and methionine, each 5 mM), organic acids (malate, fumarate, succinate and lactate, each 15 mM) or a mixture of alcohols (methanol, ethanol, *n*-propanol and *n*-butanol, each 10 mM) as substrates. For each substrate three replicates and a substrate free control were incubated at 20°C for six weeks. Growth was monitored by microscopy. Positive glucose tests were analyzed by reversed phase HPLC using a Waters HPLC system (Waters, Milford, MA) equipped with a Syneri 4µ Hydro-RP column (Phenomex, Aschaffenburg, Germany) and using phosphate buffer (50 mM KH_2PO_4, pH 2.9) as eluent. As standards 12 different carboxylic acids (oxalate, gluconate, formate, pyruvate, malate, 2-oxoglutarate, lactate, acetate, citrate, fumarate, succinate and propionate) were used. Data analysis was done using Millenium^{32} 3.05.01. Software (Waters, Eschborn, Germany).

Similarity analysis of physiological data

The results of all physiological tests were used for the construction of a matrix with a binary code for the presence or absence of each phenotypic trait. Similarity check was performed using the MVSP 3.1 Software (Kovach Computing Services, Pentreath, UK) and the Dice coefficient for calculation of the distance matrix.

Determination of intact phospholipids and phospholipid fatty acids

Cells were grown in oxic media amended with lactate (10 mM), harvested at the end of the exponential growth phase by centrifugation, washed with phosphate buffer (130 mM NaCl, 5 mM NaH_2PO_4, 5 mM Na_2HPO_4, pH 7.4), freeze-dried and stored at -20°C. Aliquots (30-100 mg) of the freeze-dried cells were extracted ultrasonically up to ten times for 10 minutes using a solvent mixture of methanol/dichloromethane/ammonium acetate buffer pH 7.6 (2:1:0.8 by volume) in centrifuge tubes (modified after Vancanneyt et al. [52]) After centrifugation at 2200 g at 15°C for 10 min the supernatants were removed and collected in a separatory funnel. Dichloromethane and ammonium acetate buffer (pH 7.6) were added to the combined extracts to achieve a final ratio of methanol/dichloromethane/ammonium acetate buffer of 1:1:0.9 (by volume). After phase separation, the organic phase was removed and the aqueous phase was re-extracted with dichloromethane five times. Combined extracts were dried over anhydrous sodium sulfate, evaporated to dryness and stored at -20°C.

Lipid extracts were dissolved in 1 ml dichloromethane/methanol 9:1 (by volume) and then chromatographically separated according to Zink and Mangelsdorf [60]. Two glass columns in sequence filled with pure silica (1 g silica 60, 63-200 µm, dried at 110°C for 16 h) and Florisil (1 g magnesium silica gel 150-250 µm, Merck, Darmstadt, Germany) were used to gain four fractions: (1) neutral lipids (eluted with 20 ml dichloromethane); (2) free fatty acids (50 ml methyl formate with 0.025 % v/v pure acetic acid); (3) glycolipids (20 ml acetone), and (4) phospholipids. To obtain the phospholipid fraction, the Florisil column was removed and only the silica column was eluted with 25 ml methanol. All fractions were evaporated to dryness and stored at -20°C.

Aliquots of the phospholipid fractions were transesterified with trimethylsulfonium-hydroxide as described by Müller et al. [30]. The methyl esters obtained were quantified by GC-FID (Hewlett Packard HP 5890 Series II gas chromatograph, Hewlett Packard, Waldbronn, Germany) equipped with a DB-5HT capillary column (30 m 0.25 mm, 0.1 µm film thickness, J&W, Folsom, CA) and identified by GC-MS using a Finnigan MAT SSQ 710B mass spectrometer (Finnigan-Thermoquest, San Jose, CA). The carrier gas was helium (constant pressure of 12 psi). The oven temperature was raised from 60°C (isothermal for 2 min) to 360°C at a rate of 3°C min^{-1} and hold for 5 min. Mass spectra were collected in full scan mode (*m/z* 50-650, ionization energy 70 eV). Positions of double bonds were tentatively assigned by comparison with retention times of standards (Bacterial Acid Methyl Esters CP Mix; Supelco, Bellefonte, PA).

Results

Phylogenetic affiliation of the Photobacterium *strains*

Based on partial 16S rRNA gene sequences all strains were previously found to be closely related to *Photobacterium profundum*. However, several novel *Photobacterium* species have been described since then (e.g. *P. aplysiae*, [44]; *P. frigidiphilum*, [43]; *P. lipolyticum*, [59] and *P. rosenbergii*, [48]), while the former *Hyphomicrobium indicum* was transferred to this genus [58]. These sequences, as well as almost complete 16S rRNA gene sequences (about 1300 bp) of the Mediterranean isolates were now included into the phylogenetic analysis.

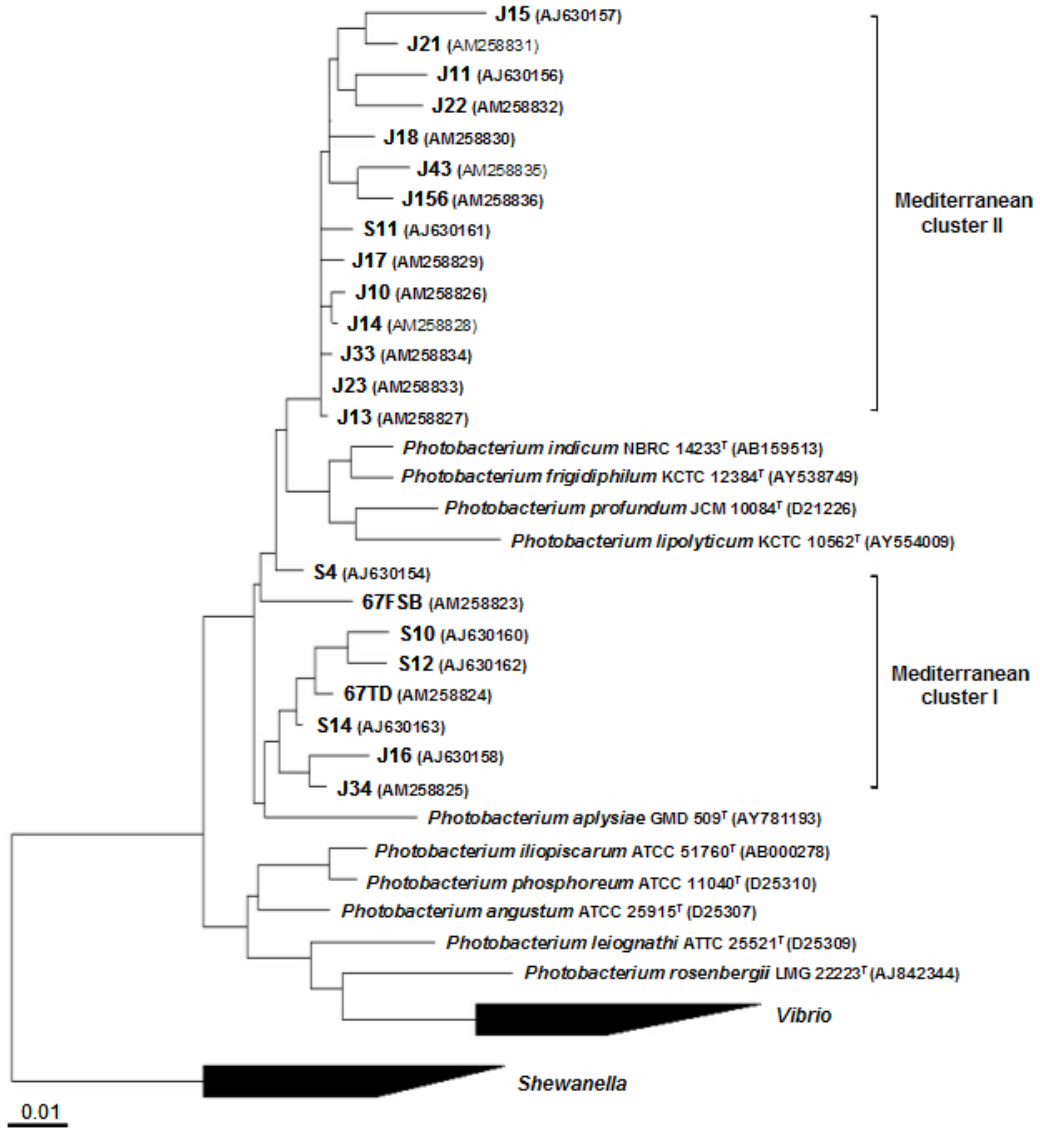

Fig. 1. Maximum likelihood tree showing the phylogenetic positions of the two *Photobacterium* clusters and closely related taxa. Sequences of *Shewanella* (7) and *Vibrio* (7) were taken as outgroups. 16S rRNA gene sequences of Mediterranean isolates were added to a back-bone tree consisting of validly described species by maximum parsimony using the ARB software and a specific *Photobacterium* filter [27].

It turned out that the isolates shared between 96 and 100 % sequence similarity of the 16S rRNA gene with each other. Their sequences affiliated with a phylogenetic branch containing *P. aplysiae*, *P. frigidiphilum*, *P. indicum*, *P. lipolyticum* and *P. profundum* (Figure 1). The strains were most closely related to either *P. frigidiphilum* or *P. aplysiae* (sequence similarities 97.1 to 99.5 % and 96.8 to 99.5 %, respectively), suggesting that the strains belong to two separate phylogenetic clusters (Mediterranean cluster I and II, Figure 1). Cluster I comprised strains S4, S10, S12, S14, J16, J34, 67FSB and 67TD that were obtained exclusively from sapropel layers, but from all three sampling sites. In contrast, strains of cluster II were obtained from surface sediments, sapropels as well as from intermediate layers, but only from a single site (#567).

Phenotypic characteristics

Microscopic inspection revealed that cells of all strains were rod-shaped, as considered to be typical for the genus *Photobacterium.* However, cells were often enlarged and irregularly shaped due to the accumulation of huge amounts of endogenous storage granula (Figure 2). Sudan-black staining (0.05 % w/v in EtOH abs) revealed that these consisted of poly-3-hydroxybutyrate. The cells were motile by monopolar flagella. Luminescence was not observed.

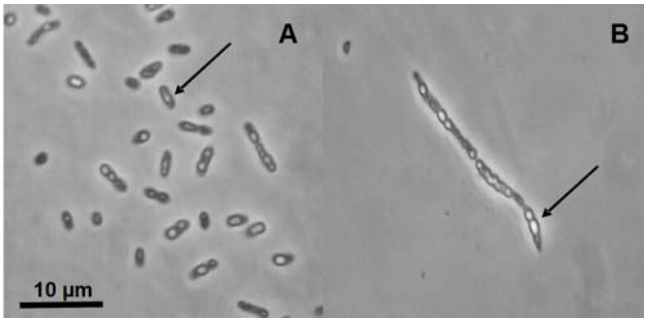

Fig. 2. Phase-contrast photomicrograph of *Photobacterium* sp. J17 after four days (left) and two weeks (right) of incubation in oxic liquid medium with glucose as substrate. Arrows indicate endogenous storage granules consisting mainly of poly-3-hydroxybutyrate.

According to their growth behavior on yeast extract-peptone-glucose agar plates the strains could be separated into two groups that reflected the two phylogenetic clusters. Cluster I strains were characterized by large cream-colored colonies that were formed normally within two days of incubation at 20ºC. The remaining strains grew distinctly slower and needed several days to weeks of incubation at 20ºC to form whitish small colonies (diameter generally less than 0.2 mm).

Molecular characterization

For all strains, genomic fingerprinting based on ERIC-PCR yielded a clear and unique pattern of amplified DNA fragments of different lengths that could be used for cluster analysis (Figure 3). Three groups of patterns were recognized. Strains of cluster I had very uniform ERIC band patterns clearly distinct from the other strains. Within this cluster the strains grouped corresponding to the sampling site they were isolated from.

The cluster II yielded two separate subclusters based on ERIC band patterns (Mediterranean cluster II subgroup A and B, Figure 3). Subgroup A comprised most of the strains (five of seven) originating from sapropel 1 and two strains (J43 and J156) isolated from the intermediate layer beneath (Z1). The second subcluster (B) consisted of strains originating from all four depths strains were isolated from. This subcluster exhibited the highest variability in band patterns.

DGGE analysis revealed that all *Photobacterium* strains possessed up to five different *rrn* gene copies. Based on the presence or absence of certain bands in the DGGE gel again three subgroups were identified, reflecting those defined on basis of the ERIC-PCR results (data not shown).

Aerobic substrate utilization

Generally, under oxic conditions the strains grew with a broad range of substrates. The vast majority utilized the complex substrates yeast extract, casamino acids and peptone as well as poly (starch, chitin) and monosccharides (glucose, fructose, mannose), monocarboxylic acids (pyruvate, lactate) and tricarboxylic acid cycle intermediates (citrate, 2-oxoglutarate, succinate) (Table 2). Only a very few strains (< 4) grew with *n*-propanol, ethylene glycol, aromatic compounds, certain carboxylic and amino acids (glycolate, tartrate, crotonate, isoleucine, phenylalanine), or with C_1 compounds (formate, methanol).

With respect to their metabolic capacities significant differences between cluster I and cluster II strains were observed. Strains of cluster I exhibited relatively uniform substrate utilization patterns (averaged pairwise similarities: 0.87±0.05, Figure 4) growing with 27 to 36 of the provided carbon sources. All of them grew on the amino acids alanine, arginine, asparagine, glutamine, proline, tryptophane, as well as on fumarate, malate, acetate, capronate and glucosamine, while these substrates were utilized only by a very few strains of cluster II.

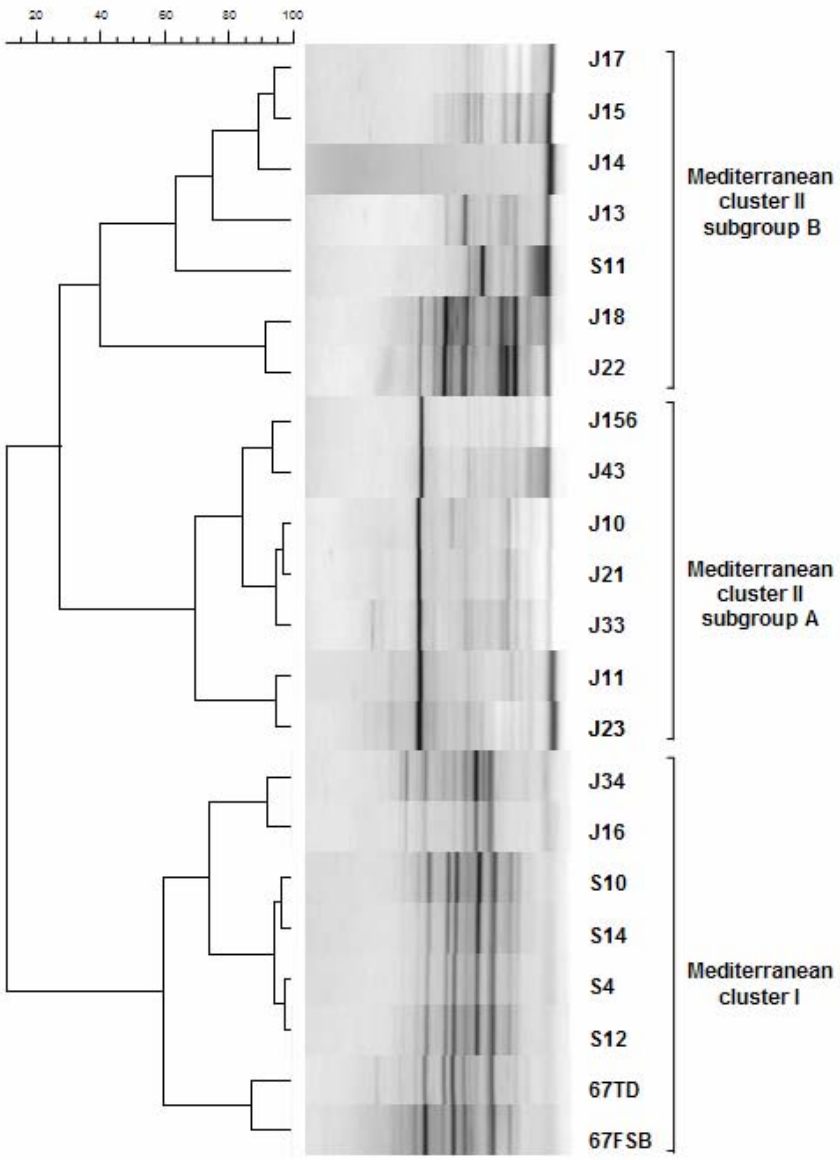

Fig. 3. Cluster analysis of ERIC-PCR fingerprinting band patterns of the *Photobacterium* strains confirmed the two clusters found on the 16S rRNA gene level (Mediterranean cluster I and II). Strains of Mediterranean cluster II formed two subgroups (A and B). The dendogram was calculated based on negative images of ethidium bromide stained agarose gels using Pearson correlation and UPGMA.

Mediterranean cluster II strains showed relatively non-uniform results (Figure 4). Each strain exhibited its unique set of substrates that could be used for growth. When compared to the majority of the strains, a few isolates (J10, J33 and J15) appeared to be rather restricted in their metabolic capacities. However, despite aerobic growth on only 10 to 13 substrates, including yeast extract, glucose and asparagine, these three strains nonetheless readily fermented carbohydrates, amino acids and carboxylic acids (Table 1).

Table 1. Origin, isolation conditions, phenotypic traits and anaerobic growth capacities of the examined *Photobacterium* strains. The location of the sampling sites and the depth of the sampled sediment are given in Süß et al. [46].

Strain	Origin		Isolation		Growth at			Reduction of			Fermentation of			
	Site	Layer		Substrate	Temperature [°C]	pH	Salinity [PSU]	NO_3^-	DMSO	AQDS HA	Glc	AS	TCA	ALC
Mediterranean cluster I														
FSB	67	S1	anox	FS	4-33	5.5-9.3	10-75	+	+	+	+	+	+	+
TD	67	S1	anox	Ac/thios	4-33	5.8-9.2	10-75	+			+		+	
S4	567	S1	ox	MKS	4-33	5.8-9.2	10-50	+	+		+	+	+	
S12		S1	ox	MKS	4-35	5.5-9.3	10-75	+	+		+	+	+	
S10		S5	ox	MKS	4-33	5.8-9.2	10-75	+		+	+	+	+	
S14		S5	ox	MKS	4-35	5.5-9.3	10-75	+	+		+		+	
J16	575	S3	anox	AS	4-30	5.5-9.3	10-75	+			+	+	+	
J34	575	S3	anox	AS	6-30	5.8-9.2	10-50	+	+	+[1]	+	+	+	
Mediterranean cluster II														
J15	567	Z0	anox	MKS	n.d.	n.d.	n.d.	+	+		+	+		
J17		Z0	anox	MKS	4-33	5.5-9.3	5-50				+	+	+	
J10		S1	anox	AS	4-35	5.5-9.3	10-75				+	+	+	
J11		S1	anox	AS	4-35	5.5-9.3	5-75	+	+		+	+	+	
J14		S1	anox	MKS	4-30	n.d.	n.d.	+	+		+	+	+	
J21		S1	anox	AS	4-33	n.d.	10-75	+			+	+	+	
J22		S1	anox	AS	4-33	5.5-9.3	10-75	+			+	+	+	
J23		S1	anox	MKS	4-35	5.5-9.3	10-75	+			+	+	+	
J33		S1	anox	AS	4-33	5.5-9.3	5-50				+	+	+	
S11		Z1	ox	MKS	4-33	5.5-9.3	10-75	+	+		+	+	+	
J43		Z1	anox	AS	4-33	5.8-9.2	n.d.				+	+		+
J156		Z1	anox	AS	4-33	5.8-9.2	n.d.				+	+		
J13		S5	anox	MKS	4-33	5.5-9.3	5-75		+		+			
J18		S5	anox	MKS	4-30	5.8-9.2	5-100				+			

Approximate age of sapropels according to Lourens et al. [25]: S1, 8,000 a; S3, 81,000 a; S5, 124,000 a. n.d.: not determined, Z0: surface layers, Z1: intermediate layer 1, FS: fatty acids, Ac/thios: acetate and thiosulfate, MKS: monomer mix [46], AS: amino acids, Glc: glucose, TCA: dicarboxylic acids and lactate, ALC: alcohols, HA: humic acids, [1] Use of humic acids

Anaerobic metabolism

All strains were able to grow in the absence of oxygen by fermentation or by anaerobic respiration (Table 1). All isolates of cluster I reduced nitrate to nitrite, while five grew with DMSO and two with AQDS as electron acceptor. One of the isolates grew in the assay amended with humic acids. In contrast, only eight of the fourteen strains belonging to cluster II exhibited anaerobic respiration. Seven of them used nitrate and five reduced DMSO. AQDS reduction or growth on humic acids was not observed for this cluster. Interestingly, in cluster II nitrate reduction was found for most isolates from surface and the youngest sapropel S1 (six out of nine strains) but was less common in isolates originating from the deeper layers (one of five strains). None of the strains reduced manganese oxides, ferric hydroxide, sulfite, thiosulfate or elemental sulfur.

All strains grew readily by fermentation of glucose. The mixture of dicarboxylic acids and lactate was fermented by all strains of cluster I and by nine out of fourteen strains of

cluster II. Fermentative growth on amino acids was found for the majority of strains in both clusters. Two strains (67FSB and J43) grew even in the assay containing alcohols.

The fermentation assays with glucose as substrate were analyzed by HPLC. In the supernatant four to six different acids were identified, with formate, 2-oxoglutarate, lactate, acetate, and citrate, being detected in almost all cultures and suggesting mixed acid fermentation (data not shown). With concentrations exceeding 13 $mmol \cdot l^{-1}$, formate was found to be the major fermentation product, except for four strains of cluster II. These strains (J13, J18, J43, and J156), that were isolated from the deeper layers Z1 and S5, were remarkable as they also failed to grow fermentatively with amino acids or carboxylic acids, nor they reduced nitrate.

Temperature range, salinity tolerance and pH requirements

With respect to their growth temperatures no clear differences between the two clusters were observed. With exception of isolate J34 (cluster I, T_{min}=6ºC), all strains grew at 4°C. Most of them revealed an upper temperature limit for growth at 33ºC (Table 1), while four did not grow above 30ºC and five still grew at 35ºC. The strains were quite uniform with respect to their salt and pH requirements. None of them grew at pH below 5.5, but they were tolerating rather alkaline conditions with pH values of up to 9.3. All strains required Na^+ and salinities of at least 5 PSU for growth. The upper salinity limit for growth was between 50 to 75 PSU. Strain J18 was an exception, growing at salinities of 100 PSU.

Phospolipid content

Eight strains (four of each cluster) were analyzed for their phospholipids. Generally, all strains yielded very similar results with phosphatidyl glycerol (12-20 %) and phosphatidyl ethanolamine (70-85 %) as the dominant phosopholipid classes. Analysis of the polar lipid fatty acids revealed the dominance of *n*-16:0 (up to 32 %), *n*-16:1 (up to 48 %) and *n*-18:1 (up to 16.6 %) fatty acids in both clusters (Table 2). While all strains of cluster II contained *n*-20:5ω3 fatty acids (0.3 – 2 %), traces of this fatty acid type were found only in two of the four strains of cluster I. The latter strains, in turn, contained *n*-18:2 and saturated short-chain fatty acids (*n*-9:0 to *n*-13:0).

Table 2. Selected phenotypic traits of Mediterranean *Photobacterium* strains compared to those of closely related, validly described *Photobacterium* species.

	P. profundum [32]	***P. frigidiphilum*** [43]	***P. aplysiae*** [44]	***P. indicum*** [20,58]	***P. lipolyticum*** [59]	**Mediterranean cluster I**	**Mediterranean cluster II**
Origin	Pacific deep-sea sediment	Pacific deep-sea sediment	Eggs of sea hare	Sea mud	Korean Intertidal sediments	Mediterranean sapropels	Mediterranean sediments
Water depth	5110 m	1450 m	12 m	400 m	surface	2150-2330 m	
Growth at 4°C	+	-	-	+	+	+	+
Growth at 30°C	-	-	+	-	+	+	+
Major fatty acids	C16:1, C16:0, C16:0 iso, C18:1, C20:5ω3	C16:1, C16:0, C18:1, C20:5ω3	C16:1, C16:0, C18:1	C16:1, C16:0, C18:1	C16:1, and/or iso C15:0 2-OH, C16:0, C18:1	C16:1, C16:0, C18:1	C16:1, C16:0, C18:1
Presence of C20:5ω3[1]	+	+	n.d.	-	-	(+)	(+)
NO_3^- reduction	+	+	+	+	+	+	v
Fermentation of glucose	+	+	+	+	+	+	+
Utilization of							
N-acetyl-glucoseamin	-	+	+	n.d.	-	+	v
Cellobiose	-	-	+	n.d.	-	+	v
Fructose	-	+	+	n.d.	+	+	v
Maltose	+	+	+	+	+	+	+
Mannose	+	+	+	n.d.	-	+	+
Sucrose	-	+	+	+	+	+	+
Trehalose	+	+	+	n.d.	-	+	v
Arabinose	-	-	n.d.	-	-	-	4/15*
Glucose	+	+	n.d.	+	+	+	+
Rhamnose	-	n.d.	n.d.	n.d.	n.d.	-	v
Mannitol	+	+	+	n.d.	n.d.	+	v
Lactate	n.d.	+	+	+	-	+	+
Citrate	n.d.	n.d.	n.d.	+	-	+	+
Succinate	n.d.	+	+	+	+	+	+
Alanine	n.d.	+	+	-	n.d.	+	v
Tween 80	+	+	+	n.d.	+	v	4/15*
Glycerol	+	+	+	+	n.d.	+	v

n.d.: no data available, + growth of more than 60 % of the strains, v: growth of 40-60 % of the strains, * number of positive versus total number of strains. [1] C20:5ω3: eicosapentanoic acid, typical for psychrophilic and piezophilc bacteria, (+) traces in the phospholipid fatty acid fraction.

Discussion

In the present study we have described a *Photobacterium* population (Mediterranean cluster I) that is obviously specialized to life in sapropel layers. The comparison to the second population (Mediterranean cluster II) that appears to occupy different ecological niches in the subsurface of Mediterranean sediments opens interesting aspects of microdiversity occurring in the deep biosphere.

Photobacterium *sp. as indigenous subsurface microbes*

Although *Photobacterium* species were so far only known from surface habitats, this genus apparently forms subsurface populations that thrive in this challenging environment. It can be regarded as virtually certain that the isolates are indigenous deep-biosphere representatives, as they were obtained during different sampling campaigns and applying various cultivation conditions (Table 1). The high viable counts (up to 3.3 % of the total count) achieved by an improved MPN technique [46], indicate that *Photobacterium* sp. are important and metabolically active members of the sediment communities. If they would not be active, it could be expected that their numbers strongly decrease with depth: that was not the case. In addition, dormant stages such as spores or cysts are not described for this genus [4].

If the *Photobacterium* strains represent indigenous subsurface microorganisms they must be able to grow under *in situ* conditions. In fact, the isolates exhibited anaerobic pathways so far unknown for *Photobacterium* spp. [4] like fermentation of dicarboxylic or amino acids. The latter capacity was recently inferred from genome sequencing data of the sediment-dwelling *P. profundum* [54]. In Mediterranean sediments the presence of nitrate and oxidized metal species indicates relatively oxidized conditions in the upper layers down to the top sapropel S1 [45]. Moreover, easily degradable substrates like carbohydrates or peptides are present at very low concentrations or adsorbed to the kerogen matrix of the sapropels [8]. The low supply of electron donors prevents the development of strongly reducing conditions and might favor growth of facultative anaerobes such as *Photobacterium* sp.

The finding that our isolates degrade a broad spectrum of monomeric and polymeric substrates classifies them as generalists. The ability to utilize many different carbon sources appears to be typical for indigenous subsurface bacteria [47]. However, the degradation capacities of our isolates might be even underestimated as all growth tests were performed under atmospheric pressure. Gene expression analyses of the closely related deep-sea strain *P. profundum* SS9 indicated that certain metabolic pathways, such as fermentation of amino acids or the degradation of biopolymers, might be expressed only at elevated hydrostatic pressure [54].

Specificity of Mediterranean cluster I for sapropel layers

The *Photobacterium* strains investigated in this study belonged to two discrete populations that differed in spatial distribution and microdiversity. The population detected in the sapropel layers exclusively, maintained a stable genome organization on a geological time scale, not found in the second cluster. This stability can be interpreted as a hint on metabolic activity,

because resting states were shown to enhance the generation of diversity [2]. The genome stability was reflected by minor variations on the physiologic level. Members of this cluster showed a broad substrate versatility, and so far it remains unknown which carbon sources they are utilizing *in situ.* However, we assume that they rely on substrates specifically released within the organic-rich layers. Ecologically successful populations do not necessarily need to develop a high genetic diversity [18]. Hence, the high stability of the cluster I population can be seen as an adaptation to life in sapropels.

Compared to the population confined to the sapropel layers, strains of cluster II that originate from surface sediments, sapropels and carbon-lean intermediate layers were rather heterogeneous. The increased variability within this population can be regarded as another strategy for long-term survival, providing the assumption that the distribution of multiple ecotypes allows an immediate response to a wide range of environmental conditions [29]. Whether this is related to nutrient partitioning [19,22] and/or changes in redox conditions [17] remains speculative.

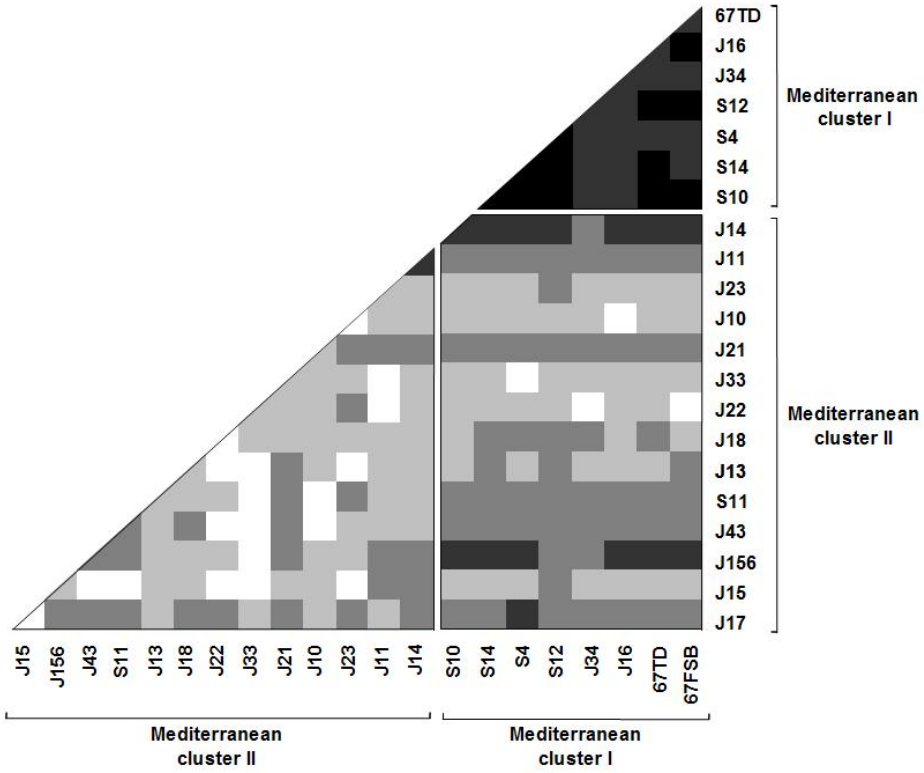

Fig. 4. Binary similarity triangle based on aerobic and anaerobic growth characteristics of the strains. The distance matrix was calculated using the Dice coefficient (DC). The following color code was used: ■ DC > 0.9 ■ DC 0.75- 0.9 ■ DC 0.6-0.75 ■ DC 0.45-0.6 □ DC < 0.45

Microdiversity in subsurface environments

The heterogeneity detected among the *Photobacterium* strains was much lower than that found in pelagic bacteria [19,39] or those inhabiting surface sediments [3,40], and might be explained by the relatively constant conditions found in subsurface environments. Seasonal changes of physicochemical gradients and temperature [50], varying light conditions [29] or increasing pressure in the deep-sea [24] were shown to stimulate the development of specifically adapted subpopulations within single species and even protozoan grazing or virus infection [14] were supposed to increase microdiversity. These stimuli in combination with short generation times may stimulate genotypic and phenotypic diversification in surface bacteria but cannot explain existence of microdiversity found in the *Photobacterium* strains.

While the concept of periodic selection predicts that two bacterial strains cannot occupy the same ecological niche and that one type would outcompete the other [33], Thompson and coworkers [49] reported on the consistent co-occurrence of a large diversity of *Vibrio* genomovars within a natural community. They concluded that most of the genotypic diversity they detected must be "ecologically neutral" and explained the co-occurrence with the erratic and unpredictable appearance of resources and grazing that could eliminate dominant subpopulations. These factors can be ruled out for subsurface bacteria, while on the other hand the low *in situ* growth rates might enable co-existence. It can be expected that the highly complex but diverse structure of the sapropelic kerogen (e.g. polyphenylic and aliphatic residues, [21]) requires specialized bacterial types for degradation. Since *Photobacterium* cluster I was detected only in the sapropels, this indicates that the two *Photobacterium* clusters do occupy distinct ecological niches.

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3. Gesamtbetrachtung und Ausblick

3.1 Kultivierung von Mikroorganismen aus Sapropelen des östlichen Mittelmeeres

Die Kultivierung von Mikroorganismen ist bis heute eine wichtige Vorraussetzung, um ihre ökologische Rolle zu verstehen (Leadbetter 2003). Kultivierungsabhängige Analysen bergen jedoch das Problem, dass durch die Selektivität der Anreicherungsbedingungen nur ein geringer Teil der an einem Standort vorhandenen mikrobiellen Diversität erfasst werden kann.

In der vorliegenden Arbeit wurden verschiedene Strategien verfolgt, um abundante Vertreter der mikrobiellen Gemeinschaften in Sapropelen des östlichen Mittelmeeres anzureichern und zu isolieren. Diese erwiesen sich als sehr erfolgreich und enthüllten in Kombination mit molekularbiologischen Analysen die erstaunliche Diversität der Prokaryoten in diesen ungewöhnlichen Sedimentschichten.

Durch den Einsatz vieler verschiedener Substrate in geringen Konzentrationen konnte die Kultivierungseffizienz im Vergleich zu vorangegangenen Studien an Sapropelen (Coolen & Overmann 2000) um mehrere Größenordnungen gesteigert werden. Aus den MPN-Reihen und alternativen Anreicherungskulturen wurde eine einzigartige Stammsammlung etabliert, die 27 Phylotypen aus sechs bakteriellen Großgruppen umfasst.

Die molekulare Analyse ausgewählter Originalkulturen zeigte, dass noch wesentlich mehr Phylotypen zum Wachstum stimuliert, jedoch nicht in Reinkultur gebracht werden konnten. Demnach werden kultivierungsbasierte Analysen nicht nur durch die Anreicherungsbedingungen, sondern in erheblichem Maß durch die Selektivität des Isolierungsprozesses limitiert. In zukünftigen Studien sollte dieser Prozess deshalb durch molekulare Techniken begleitet werden. Es wurde deutlich, dass Verdünnungsreihen zwar den Isolierungsprozess fördern, jedoch zur wiederholten Kultivierung einzelner Arten führen. Durch den Einsatz von Sedimentgradientenkulturen, in denen Mikroorganismen in ungestörten Sedimentproben angereichert wurden, konnten dagegen nicht nur sehr viele verschiedene Phylotypen sondern auch dominante Vertreter der Sapropelgemeinschaften (*Chloroflexi*) zum Wachstum stimuliert werden. Diese Technik besitzt somit ein großes Potenzial Vertreter ubiquitärer, aber selten kultivierter Phyla zu isolieren und sollte in zukünftigen Studien weiterhin eingesetzt werden.

Einfluss des Alters der Sapropele auf die Kultivierung indigener Mikroorganismen. Obwohl auch aus 200.000 Jahre alten Sapropelen Reinkulturen gewonnen werden konnten,

nahm die Anzahl an kultivierten Arten mit zunehmendem Alter der Sapropele deutlich ab. Dies war insofern erstaunlich, dass auch in älteren Sapropellagen (z.B. S5, 120.000 Jahre) ähnlich hohe Lebendzellzahlen erzielt wurden, wie an der Sedimentoberfläche und in Sapropel S1 (siehe Kapitel 2.1). Ein möglicher Erklärungsansatz für dieses Phänomen ist eine altersbedingte Änderung in der Zusammensetzung der Sapropelgemeinschaften. Ein solcher mit der Tiefe der Schichten einhergehender *Populationsshift*, der sich auch in der Zusammensetzung der kultivierbareren Fraktion widerspiegelte, wurde ebenfalls in Wattsedimenten detektiert (Köpke et al. 2005, Wilms et al. 2006). Dies scheint auf Sapropele jedoch nicht in diesem Maße zuzutreffen, da sich z.B. die DGGE-Profile der einzelnen Schichten nicht wesentlich voneinander unterschieden (Coolen et al. 2002, Supplementary material). Es ist jedoch zu bedenken, dass geringfügige Änderungen in der Zusammensetzung der Gemeinschaften mit molekularen Methoden nicht erfasst werden können (Muyzer et al. 1993).

Obwohl auch in tiefen Sapropelschichten erhöhte mikrobielle Aktivitäten detektiert wurden (Coolen et al. 2002), ist anzunehmen, dass viele Zellen in einem Zustand verringerter metabolischer Aktivität vorliegen, was ihr Anwachsen im Labor verhindern kann (Bernard et al. 2000, Barer 2003). Ruhestadien im eigentlichen Sinn (Sporen) scheinen in Sapropelen jedoch eine vergleichsweise geringe Bedeutung zu haben. Aus den oben erwähnten Wattsedimenten wurden ab einer Tiefe von 2 m fast ausschließlich Vertreter der *Firmicutes* isoliert (Köpke et al. 2005). Diese typischen Sporenbildner nahmen auch in einer Kultursammlung aus Pazifiksedimenten einen erheblichen Anteil ein (D'Hondt et al. 2004), was in dieser Studie nicht der Fall war (Kapitel 2.1, 2.2).

Kultivierung unter atmosphärischem Druck. Die Ergebnisse dieser Studie zeigen, dass an den Standort angepasste Anreicherungsbedingungen den Kultivierungserfolg deutlich steigern können. Ein Parameter, der nicht berücksichtigt wurde, sind die in Tiefseesedimenten vorherrschenden hohen Drücke. Allein durch die Dekompression während der Probenahme könnten viele Organismen irreversibel geschädigt worden sein. Es ist außerdem bekannt, dass barophile Mikroorganismen kein Wachstum bei Drücken unter 40 MPa zeigen (Kato & Bartlett 1997, Kato et al. 1998). Andere Arten sind dagegen moderat barophil oder barotolerant und können auch unter atmosphärischem Druck wachsen. Häufig unterliegen die Stoffwechselleistungen dieser Mikroorganismen jedoch einer Druckregulation (Kato & Bartlett 1997, Vezzi et al. 2005). In jedem Fall wirken sich die für die Inkubation von Tiefseeproben gewählten Druckbedingungen wesentlich auf die Diversität der kultivierbaren

Fraktion aus (Kato et al. 1998, Yanagibayashi et al. 1999). Deshalb sollten Druck-inkubationen Bestanteil zukünftiger Untersuchungen sein.

Lebensweise kultivierter *Chloroflexi* und zukünftige Isolierungsansätze. Da wenig über die Ansprüche der in den Sapropelen dominanten Phyla bekannt ist, wurden in dieser Studie unterschiedliche Substrate in verschiedenen Kombinationen eingesetzt. Tatsächlich wurden Vertreter der *Chloroflexi* in einer Sedimentgradientenkultur detektiert, die mit Intermediaten des Citratzyklus versetzt war. Da diese Mikroorganismen nicht in Reinkultur gebracht wurden, bleibt nach wie vor unklar, warum sie in Sapropelen so erfolgreich sind.

Vergangene Studien machen deutlich, warum *Chloroflexi* so schwierig zu kultivieren sind, liefern jedoch zusammen mit den Ergebnissen der vorliegenden Arbeit, wertvolle Hinweise für zukünftig anzuwendende Strategien. Alle kultivierten Vertreter dieses Phylums verfügen über ein sehr begrenztes Repertoire an biosynthetischen Kapazitäten und benötigen deshalb sehr spezifische Kulturbedingungen. *Dehalococcoides ethenogenes*, der nächste kultivierte Verwandte der in den Sapropelen detektierten *Chloroflexi*, nutzt ausschließlich Wasserstoff zur Reduktion halogenierter Verbindungen (Maymó-Gatell et al. 1997). Um in Reinkultur zu wachsen, benötigt dieser Organismus außerdem Acetat, Vitamin B12 und Extrakte aus gemischten Bakterienkulturen. Die kürzlich neu beschriebenen Arten, *Levilinea saccharolytica*, *Leptolinea tardivitalis* und *Anaerolinea thermolimosa*, fermentieren verschiedene Zucker allerdings nur in Anwesenheit von Hefeextrakt und reagieren empfindlich gegenüber Wasserstoff (Yamada et al. 2006). Generell scheinen *Chloroflexi* auf ausgeprägte Interaktionen mit anderen Mikroorganismen angewiesen zu sein (Seshadri et al. 2005) und wachsen deshalb in Reinkulturen extrem langsam (Sekiguchi et al. 2001, Yamada et al. 2005). Die Isolierung dieser Mikroorganismen gelang nur durch den gezielten Einsatz spezifischer Sonden, den Ausschluss anderer schnell wachsender Mikroorganismen, systematische Mediumvariationen und adäquate Inokula in Subkulturen.

3.2 Der Nachweis typischer Bodenbakterien in der Tiefen Biosphäre

Erstaunlicherweise wurden aus allen beprobten Sedimentschichten nahe Verwandte des Bodenbakteriums *Rhizobium radiobacter* isoliert. Ein erster Schritt zur Aufklärung der Rolle dieser Mikroorganismen war die Bestimmung ihrer *in situ* Abundanz mit Hilfe eines spezifischen, hochsensitiven *real-time* PCR-Protokolls. Die Ergebnisse dieser Analyse

zeigten, dass *R. radiobacter* in den Mittelmeersedimenten weit verbreitet ist und bis zu 5 % der Bakteriengemeinschaften in Sapropelen einnimmt.

Die Detektion von *R. radiobacter* in den Sedimenten des Mittelmeeres ist kein Einzelfall. Die gleichen Mikroorganismen wurden auch aus bis zu 380 m tiefen Sedimenten des Pazifik isoliert (D'Hondt et al. 2004) und kürzlich erneut in Klonbanken aus Tiefseesedimenten detektiert (Inagaki, persönliche Mitteilung). Demnach scheinen diese bislang als typische Bodenbakterien angesehenen Organismen nicht nur in Sapropelen, sondern auch in anderen Bereichen der Tiefen Biosphäre wesentlicher Bestandteil der mikrobiellen Gemeinschaften zu sein. Wie diese Mikroorganismen an das Leben in der Tiefen Biosphäre bzw. in Sapropelen angepasst sind, ist Gegenstand derzeitiger Untersuchungen.

Methodische Aspekte. Generell lassen DNA-basierte Analysen keine Rückschlüsse auf die Lebensfähigkeit oder die Aktivität eines Mikroorganismus am Standort zu. Trotzdem besteht berechtigter Grund zu der Annahme, dass in den Sapropelen aktiv wachsende *R. radiobacter*-Populationen existieren (Kapitel 2.3). Durch die Analyse ribosomaler RNA werden dagegen bevorzugt metabolisch aktive Organismen erfasst (Ward et al. 1992). Da der Ribosomensatz einer Zelle in Abhängigkeit von ihrem physiologischen Zustand variiert, bietet diese Methode jedoch nicht die Möglichkeit der Zellzahlbestimmung. Auch intakte Phospholipide sind Biomarker für lebende Organismen, da sie innerhalb weniger Tage nach dem Tod einer Zelle abgebaut werden (White et al. 1979). Diese Methode wurde bereits zur Quantifizierung sulfatreduzierender Bakterien in Wattsedimenten herangezogen (Rütters et al. 2002). Derzeit werden die Phospholipide der Sapropelisolate charakterisiert, um ähnliche Untersuchungen an Mittemeersedimenten durchzuführen.

Zukünftige Anwendung des *R. radiobacter* spezifischen Nachweissystems. Trotz der oben genannten Einschränkung sind DNA-basierte Nachweissysteme sehr gut zur Untersuchung der biogeografischen Verbreitung einzelner phylogenetischer Gruppen oder Arten geeignet. Auf diese Weise wurde z.B. die globale Verteilung des *Roseobacter-Clusters* in verschiedenen Ozeanregionen (Selje et al. 2004) und die Verbreitung des *subsurface*-spezifischen JS1-*Clusters* in Oberflächenhabitaten analysiert (Webster et al. 2004). Das in dieser Studie entwickelte Protokoll zur spezifischen Detektion von *R. radiobacter* könnte zur Beantwortung grundlegender Fragen hinsichtlich der Verbreitung dieser Organismen in marinen Sedimenten und ihrer Herkunft herangezogen werden. Die Untersuchung von Sediment- und Wasserproben des Nils lieferte erste Hinweise darauf, dass *R. radiobacer* über

den Nil in das östliche Mittelmeer eingetragen worden sein könnte. Die systematische Untersuchung der Verteilung dieser Bakterien entlang des Nildeltas und in der Wassersäule des Mittelmeeres könnte Auskunft darüber geben, ob dies tatsächlich der Fall ist.

3.3 Physiologische Kapazitäten indigener Sapropelbakterien

Ein wichtiges Ziel dieser Arbeit war es, Einblicke in die physiologischen Kapazitäten und die Anpassungen indigener Mikroorganismen an die Bedingungen in den Sapropelen zu erhalten. Für diese Untersuchung wurden Vertreter der Gattung *Photobacterium* herangezogen, die ausschließlich aus MPN-Reihen isoliert wurden und deshalb als abundante Vertreter der Sapropelgemeinschaften anzusehen sind. Physiologische Untersuchungen zeigten, dass es sich bei den Isolaten um Generalisten handelt. Unter anoxischen Bedingungen nutzten sie alternative Elektronenakzeptoren oder betrieben Gärung und sind somit grundsätzlich in der Lage in den Mittelmeersedimenten zu leben. Es wurde außerdem gezeigt, dass diese Isolate zwei unterschiedliche Populationen repräsentieren, die deutliche Unterschiede hinsichtlich ihrer Verbreitung in den Sedimenten und ihrer Mikrodiversität zeigen. Eine dieser Populationen (*Mediterranean cluster* I) scheint speziell an das Leben in Sapropelen angepasst zu sein.

Anpassungen an die Bedingungen in Sapropelen. Nicht nur die *Photobacterium*-Isolate, sondern auch viele andere der hier kultivierten Phylotypen scheinen die Fähigkeit zu besitzen, ein weites Spektrum an Kohlenstoffquellen nutzen zu können (Kapitel 2.2, 2.3). Auch die bisher untersuchten *Rhizobium*-Isolate erwiesen sich als ausgesprochene Generalisten (Overmann, persönliche Mitteilung). Dies trifft auch für viele Mikroorganismen, die in einer parallelen Studie aus Pazifiksedimenten gewonnen wurden, zu (Batzke, persönliche Mitteilung). Generalisten sind in der Lage unter nährstofflimitierten Bedingungen viele Kohlenstoffquellen gleichzeitig zu verwerten (Kovárová-Kovar & Egli 1998). Diese Art von Co-Metabolismus ermöglicht die Aufnahme von Substraten, die in so geringen Konzentrationen vorliegen, dass sie als Einzelkomponente das Wachstum nicht fördern (van der Kooij et al. 1982). Demnach handelt es sich bei dieser Eigenschaft um eine Anpassung an die geringe Nährstoffverfügbarkeit in Sapropelen bzw. in der Tiefen Biosphäre allgemein. Die Mechanismen, die Mikroorganismen befähigen unter lang anhaltendem Substratmangel zu leben, sind jedoch wenig verstanden (Ferenci 1999).

Da das Kerogen der Sapropele selbst als Kohlenstoff- und Energiequelle dient (Coolen et al. 2002), kann davon ausgegangen werden, dass indigene Mikroorganismen auch schwer abbaubare Substrate verwerten können. Tatsächlich wurden mit dem eingesetzten Aromatenmedium recht hohe Lebendzellzahlen erzielt (Kapitel 2.1) und viele verschiedene Phylotypen isoliert (Kapitel 2.2). Die meisten kultivierten Arten zeigten zudem hohe Sequenzähnlichkeiten zu Organismen, die Huminsäuren oder aromatische Verbindungen zum Wachstum nutzen (Kapitel 2.1). Auch einige der *Photobacterium*-Isolate des *Mediterranean cluster* I wuchsen unter anoxischen Bedingungen mit Huminsäuren bzw. dem Huminstoffanalogon AQDS. Da diese Mikroorganismen speziell an Sapropele angepasst zu sein scheinen, hätte man einen eindeutigeren Trend in diese Richtung erwartet. Es ist jedoch anzunehmen, dass viele metabolische Fähigkeiten, die das Wachstum unter komplexen Bedingungen *in situ* ermöglichen, im Labor (Einzelsubstrat, Reinkultur) nicht detektiert werden können (Kovárová-Kovar & Egli 1998).

Wie bei den meisten der kultivierten Phylotypen handelte es sich bei den *Photobacterium*-Isolaten um fakultativ anaerobe Organismen. Die Dominanz fakultativer Anaerobier in der Kultursammlung war überraschend, deckt sich jedoch mit den Ergebnissen anderer Studien (Inagaki et al. 2003, D'Hondt et al. 2004). Demnach scheinen die geringe Substratverfügbarkeit und die damit einhergehenden schwach reduzierten Bedingungen in Tiefseesedimenten bzw. in Sapropelen diese Mikroorganismen zu fördern. Hohe MPN-Zahlen, die mit oxischen Ansätzen auch in dem 5 m tiefen Sapropel S5 erzielt wurden (Kapitel 2.1), bestätigen diese Annahme. Zum Vergleich seien Wattsedimente genannt, in denen oxische MPN-Zahlen, bei gleicher Medienzusammensetzung, bereits in einer Tiefe von einem Meter um mehrere Größenordnungen niedriger lagen (Köpke et al. 2005).

Mikrodiversität als Überlebensstrategie. Neben der oben erwähnten, auf Sapropele spezialisierten Population, existiert in den Mittelmeersedimenten eine zweite, recht diverse *Photobacterium*-Population, die die gesamte Sedimentsäule besiedelt (*Mediterranean cluster* II). Die Ausbildung genetisch und physiologisch unterschiedlicher Subpopulationen bzw. Ökotypen innerhalb einer bzw. eng verwandter Arten ist aus Oberflächenhabitaten bekannt. Dieses Phänomen wird gemeinhin als Strategie zum Erhalt einer Population über einen weiten Bereich an Umweltbedingungen gesehen. So ermöglicht die Koexistenz Schwachlicht- und Starklicht-adaptierter *Prochlorococcus*-Populationen im offenen Ozean, die die Ausbreitung dieser Art bis in sehr große Tiefen (Moore et al. 1998). Es wurde auch gezeigt, dass sich eng verwandte Cyanobakterien-Populationen entlang des Temperaturgradienten in heißen Quellen

einnischen (Ferris et al. 1996). Ein weiteres Beispiel sind diazotrophe Gemeinschaften in Salzmarschen, die durch die Verwertung unterschiedlicher Substrate den Prozess der Stickstofffixierung über ein breites Spektrum an Umweltbedingungen aufrechterhalten (Bagwell & Lovell 2000). Den Ergebnissen der vorliegenden Studie zu Folge scheint dieses Phänomen auch in der Tiefen Biosphäre von Bedeutung zu sein.

Bisher kann nur spekuliert werden, welche Faktoren für die Diversifizierung von Mikroorganismen in diesen Bereichen ausschlaggebend sind. Untersuchungen an limnischen Sedimenten zeigten, dass verschiedene Subpopulationen von *Achromatium oxaliferum* entlang des Redoxgradienten in der Sedimentsäule auftreten (Gray et al. 1999). In den meisten Fällen scheint die Ausbildung distinkter Subpopulationen jedoch an die Aufteilung verfügbarer Substrate gekoppelt zu sein (Wilson & Lindow 1994, Sass et al. 1998, Sass et al. 2001, Jaspers & Overmann 2004). Dies scheint angesichts der geringen Verfügbarkeit an organischen Substraten auch in Sapropelen plausibel zu sein. Obwohl viele Fragen offen bleiben wird deutlich, dass Mikroorganismen unterschiedliche Strategien verfolgen, um ihr Überleben in der Tiefen Biosphäre zu sichern.

Fazit

In der vorliegenden Arbeit konnten mittels verbesserter Kulturmedien und neuer Kultivierungstechniken viele verschiedene Vertreter der Sapropelgemeinschaften angereichert und isoliert werden. In nachfolgenden Untersuchungen wurde erstmals die numerische Dominanz eines kultivierten Vertreters der Tiefen Biosphäre (*Rhizobium radiobacter*) am Standort nachgewiesen. Die hohe Abundanz dieses typischen Bodenbakteriums in Sapropelen und seine weite Verbreitung in marinen Sedimenten wirft grundsätzliche Fragen nach dem Einfluss von Oberflächenhabitaten auf die Tiefe Biosphäre auf, die es in folgenden Untersuchungen zu klären gilt.

Zudem konnten wichtige Einblicke in die physiologischen Kapazitäten, Anpassungen und Überlebensstrategien indigener Sapropelbakterien gewonnen werden, die zum grundlegenden Verständnis mikrobiellen Lebens in der Tiefen Biosphäre beitragen. Am Ende dieser Arbeit bleiben jedoch viele Fragen offen und es sind weitere Studien nötig, um die Lebensweise von Mikroorganismen in Sapropelen zu verstehen.

Die Isolierung dominanter Vertreter der Sapropelgemeinschaften (*Chloroflexi* usw.) sollte ein wichtiges Ziel bleiben. Die vorliegende Studie liefert wertvolle Hinweise für zukünftige Ansätze. Der Erfolg kann jedoch nur durch den Einsatz molekularer Techniken

und modifizierte, an die Bedürfnisse der Mikroorganismen angepasste Isolierungstechniken gewährleistet werden. Um Auskunft darüber zu erhalten, welche Mikroorganismen in Sapropelen aktiv sind und wie sie miteinander interagieren sollten kultivierungsabhängige Analysen durch *in situ* Experimente ergänzt werden (*Stable Isotope Probing* oder MAR-FISH).

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Lebenslauf

Jacqueline Süß

geboren am 28.11.1972 in Cottbus

1979-1989	3. Polytechnische Oberschule „Hermann Matern“ Cottbus
1989	1. Erweiterte Oberschule „Theodor Neubauer“ Cottbus
1990-1992	Gymnasium der Stadt Wesseling, Abschluss mit dem Abitur
1993-1994	Studium Chemieingenieurwesen/Biotechnologie FH Aachen /Jülich
1994-2001	Studium der Biologie an der Universität zu Köln Anfertigung der Diplomarbeit: "Diversity and abundance of heterotrophic protists in selected German aquifers" in der AG Allgemeine Ökologie und Limnologie unter der Betreuung von Herrn Prof. Dr. Harmut Arndt Abschluss mit dem Diplom
2002-2006	Wissenschaftliche Mitarbeiterin an der Universität Oldenburg Experimentelle Arbeiten zur vorliegenden Dissertation in der AG Paläomikrobiologie unter der Betreuung von Herrn Prof. Dr. Heribert Cypionka

Hiermit versichere ich, dass ich die vorliegende Arbeit selbstständig verfasst und nur die angegebenen Hilfsmittel verwendet habe.

Oldenburg, im August 2006 Jacqueline Süß