Influence of Crustal Fluids on Growth and Activity of Marine Deep Biosphere Microbial Populations

Einfluss von Fluiden der Ozeankruste auf Wachstum und Aktivität mikrobieller Populationen der marinen Tiefen Biosphäre

Von der Fakultät für Mathematik und Naturwissenschaften
der Carl von Ossietzky Universität Oldenburg
zur Erlangung des Grades und Titels einer Doktorin der
Naturwissenschaften
– Dr. rer. nat. –
angenommene Dissertation von

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Tag der Disputation: 11.12.2014

Für meine Familien.
Insbesondere für Franka und Luis.

Das Leben liegt Euch zu Füßen.

Vorwort

Die vorliegende Doktorarbeit wurde im August 2005 am Institut für Chemie und Biologie des Meeres (ICBM) der Carl von Ossietzky Universität Oldenburg in der Arbeitsgruppe Paläomikrobiologie unter der Leitung von Herrn Prof. Dr. Heribert Cypionka begonnen. Alle praktischen Arbeiten wurden bis März 2009 in Oldenburg, der schriftliche Teil im Oktober 2014 in Burghaslach abgeschlossen. Das Projekt wurde von Herrn Dr. Bert Engelen und Herrn Prof. Dr. Cypionka vorgeschlagen und von der Deutschen Forschungsgemeinschaft (DFG) im Rahmen des Schwerpunktprogrammes 527 *Integrated Ocean Drilling Program* (IODP) gefördert (DFG Förderung EN477/1-1 und EN477/1-2). Die Arbeit wurde von Herrn Dr. Engelen und Herrn Prof. Dr. Cypionka betreut.

Aus dem Projekt gingen zwei Veröffentlichungen hervor. Ein drittes Manuskript entstand in Kooperation mit Herrn Jörn Logemann aus der Arbeitsgruppe Organische Geochemie unter der Leitung von Herrn Prof. Dr. Jürgen Rullkötter, ICBM Oldenburg. Das Manuskript ist in Vorbereitung zur Einreichung bei einem Fachjournal. Herr Logemann hat es in ähnlicher Form in seiner Dissertation veröffentlicht (2013, Kapitel 4-5).

What would life in the surface biosphere look like if the deep subsurface biosphere did not exist?

D'Hondt et al., 2007

One of the biggest mysteries of life below the sea floor
is that although there are microbes down there
it's really hard to understand
how they have enough energy to live
and how incredibly slowly they are growing.

Beth Orcutt in Redfern, 2013

Inhaltsverzeichnis

Beiträge zu den Publikationen

Die vorliegende Arbeit enthält drei Manuskripte (Kapitel 2-4), an deren Entstehung mehrere Personen mitgewirkt haben. Meine Beiträge sowie die Beiträge der Koautoren und weiterer Mitarbeiter werden im Folgenden erläutert.

1. Engelen, B., **Ziegelmüller, K.**, Wolf, L., Köpke, B., Gittel, A., Cypionka, H., Treude, T., Nakagawa, S., Inagaki, F., Lever, M. A., Steinsbu, B. O., 2008. Fluids from the oceanic crust support microbial activities within the deep biosphere. *Geomicrobiol. J.* 25, 56-66.

Probenahme u. Porenwasser-Analysen: Wissenschaftl. Personal der IODP Exp. 301; **Konzept, Probenahme, MPN-Serien:** Engelen; **Auswertung der MPN-Serien:** Köpke, Schlingloff, Ziegelmüller; **Sulfatreduktionsraten:** Köpke, Gittel; **Zellzahlen:** Engelen, Inagaki, Ziegelmüller; **PCR:** Ziegelmüller; **AOM-Raten:** Treude, Niemann, Ziegelmüller; **Phosphatase-Aktivität:** Wolf; **Erstellung des Manuskripts:** Engelen, Ziegelmüller; **Überarbeitung durch:** Cypionka, Gittel, Lever, Steinsbu, Nakagawa, Treude, Inagaki.

2. Fichtel, K., Mathes, F., Könneke, M., Cypionka, H., Engelen, B., 2012. Isolation of sulfate-reducing bacteria from sediments above the deep-subseafloor aquifer. *Front. Microbiol.* 3, Artikel 65. doi: 10.3389/fmicb.2012.00065.

Erstanreicherungen: Engelen, Wolf, Schlingloff, Mathes; **Molekularbiolog. Untersuchung der Erstanreicherungen und Subkultivierung:** Fichtel; **Isolierung:** Fichtel, Schlingloff; **Phylogenetische Analysen:** Fichtel, Schlingloff; **Physiologische Analysen:** Fichtel, Könneke; **TEM-Photographie:** Rhiel; **Erstellung des Manuskripts:** Fichtel, Engelen; **Überarbeitung durch:** Cypionka, Mathes, Könneke.

3. Fichtel, K., Logemann, J., Fichtel, J., Rullkötter, J., Cypionka, H., Engelen, B. Temperature and pressure adaptations of a piezothermophilic sulfate reducer from the deep marine subsurface. Vorbereitet für die Einreichung bei *The ISME Journal*.

Idee u. Konzept: Engelen, Cypionka, K. Fichtel, Logemann; **Druckexperimente:** K. Fichtel; **Lipidanalysen:** Logemann, J. Fichtel; **Erstellung des Manuskripts:** K. Fichtel, Logemann; **Überarbeitung durch:** J. Fichtel, Rullkötter, Engelen, Cypionka.

Zusammenfassung

Meerwasser zirkuliert in großen Mengen durch die oberen, porösen Schichten der Ozeankruste. Die hydrothermalen Fluide enthalten Elektronenakzeptoren, wie beispielsweise Sulfat, die für mikrobielle Atmungsprozesse zur Verfügung stehen. In der vorliegenden Arbeit wurde untersucht, welchen Einfluss die sulfathaltigen Fluide auf das Vorkommen, die Aktivität und die Diversität mikrobieller Populationen der Tiefen Biosphäre in Meeressedimenten haben. Hierzu wurde eine 265 m lange Sedimentsäule analysiert, die während der IODP Expedition 301 über einer Flanke eines mittelozeanischen Rückens beprobt wurde. Aufgrund der hydrothermalen Zirkulation ist der Untersuchungsstandort durch einen steilen Temperaturgradienten von 2 °C in den Oberflächenschichten und über 60 °C in Krustennähe sowie durch eine zweite Sulfat-Methan-Übergangszone charakterisiert. Unsere Untersuchungen richteten sich auf eine molekularbiologische und kultivierungsabhängige Quantifizierung und Charakterisierung indigener Mikroorganismen, insbesondere sulfatreduzierender Bakterien, sowie auf die Messung mikrobieller Aktivitäten entlang der Sedimentsäule. Erhöhte Zellzahlen, Exoenzymaktivitäten und Stoffumsatzraten in Schichten, die durch Fluide beeinflusst werden, deuteten auf lebendige und metabolisch aktive Gemeinschaften in der Tiefe hin. Aus krustennahen Sedimenten wurden nichtsporenbildende, sulfatreduzierende Deltaproteobakterien isoliert, die chemoheterotroph, chemolithotroph mit Wasserstoff und fermentierend wuchsen. Einem Stamm (P23), der der Gattung *Desulfovibrio* zuzuordnen ist, wurde autotrophes Wachstum mit Wasserstoff, Kohlendioxid und Sulfat nachgewiesen. Diese Ergebnisse unterstützen die Annahme, dass Wasserstoff in Sedimentschichten mit basaltischer Umgebung ein wichtiger Elektronendonator ist. *In situ* ist Stamm P23 sowohl hohen Temperaturen als auch einem hohen hydrostatischen Druck ausgesetzt. Wachstumsversuche mit Stamm P23 unter diesen Bedingungen und eine nachfolgende Lipidanalyse haben gezeigt, dass die Bakterienart moderat piezothermophil ist und ihre Membranviskosität und -funktion über den Ornithingehalt und durch strukturelle Veränderungen der Hauptphospholipide stabilisiert. Insgesamt zeigen unsere Untersuchungsergebnisse, dass Fluide der Ozeankruste mikrobielles Leben in tiefen Meeressedimenten unterstützen. In Anbetracht der globalen Ausdehnung des Grundwasserleiters in der Ozeankruste ist anzunehmen, dass zirkulierendes Meerwasser weltweit und über die geologische Zeit hinaus eine wichtige Antriebsquelle für die marine Tiefe Biosphäre ist.

Summary

Huge amounts of seawater circulate through the upper porous layers of the ocean crust. The hydrothermal fluids contain electron acceptors such as sulfate for microbial respiration. In the present thesis, we studied the influence of diffusive flows, especially that of sulfate, on the abundance, activity and diversity of deep-biosphere microbial populations in marine sediments. Samples were analysed from a 265 m long sediment column drilled during IODP Expedition 301 at a mid-ocean ridge flank. Due to the hydrothermal circulation, the sampling site is characterized by a steep temperature gradient of 2 °C within the surface layers to over 60 °C towards the basement and a second sulfate-methane transition zone. Our investigations aimed at the molecular biological and cultivation-dependent quantification and characterization of indigenous microorganisms, with special interest on sulfate-reducing bacteria, and microbial activity measurements along the sediment column. Elevated cell numbers, exoenzyme activities and turnover rates in fluid-influenced layers indicated the presence of vital and metabolically active communities thriving near the bedrock. From crust-near layers, non-sporeforming sulfate-reducing Deltaproteobacteria were isolated into pure cultures, turning out to grow either chemoheterotrophically, chemolithotrophically using hydrogen or by fermentation. One *Desulfovibrio*-affiliated isolate, strain P23, even grew autotrophically on sulfate, carbon dioxide and hydrogen. This finding supports the assumption that hydrogen may act as a key electron donor in subseafloor sediments with basaltic environments. *In situ*, the bacteria living in deep, crust-near sediments are exposed to both, high temperature and high hydrostatic pressure. Growth experiments of strain P23 under such conditions revealed its moderate piezothermophilic nature. A subsequent lipid analysis has shown that strain P23 was able to stabilize its membrane viscosity and function by regulating the ornithine content, and by structural changes of main phospholipids. In conclusion, our studies confirm the hypothesis that crustal fluids support microbial life in marine subseafloor sediments. Regarding the worldwide expansion of the crustal fluid aquifer, we assume that this impact is one major driving force for marine deep biosphere populations on a global scale and over geological time scales.

Abkürzungen

AEG	*Acyl-ether glycerol*
ai	*anteiso*
ANME	*Anaerobic methanotrophic archaea*
AODC	*Acridin orange direct counts*
AOM	*Anaerobic oxidation of methane*
APC	*Advanced piston coring*
BLASTN	*Nucleotide Basic Logical Alignment Search Tool*
bp	*Base pair*
Bq	Becquerel
BSA	*Bovine serum albumin*
CARD-FISH	*Catalyzed reporter deposition–fluorescence in situ hybridization*
CORK	*Circulation–Obviation–Retrofit–Kit*
Da	*Dalton*
DAG	Diacylglycerol
DAPI	4',6-Diamidino-2-phenylindol
DFG	Deutsche Forschungsgemeinschaft
DGGE	*Denaturing gradient gel electrophoresis*
DGTS	*Diacylglycerol trimethylhomoserine*
DNA	*Deoxyribonucleic acid*
dNTP	*Deoxyribonucleoside triphosphate*
DOC	*Dissolved organic carbon*
DSDP	*Deep Sea Drilling Program*
DSMZ	Deutsche Sammlung von Mikroorganismen und Zellkulturen GmbH
dsr	*Dissimilatory sulfite reductase*
Dv.	*Desulfovibrio*
EDTA	Ethylendiamintetraacetat
ESI	*Electrospray ionization*
Exp.	*Expedition*
FA	*Fatty acid*
FtsZ	*Filamenting temperature-sensitive mutant Z*
g	Erdbeschleunigung
GC-FID	*Gas chromatography–flame ionization detector*
HEPES	4–(2–Hydroxyethyl)piperazin–1–ethansulfonsäure
HPLC	*High–performance liquid chromatography*
i	*iso*
IODP	*International Ocean Discovery Program*
IPL	*Intact polar lipids*
LC-MS	*Liquid chromatography mass spectrometry*

mbsf	*Meters below seafloor*
MPa	Megapascal
MPN	*Most probable number*
MS	*Mass spectrometry*
m/z	*Mass to charge ratio*
MUF-P	*4-Methylumbelliferyl-phosphate*
n	*normal*
ODP	*Ocean Drilling Program*
OL	*Ornithine lipid*
OTU	*Operational taxonomic unit*
PA	*Phosphatidic acid*
PCR	*Polymerase chain reaction*
PE	*Phosphatidylethanolamine*
PFT	*Perfluorocarbon tracer*
PG	*Phosphatidylglycerol*
qPCR	*Quantitative polymerase chain reaction*
Rec	*recombination*
rpm	*Rounds per minute*
rRNA	*Ribosomal ribonucleic acid*
SGI	*SybrGreenI*
SMTZ	*Sulfate–methane–transition zone*
SQDG	*Sulfoquinovosyldiacylglycerol*
SR	*sulfate reduction*
SRB	*Sulfate–reducing bacterium/bacteria*
TAE	Tris–Acetat–EDTA
TEM	*Transmission electron microscopy*
TOC	*Total organic carbon*
Tris	Tris–(hydroxymethyl)–aminomethan
TRIS	*Total amount of reduced inorganic sulfur*
U	*Unit*

1 Einleitung

1.1 Die marine Tiefe Biosphäre

Mikroorganismen sind die am weitesten verbreiteten und artenreichsten Lebewesen auf unserer Erde. Als Motor der globalen biogeochemischen Kreisläufe nehmen insbesondere marine Arten durch ihre Stoffwechselaktivitäten Einfluss auf die Chemie in den Ozeanen und der Atmosphäre, und damit letztlich auf unser Klima.

Unsere Wahrnehmung von Leben auf unserem Planeten hat sich in den letzten drei Jahrzehnten grundlegend verändert. Im Rahmen von internationalen Meeresbohrprogrammen hat sich herausgestellt, dass Mikroorganismen nicht nur die Meeresbodenoberfläche bewohnen, sondern auch über weite Teile tief im Sediment (Parkes et al., 1994; Whitman et al., 1998; D'Hondt et al., 2004; Schippers et al., 2005) und sogar in Gesteinen der Ozeankruste (Santelli et al., 2008; Lever et al., 2013) vorhanden sind. Durchschnittlich werden selbst in 1 km Sedimenttiefe noch eine Million intakte Zellen pro Kubikzentimeter Sediment gefunden (Parkes et al., 2000). Jüngsten Hochrechnungen zufolge sind in den Meeressedimenten mit 2.9×10^{29} prokaryotischen Zellen ähnlich viele Einzeller wie im gesamten Meerwasser oder in den terrestrischen Böden enthalten, umgerechnet also 9-31 % der weltweit vorhandenen mikrobiellen Biomasse (Kallmeyer et al., 2012).

Diese sogenannte *Tiefe Biosphäre* (Gold, 1992) überrascht sehr, wenn man bedenkt, dass die dort vorkommenden Mikroorganismen unter extremen Bedingungen leben oder zumindest überdauern. Im marinen Untergrund herrschen fernab vom Sonnenlicht absolute Dunkelheit, durch die darüberstehende Wassersäule hohe hydrostatische Drücke, bedingt durch geologische Prozesse teils extreme Temperaturen und mit der Tiefe zunehmende Substrat- und Energielimitierung. Um das Habitat in seiner Funktion und Auswirkung besser verstehen zu können, wird es weltweit intensiv erforscht (siehe *Review*-Artikel z.B. von Parkes et al., 2000; Edwards et al., 2005; Jørgensen & Boetius, 2007; Fry et al., 2008; Orcutt et al., 2011; Fang & Zhang, 2011; Orcutt et al., 2013).

Eine wichtige Kernfrage, die auch für meine Arbeit entscheidend war, ist: Wovon ernähren sich die Bewohner der Tiefen Biosphäre? Welche Energiequellen unterstützen mikrobielles Leben im marinen Untergrund? Neben dem Abbau des abgelagerten organischen Materials durch unterschiedliche Remineralisierungsprozesse in der oberen Sedimentsäule werden in verschie-

denen Studien thermogene und geologische Prozesse diskutiert, welche die Mikroorganismen in den tiefsten Sedimentschichten mit Energie versorgen könnten (Kapitel 1.4 bis 1.6). In der vorliegenden Arbeit wurde der Einfluss des basaltischen Grundwasserleiters untersucht. Das durch die Ozeankruste zirkulierende Meerwasser könnte eine mögliche weitere Energiequelle sein, welche die Tiefe Biosphäre in den Sedimenten von unten mit Elektronenakzeptoren für mikrobielle Atmungsprozesse versorgt (siehe auch Kapitel 1.7).

1.2 Vertikale Ausdehnung

Meeressedimente bedecken rund zwei Drittel der Erdoberfläche. Sie können mehr als zehn Kilometer tief und über 100 Millionen Jahre alt sein (Parkes et al., 2000). Die marine Tiefe Biosphäre beginnt unterhalb der hochaktiven Oberflächenschicht (Orcutt et al., 2011), je nach Ozeanregion ab einer Sedimenttiefe zwischen 10 cm und 10 m (Whitman et al., 1998; D'Hondt et al., 2009). Informationen über die Häufigkeit und die vertikale Verteilung von Mikroorganismen im Meeresuntergrund liefern mikroskopische Zellzählungen. Hierfür werden die Zellen in den Sedimentproben mit Hilfe von DNA-interkalierenden Fluoreszenzfarbstoffen wie Acridinorange oder SybrGreen sichtbar gemacht und unter dem Mikroskop gezählt (Cragg et al., 1990; Lunau et al., 2005; Santelli, 2007).

In Abhängigkeit von den Umgebungsbedingungen variieren die Zelldichten in den Sedimentsäulen zwischen 10^3 und 10^9 Zellen pro Kubikzentimeter Sediment (Parkes et al., 1994; D'Hondt et al., 2009), wobei die Anzahl der Zellen generell kontinuierlich mit der Tiefe abnimmt (Parkes et al., 2000). Begründen lässt sich dies mit den Folgen der hohen mikrobiellen Aktivität in den obersten Sedimentschichten. Hier wird der größte Teil des frisch abgelagerten, organischen Materials unter Verbrauch terminaler Elektronenakzeptoren, die im Meerwasser enthalten sind, abgebaut (siehe Kapitel 1.4). Infolgedessen ist die Kohlenstoff- und Energieverfügbarkeit und damit das Wachstum der Mikroorganismen in den tiefer vergrabenen und gealterten Sedimentablagerungen eingeschränkt (Parkes et al., 2000; Jørgensen & Boetius, 2007).

Porosität und Permeabilität des Sediments und die Verfügbarkeit von Wasser sind weitere wichtige Faktoren, die den Transport chemischer Verbindungen, beispielsweise gelöstes organisches Material (DOC), und damit die Komplexität und Zelldichte von mikrobiellen Gemeinschaften in der Tiefe be-

einflussen (Parkes et al., 2000). Erhöhter Druck und zunehmende Erwärmung mit der Tiefe erschweren ebenfalls das Wachstum, da sie die Anpassung der mitvergrabenen Mikroorganismen erfordern (Parkes et al., 2000; Jørgensen & Boetius, 2007). So vermögen an heißen Standorten nur (hyper)thermophile, an hohe Temperaturen angepasste Mikroorganismen zu überleben, die in der Lage sind, durch Hitze verursachte Schäden an zellulären Komponenten zu reparieren (Parkes et al., 2000). Es gilt als wahrscheinlich, dass dem Leben in der Tiefen Biosphäre letztlich durch die Temperatur eine Grenze gesetzt ist (Parkes et al., 2000; Jørgensen & Boetius, 2007).

Nach derzeitigem Erkenntnisstand ist mikrobielles Wachstum bis 121 °C nachgewiesen (Kashefi & Lovley, 2003). In einer typischen marinen Umgebung mit einem geothermalen Gradienten von 3-5 °C pro 100 m wird diese Temperatur erst in einer Sedimenttiefe von zwei bis vier Kilometern erreicht (Whitman et al., 1998; Parkes et al., 2000; Lipp, 2008). Die bisher tiefste dokumentierte marine Stelle, in der noch zahlreiche intakte Zellen nachgewiesen wurden, liegt vor Neufundland in 1,6 km Tiefe unter dem Meeresboden (Roussel et al., 2008). In dieser Tiefe wird an einer dicken Paläobasaltschicht thermogen entstandenes, aufsteigendes Methan zurückgehalten und von Archaeen zur Energiegewinnung genutzt. In Subduktionsgebieten wie zum Beispiel am nordostpazifischen Juan-de-Fuca-Rücken, unserem Untersuchungsgebiet, kann diese Temperatur jedoch schon in geringen Tiefen erreicht werden (Parkes et al., 2000; Lipp, 2008). Aufgrund der aktiven Fluidzirkulation in diesen Gebieten wurde in den hydrothermalen Sedimenten ein Temperaturanstieg von bis zu 12 °C pro Meter gemessen.

Solange die Umgebungstemperatur im Habitat unter 120 °C liegt, ist das Vorkommen der tiefen Biozönose nicht auf die Sedimente beschränkt. Mittlerweile gibt es zahlreiche strukturelle, isotopische und molekularbiologische Beweise, dass sich die Tiefe Biosphäre bis in den Gesteinssockel der Ozeane hinein erstreckt (z.B. Fisk et al., 1998; Bach & Edwards, 2003; Cowen et al., 2003; Ehrhardt et al., 2007).

1.3 Mikrobielle Aktivität

Marine Sedimente enthalten geochemische Hinweise auf Energieflüsse und mikrobielle Stoffwechselaktivitäten. So gelten sowohl der Fluss potenzieller Elektronenakzeptoren als auch das Vorkommen und die Verteilung biologisch erzeugter Nebenprodukte im Sediment, zum Beispiel Gase, als Marker für spezifische Stoffwechselvorgänge, die einen Einblick in das Leben im Mee-

resuntergrund geben. Zusammen mit vielfältigen Methoden liefern sie den Nachweis, dass die Tiefe Biosphäre lebt. So lassen sich Lebendzellzahlen bestimmen (Cragg et al., 1990; Schippers et al., 2005) oder hochmolekulare DNA (Webster et al., 2003), ribosomale RNA (Orsi et al., 2013) und intakte polare Lipide (Sturt et al., 2004; Lipp et al., 2008) extrahieren, von denen man jeweils annimmt, dass sie nur von lebenden Organismen stammen können. Des Weiteren lassen sich verschiedenartige, z.T. unbekannte Prokaryoten kultivieren und isolieren (Batzke et al., 2007; Smith et al., 2011), während Wachstumsexperimente mit stabilen Isotopen-markierten Substraten (Morono et al., 2011) oder unter Druck (Bale et al., 1997; Barnes et al., 1998) demonstrieren, dass Populationen an das Leben unter den Bedingungen im marinen Untergrund angepasst sind (siehe auch Orcutt et al., 2013).

Aus den Tiefenprofilen gemessener Stoffkonzentrationen lassen sich signifikante und mögliche metabolische Prozesse ableiten und in Kombination mit *Radiotracer*-Experimenten mikrobielle Umsatzraten bestimmen (z.B. Jørgensen et al., 2001; Joye et al., 2004; Lomstein et al., 2012). Die Quantifizierung mikrobieller Aktivitäten hilft, den Einfluss auf Stoffkreisläufe abzuschätzen und ist für das Verständnis notwendig, wie Mikroorganismen in der extremen Umgebung überleben.

Tendenziell nehmen mit der Zahl der Mikroorganismen auch die Aktivitäten pro Zelle mit der Tiefe ab (Parkes et al., 2000). Aufgrund der stark energielimitierenden Bedingungen zeigen die tief vergrabenen Zellen, im Vergleich zu Oberflächensedimenten und Wachstumsversuchen im Labor, jedoch nur sehr geringe metabolische Aktivität (D'Hondt et al., 2002, 2004). Das heißt, dass die Zellen nur sehr langsam wachsen und lange Hungerperioden überstehen müssen (Jørgensen & Boetius, 2007; D'Hondt et al., 2009; Jørgensen, 2011, 2012). Modellierten Aktivitätsberechnungen zufolge teilen sich die Zellen der Tiefen Biosphäre nur einmal in mehreren Tausend Jahren (Jørgensen & Boetius, 2007; Jørgensen, 2011). Und obwohl es wahrscheinlich ist, dass ein Großteil der Zellen inaktiv ist oder schlafend in Form von Sporen überdauert (Fichtel et al., 2008; Lomstein et al., 2012), muss angenommen werden, dass die metabolisch aktiven Mikroorganismen einen sehr effektiven Erhaltungsstoffwechsel haben und gewonnene Energie eher für die Aufrechterhaltung von Zellfunktionen einsetzen, als sie für die Zellteilung zu nutzen (Jørgensen, 2011).

Doch welche Energiequellen halten die Mikroorganismen der Tiefen Biosphäre über die geologische Zeit am Leben? Da die Energiefrage eine ent-

scheidende ist, habe ich mich damit auseinandergesetzt, welche Möglichkeiten indigene Populationen haben, Stoffwechsel zu betreiben.

1.4 Remineralisierung von organischem Material als Hauptenergiequelle

Der Großteil der Substrate, die in Oberflächensedimenten zur Verfügung stehen, stammt aus dem Abbau des am Meeresgrund abgelagerten organischen Materials. Die Bedingungen für die Tiefe Biosphäre hängen von der Primärproduktion in der Wassersäule, der Wassertiefe, Einträgen von Land, der Entfernung von Landmassen, der Temperatur und der Sedimentationsrate ab (Jørgensen, 1982; Jahnke, 1996; Kallmeyer et al., 2012) und variieren in den Weltmeeren sehr stark. Demzufolge variieren auch die Aktivität, der Stoffwechsel, das Vorkommen und die Zusammensetzung mikrobieller Gemeinschaften. Die mikrobiellen Remineralisierungsprozesse bestimmen wiederum die Vergrabungsrate des organischen Materials, welches mit der Tiefe rekalzitrant wird und nur schwer zu charakterisieren ist (Niggemann, 2005).

1.4.1 Atmungsprozesse

Heterotrophe Sedimentmikroorganismen remineralisieren die abgelagerten organischen Verbindungen durch Oxidation mit externen Elektronenakzeptoren, die gewöhnlich aus dem Meerwasser in die Sedimentsäule diffundieren. Die theoretische Abfolge des Verbrauchs der Elektronenakzeptoren hängt von der freien Energie ab, die durch ihre Reduktion gewonnen wird (siehe z.B. Orcutt et al., 2011). Im Sediment entstehen dadurch sukzessiv mit der Tiefe Zonen aerober und anaerober mikrobieller Atmungsprozesse, beginnend mit der Reduktion von Sauerstoff, der das höchste Redoxpotenzial besitzt, gefolgt von Nitrat, Eisen(III)- und Mangan(IV)oxiden sowie Sulfat. Die Verteilung der entsprechenden Mikroorganismen ist dabei eng an die chemischen Zonen gekoppelt. Der finale Remineralisierungsschritt ist die Veratmung von CO_2 bzw. Carbonat mit Wasserstoff unter Bildung von Acetat (Acetogenese) oder Methan (hydrogenotrophe Methanogenese). Die durch die Atmungsprozesse entstehenden Stoffwechselprodukte Acetat, Methan, Wasserstoff, reduzierte Schwefel-, Eisen- und Manganverbindungen sowie Ammonium sind weitere energiereiche, elektronenspendende Substrate, die von anderen chemoorgano- oder chemolithotrophen Mikroorganismen der Tiefen Biosphäre zur Energiegewinnung genutzt werden (Amend & Teske, 2005).

1.4.2 Gärung

Zusätzlich zu den Atmungsprozessen spielt in anoxischen Habitaten die Gärung eine wichtige Rolle (D'Hondt et al., 2002; Wellsbury et al., 2002). Die entstehenden Gärprodukte, beispielsweise kurzkettige Fettsäuren, Amine, Alkohole oder Lactat, Wasserstoff und Kohlendioxid, werden von vielen weiteren spezialisierten Organismen als Substrate verwertet. Acetat beispielsweise kann von Sulfatreduzierern oxidiert oder von Methanogenen disproportioniert, das heißt aufgespalten werden. In tiefen marinen Sedimenten spielt Acetat daher als Ausgangsstoff für die acetoklastische Methanbildung eine zentrale Rolle (Whiticar, 1999; Wellsbury et al., 2002; Parkes et al., 2005, 2007).

1.4.3 Methanbildung und anaerobe Oxidation von Methan (AOM)

In tieferen, sulfatfreien Sedimentschichten ist die Bildung von Methan durch hydrogenotrophe und methylotrophe Archaeen der dominierende terminale Remineralisierungsprozess (D'Hondt et al., 2002; Wellsbury et al., 2002). Methanogene konkurrieren zwar mit acetogenen Bakterien um einfache Substrate, zum Beispiel H_2/CO_2, Formiat ($CHOO^-$) oder Methanol (CH_3OH), aufgrund ihrer Wachstumskinetik sind sie ihnen jedoch überlegen (Whiticar, 1999). In Abwesenheit von Oxidantien kann Methan an lithologischen Grenzschichten (Roussel et al., 2008) oder in Form von Gashydraten akkumulieren (Ginsburg, 1998) und in sulfathaltige Zonen aufsteigen, wo es unter Sulfatverbrauch von mikrobiellen Konsortien (Hinrichs et al., 1999; Boetius et al., 2000) durch anaerobe Oxidation zu CO_2 abgebaut wird (AOM, Hoehler et al., 1994). Derartige Sulfat-Methan-Übergangszonen sind potenzielle „hot spots“ hoher mikrobieller Aktivität, welche wir auch an unserem Untersuchungsstandort erwartet haben. Global gesehen werden rund 90 Prozent des im Sediment befindlichen Methans noch im Sediment recycelt (Hinrichs & Boetius, 2003). Mittlerweile ist es anerkannt, dass die mikrobielle anaerobe Oxidation von Methan ein bedeutender mariner Prozess ist, der den Austritt von Methan als klimarelevantes Treibhausgas aus den Sedimenten in die Ozeane und die Atmosphäre reguliert (Knittel & Boetius, 2009; Holler et al., 2011). Nach neuestem Kenntnisstand koppeln anaerobe methanotrophe Archaeen (ANME) die Oxidation von Methan an die dissimilatorische Reduktion von Sulfat, wobei der dabei entstehende Schwefel von kommensalen sulfatreduzierenden Deltaproteobakterien zur Energiegewinnung weiter disproportioniert wird (Milucka et al., 2012). Obwohl AOM

auch mit anderen Elektronenakzeptoren möglich ist (Raghoebarsing et al., 2006; Beal et al., 2009), wird angenommen, dass in marinen Sedimenten AOM die bedeutendste Senke für gelöstes Sulfat ist (D'Hondt et al., 2002).

1.5 Energie aus geologischen Quellen

Bei der Frage, welche zusätzlichen, aus der Tiefe stammenden Energiequellen die marine Biosphäre antreiben, werden verschiedene Prozesse diskutiert. Als wichtige Treibstoffe für die Tiefe Biosphäre werden molekularer Wasserstoff, Acetat, CO_2 und Methan erachtet, welche unter hohen Temperaturen und Drücken alle abiotisch bei der Verwitterung von Basaltgestein gebildet werden können (Stevens & McKinley, 1995). Wasserstoff ist von besonderer Bedeutung. Für die metabolische Energiegewinnung kann er an alle bekannten Elektronenakzeptoren gekoppelt werden (Bach et al., 2006; Orcutt et al., 2011). Zusammen mit CO_2 unterstützt er chemoautotrophe Lebensgemeinschaften und ist innerhalb der Erdkruste Ausgangsstoff für die abiotische Fischer-Tropsch-artige Synthese von Methan und anderen Kohlenwasserstoffen (Holm & Charlou, 2001). Wasserstoff wird in großen Mengen während der Serpentinisierung von Mantelgestein freigesetzt (McCollom & Bach, 2009) oder durch die radiolytische Spaltung von Wasser erzeugt. Dieser Prozess wird vom natürlichen Zerfall radioaktiver Elemente wie beispielsweise Uran angetrieben. Uran ist ein natürlicher Bestandteil der Erdkruste (Lin et al., 2005; Blair et al., 2007) und auch in marinen Sedimenten (D'Hondt et al., 2009) enthalten.

1.6 Der Einfluss höherer Temperaturen auf die Energieversorgung in tiefen Sedimenten

Die tief vergrabenen mikrobiellen Populationen werden über die geologische Zeit hinweg wahrscheinlich auch über thermogene Prozesse aufrechterhalten. Verschiedenartige Experimente zeigen, dass schwer abbaubar gewordenes, rekalzitrantes organisches Material durch die mit der Tiefe zunehmende Erwärmung reaktiviert und dadurch für mikrobielle Abbauprozesse wieder leichter zugänglich gemacht wird (Wellsbury et al., 1997; Parkes et al., 2007). Außerdem kann die dauerhafte Einwirkung hoher Temperaturen (>100 °C) auf fossile organische Substanz leichtflüchtige Kohlenwasserstoffe als potenzielle Substrate freisetzen (Parkes et al., 1994; Wellsbury et al., 1997; Horsfield et al., 2006; Roussel et al., 2008). Eine Studie von Parkes et al. (2011)

hebt die Bedeutung von Wechselwirkungen zwischen thermogenen und biogenen Prozessen in 65-155 °C heißen Sedimenten hervor. Wasserstoff und wichtige Metabolite liefernde Reaktionen zwischen Porenwasser und Sedimentbestandteilen werden der Studie zufolge durch die Einwirkung indigener Prokaryoten sogar verstärkt.

1.7 Fluide der Ozeankruste

Auch Elektronenakzeptoren können erneut in tiefe Sedimentschichten eingetragen werden und dort mikrobielle Prozesse stimulieren. So wurde beispielsweise eine zweite, tiefere AOM-Zone auf die Diffusion von Sulfat aus alten Salzgesteinsablagerungen in methanhaltige Sedimente zurückgeführt (Parkes et al., 1990; D'Hondt et al., 2004; Parkes et al., 2005). Geochemische Tiefenprofile der Sedimentbedeckung im östlichen Äquatorialpazifik deuteten hingegen auf ein Einströmen von Sulfat aus dem basaltischen Aquifer in darüberliegende Sedimente hin (Baker et al., 1991; D'Hondt et al., 2004). Ähnliches wurde aufgrund der aktiven hydrothermalen Zirkulation von Meerwasser durch die Ozeankruste in unserem Probenahmegebiet auch an unserem Untersuchungsstandort erwartet.

Erste Hinweise auf ein Grundwassersystem in der Ozeankruste lieferten aktive hydrothermale Quellen, die infolge von vulkanischen Eruptionsprozessen oder magmatischen Ausstößen während der Krustenneubildung an Ozeanspreizungszentren entstehen (siehe Review von Kelley et al., 2002). Mittlerweile ist es gut dokumentiert, dass in der Nähe von mittelozeanischen Rücken Meerwasser durch die voluminöse und permeable obere Ozeankruste, die an den Rückenachsen neu gebildet wird, zirkuliert und zur Abkühlung der Flanken beiträgt (siehe *Reviews* von Cowen, 2004; Orcutt et al., 2011; Edwards et al., 2012). Der Fluidtransport wurde bisher am besten an der östlichen Flanke des nordostpazifischen Juan-de-Fuca-Rückens, unserem Probenahmestandort (Abb. 1), untersucht (Davis et al., 1989; Elderfield et al., 1999; Wheat et al., 2000; Fisher et al., 2003; Johnson & Pruis, 2003; Wheat et al., 2003). Die hydrothermale Zirkulation (siehe Kapitel 2, Abb. 4) wird durch den Wärmeeinfluss aus dem Erdmantel sowie durch Druck- und Temperaturunterschiede zwischen exponierten, nahezu sedimentfreien Felsformationen als Ein- und Austrittsstellen angetrieben (Fisher et al., 2003; Hutnak et al., 2006), während die Sedimentbedeckung als eine Art hydraulischer Verschluss betrachtet werden kann (Davis et al., 1999). Die Fließzeit zwischen den Seebergen beträgt schätzungsweise 4300 Jahre (Elderfield et al., 1999).

Auf diese Weise sorgt sie seit Millionen von Jahren für einen hydrogeologischen Austausch zwischen der Ozeankruste und dem Meer (Fisher et al., 2003; Fisher & Wheat, 2010).

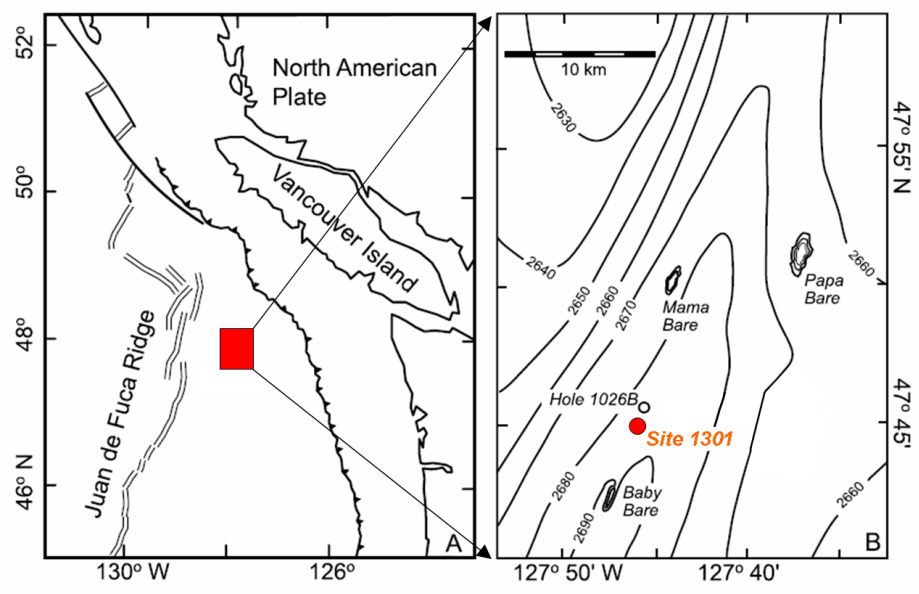

Abb. 1: Lagepläne des Probenahmegebietes und der Site 1301. A) Lage des ca. 500 km langen mittelozeanischen Juan-de-Fuca-Rückens im nordöstlichen Pazifik vor der kanadischen Küste. Die submarine Gebirgskette entstand durch Spreizung des Meeresbodens infolge der Subduktion der Juan-de-Fuca-Platte unter die Nordamerikanische Platte. B) Die bathymetrische Karte zeigt sowohl die Lage des Probenahmestandorts Site 1301 auf der östlichen Flanke unterhalb von 2656 m Wassertiefe, das benachbarte Bohrloch 1026B sowie die aus dem Meeresboden herausragenden Felsformationen („Mama Bare“, etc.), die für die hydrothermale Zirkulation des Meerwassers in diesem Gebiet eine bedeutende Rolle spielen (siehe auch Kapitel 2, Abb. 4). Modifizierte Abbildungen nach Fisher et al. (2008), Beschreibung nach Hutnak et al. (2006).

Während des Fluidflusses durch die dreieinhalb Millionen Jahre alte Kruste erwärmen sich die Fluide von ungefähr 2 °C auf ca. 65 °C und werden in ihrer geochemischen Zusammensetzung durch Wasser-Gesteins-Reaktionen verändert (Davis et al., 1999; Elderfield et al., 1999; Cowen, 2004). Trotz der Reaktion der gemäßigten hydrothermalen Fluide mit dem Krustengestein sind sie nicht vollständig reduziert und enthalten Energie für chemolithoautotrophe Gemeinschaften sowie Elektronenakzeptoren für mikrobielle Atmungsprozesse (Mottl et al., 1998; Elderfield et al., 1999; Wheat et al., 2000). Erstmalige mikrobiologische Untersuchungen von Fluiden an der benachbarten Site 1026 (Abb. 1) bestätigten die Anwesenheit verschiedener thermophiler Mikroorganismen (Cowen et al., 2003; Huber et al., 2006; Orcutt et al., 2011; Smith et al., 2011), die wahrscheinlich bei der Verwitterung des Basalts mitwirken (Fisk et al., 1998; Furnes & Staudigel, 1999; Bach & Edwards, 2003). Es lässt sich daher vermuten, dass hydrothermale

Fluide nicht nur eine aktive Biosphäre innerhalb der Ozeankruste antreiben, sondern über aufwärtsgerichtete Diffusion auch die Biosphäre in der darüberliegenden Sedimentsäule mit Energie versorgen könnten (Parkes et al., 2000; D'Hondt et al., 2004). An Site 1026 und 1027 in der Tiefe erhöhte Zellzahlen lieferten einen ersten Hinweis (Mather & Parkes, 2000). Nach DeLong (2004) hätte das basaltische Grundwassersystem möglicherweise sogar das Potenzial, am Sockel des Meeresbodens ein umgekehrtes Redox-Profil entstehen zu lassen, das die oberflächennahen mikrobiellen Abbauprozesse widerspiegelt (Abb. 2).

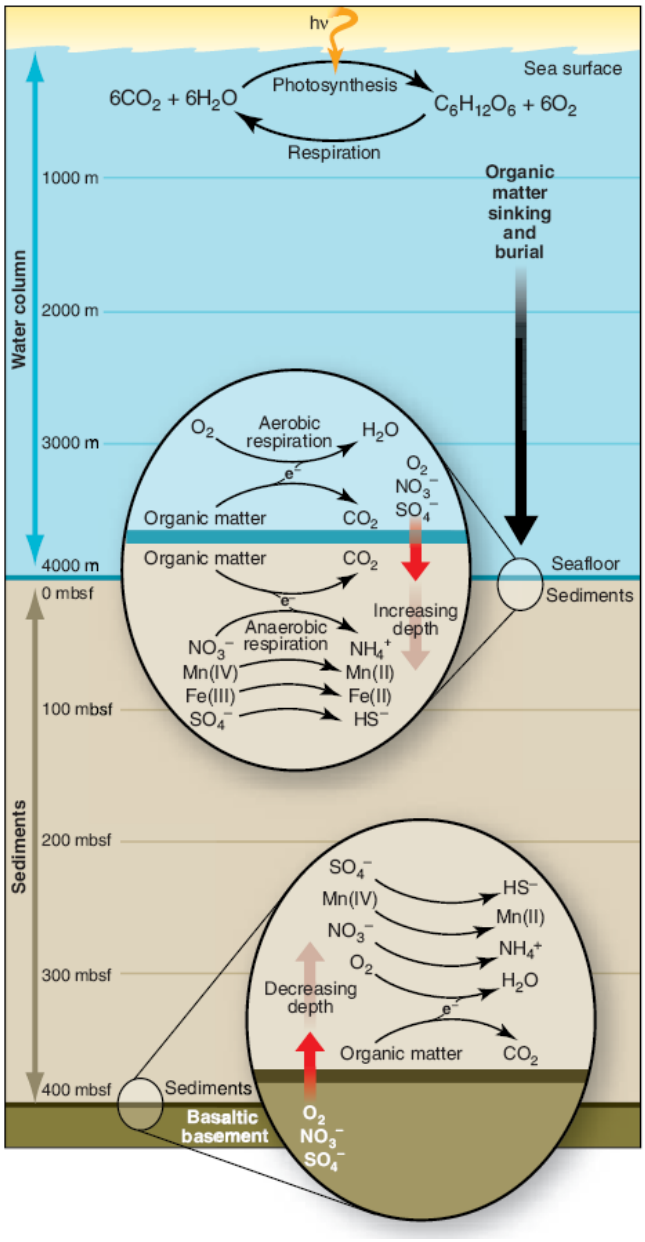

Abb. 2: Herkunft und Verwendung von Elektronenakzeptoren für mikrobielle Atmungsprozesse im Meeresboden (Grafik aus DeLong, 2004).

1.8 Sulfatreduzierende Mikroorganismen

Da die dissimilatorische Sulfatreduktion aufgrund der hohen Verfügbarkeit von Sulfat (28 mM) in Meerwasser einer der wichtigsten anaeroben Atmungsprozesse in marinen Sedimenten ist (Jørgensen, 1982; Reeburgh, 1983; Henrichs & Reeburgh, 1987; D'Hondt et al., 2004), standen sowohl die Messung potenzieller Sulfatreduktionsraten als auch die Kultivierung und physiologische Charakterisierung sulfatreduzierender Bakterien, insbesondere aus fluid-beeinflussten Sedimentschichten, im Mittelpunkt der vorliegenden Arbeit.

1.8.1 Diversität und Physiologie von Sulfatreduzierern

Die energiekonservierende dissimilatorische Sulfatreduktion wird einzig von einer metabolisch vielseitigen Gruppe von Sulfatreduzierern geleistet, die weltweit in anoxischen Habitaten zu finden ist und eine bedeutende Rolle im Schwefel- und Kohlenstoffkreislauf einnimmt (siehe *Review* von Muyzer & Stams, 2008). Derzeit werden auf Basis vergleichender 16S rRNA-Gensequenzen phylogenetisch gramnegative *Deltaproteobacteria*, grampositive, sporenbildende *Firmicutes* sowie thermophile Bakterien der Klassen *Thermodesulfobacteria*, *Nitrospira* und der Familie *Thermodesulfobiaceae* unterschieden als auch thermophile *Eury-* und *Crenarchaeen* (Castro et al., 2000; Rabus R., 2006; Muyzer & Stams, 2008), die Sulfat als Elektronenakzeptor nutzen.

Aus Wachstumsversuchen mit isolierten Arten weiß man, dass Sulfatreduzierer hauptsächlich heterotroph sind und insbesondere Gärprodukte als Substrate nutzen. Physiologisch werden Sulfatreduzierer anhand ihres Oxidationsprofils unterschieden. Einige Arten oxidieren organische Kohlenstoffverbindungen vollständig zu CO_2, andere nur unvollständig zu Acetat. Durch den Redoxprozess wird Schwefelwasserstoff freigesetzt, welcher in marinen Habitaten zu großen Vorkommen von Sulfidmineralien wie Pyrit (FeS_2) führt oder, je nach oxischer oder anoxischer Bedingung, von chemolithotrophen bzw. phototrophen Schwefelbakterien zurück oxidiert werden kann (Kasten & Jørgensen, 2000). Mittlerweile sind auch Arten bekannt, die auf den Abbau von Kohlenwasserstoffen wie Alkanen, Alkenen oder aromatischen Substanzen spezialisiert sind (Widdel & Bak, 1992; Rabus R., 2006, und darin enthaltene Referenzen). Einige wenige bisher bekannte Sulfatreduzierer wachsen chemolithoautotroph mit Wasserstoff,

Kohlendioxid und Sulfat als einzige Energiequellen (Klemps et al., 1985; Brysch et al., 1987; Widdel, 1987; Strittmatter et al., 2009; Steinsbu et al., 2010). In Abwesenheit von Sulfat sind viele Sulfatreduzierer fähig, für die Energiegewinnung verschiedene Substrate, zum Beispiel Pyruvat, zu fermentieren, Thiosulfat, Sulfit, Schwefel oder Fumarat zu disproportionieren oder alternative Elektronenakzeptoren zu verwenden (Widdel & Bak, 1992, und darin enthaltene Referenzen). Andere Arbeiten zeigen, dass manche Arten sogar zur homoacetogenen Gärung oder Acetogenese befähigt sind (Jansen et al., 1984; Klemps et al., 1985; Schink et al., 2002; Sass et al., 2004a; Rabus R., 2006). In diesem Fall vergären Sulfatreduzierer in sulfatlimitiertem oder -freiem Medium beispielsweise Formiat, Methanol oder Lactat unter Bildung von Wasserstoff und CO_2, welche in einer Art CO_2-Atmung weiter zu Acetat als einzigem Endprodukt umgesetzt werden.

1.8.2 Molekularbiologischer Nachweis und Kultivierung von Sulfatreduzierern

Aufgrund ihrer ökophysiologisch wichtigen Rolle in marinen Sedimenten könnte man annehmen, dass Sulfatreduzierer zu den am häufigsten gefundenen Gruppen gehören. Tatsächlich jedoch sind die Identität und physiologischen Eigenschaften der Mikroorganismen, die für die Sulfatreduktionsraten verantwortlich sind, weitestgehend unbekannt (DeLong, 2004). Ihre Abundanz und Verteilung im Sediment entlang des Sulfatkonzentrationsprofils kann mit Hilfe von molekularbiologischen Methoden abgeschätzt werden. Hierfür verwendet man zum Beispiel die quantitative Polymerasekettenreaktion des dissimilatorischen Sulfitreduktase-Gens (*dsr*AB) als Schlüsselgen für Sulfatreduzierer (Wagner et al., 1998). Aus einem Vergleich von entsprechenden Studien am Kontinentalhang vor Peru (Schippers & Neretin, 2006; Webster et al., 2006) schlossen Fry et al. (2008), dass entweder nur eine sehr geringe Zahl sulfatreduzierender Populationen aktiv ist oder die Sulfatatmung von bisher unbekannten Sulfatreduzierern geleistet wird, die mit gegenwärtigen Methoden nicht detektierbar sind (Parkes et al., 2005; Teske, 2006; Fry et al., 2008). Tatsächlich sind selbst in umfangreichen Klonbanken, die auf Basis von molekularen 16S rRNA-Gen-Analysen erstellt wurden, nur wenige Sequenzen von Sulfatreduzierern enthalten. Viele davon sind mit der DNA von kultivierten Sulfatreduzieren aus verschiedenen Habitaten nur entfernt verwandt und deuten möglicherweise auf neue Linien mit unbekannter

Physiologie hin (Dhillon et al., 2003; Inagaki et al., 2003; Kormas et al., 2003; Parkes et al., 2005; Inagaki et al., 2006; Webster et al., 2006). Ein Rückschluss auf deren metabolische Fähigkeiten oder Anpassungsstrategien ist daher nicht gegeben. Hierfür ist die Gewinnung von Reinkulturen unerlässlich.

Die Anreicherung und Kultivierung physiologisch bedeutsamer, repräsentativer Bakterien aus tiefen Sedimentschichten ist jedoch allgemein schwierig. Die strikt anaerobe Lebensweise, die Anpassung an langsames Wachstum und fehlendes Wissen über essenzielle Bedürfnisse der Populationen sowie syntrophe Partner scheinen Kultivierungs- und Isolierungsversuche zu erschweren. In Relation zur Diversität wird häufig nur ein geringer Anteil an Mikroorganismen kultiviert (Parkes et al., 2000; Huber et al., 2002; Wellsbury et al., 2002). Ein weiterer Grund hierfür könnte sein, dass die Sedimentproben während der Probenahme generell dekomprimiert werden und unter Atmosphärendruck weiterverarbeitet werden. Auch bleibt der in der Tiefe vorherrschende hydrostatische Druck bei den meisten Kultivierungsexperimenten aus technischen Gründen unberücksichtigt. Dekompressionsempfindliche, piezophile Mikroorganismen werden dadurch ihrer Analyse entzogen (Parkes et al., 2009). Piezophilie ist jedoch ein wichtiges Anpassungsmerkmal tief vergrabener Populationen an ihr extremes Habitat (Parkes et al., 1995; Yayanos, 1995; Parkes et al., 2000).

Bei den bislang einzigen, aus bis zu 500 m tiefen Meeresbodenschichten isolierten Sulfatreduzierern handelt es sich um den Typenstamm *Desulfovibrio profundus* (Parkes et al., 1995; Bale et al., 1997) sowie *Desulfovibrio profundus*-verwandte Arten (Barnes et al., 1998). Physiologische Untersuchungen unter Druck zeigten, dass die Bakterien piezophil sind, das heißt, dass sie unter Druck besser wachsen als bei Atmosphärendruck.

1.9 Wachstumsversuche unter hydrostatischem Druck

In der vorliegenden Arbeit wurden mit einem Isolat von unserem Untersuchungsstandort Wachstumsversuche unter Druck durchgeführt. Die zusätzliche Analyse der Membranlipide sollte einen Einblick geben, welche Anpassungsmechanismen genutzt werden, um unter hohem Druck und hoher Temperatur in tiefen Meeressedimenten zu überleben.

Die Höhe des Druckes im Sediment wird hauptsächlich durch das Gewicht der Wassersäule bestimmt. Da die Dichte von Wasser durch die Druckzunahme wenig beeinflusst wird, erhöht sich der hydrostatische Druck kontinuier-

lich um 0,1 MPa pro 10 m Wassertiefe (Yayanos, 1995). Obwohl poröse Sedimente durch die Einwirkung des Gewichts der darüberstehenden Wassersäule verdichtet werden, spielt in Tiefseesedimenten die Änderung des lithostatischen Druckes mit der Tiefe ebenso wie der mittlere Atmosphärendruck der Erde auf den Meeresspiegel (0,1 MPa) kaum eine Rolle (Vossmeyer, 2008).

Es ist bekannt, dass der Druck und die Temperatur wesentlich die physikalischen Eigenschaften von Lipiden beeinflussen (Somero, 1992). Interessanterweise können viele Mikroorganismen ihre Zellmembranlipide über z.T. weite Temperatur- und Druckbereiche nahezu konstant fluid halten. Dies geschieht hauptsächlich durch Änderungen in der Fettsäurenzusammensetzung in den Phospholipiden, die wesentliche Bestandteile der mikrobiellen Cytoplasmamembran sind. Diese Fähigkeit wird als „homeoviskose Anpassung“ bezeichnet und ist für die effektive Funktion einer Zellmembran von essenzieller Bedeutung (Sinensky, 1974).

Physiologische Untersuchungen zur Tiefenanpassung wurden bisher vorwiegend mit psychrophilen Bakterien aus permanent kalten Tiefseehabitaten durchgeführt (siehe Review von Bartlett, 2002). Es hat sich gezeigt, dass erhöhter Druck ebenso wie kalte Temperaturen dazu führen, dass in einer Zelle mehr Membranlipide mit geringeren Schmelzpunkten und Packungsdichten synthetisiert werden, um die Viskosität der Zellmembran aufrecht zu halten. Dies wird hauptsächlich durch den Einbau höherer Anteile ungesättigter Fettsäuren in die Phospholipide erreicht (DeLong & Yayanos, 1985; Yayanos, 1995). Weitere Mechanismen zur homeoviskosen Anpassung der Lipiddoppelschicht scheinen sich von Art zu Art zu unterscheiden. Manche psychropiezophile Arten erhöhen zusätzlich die Anteile kurzkettiger Fettsäuren (Zhang & Rock, 2008; Wang et al., 2009). Wiederum andere marine Arten produzieren verstärkt langkettige, mehrfach ungesättigte Fettsäuren (DeLong & Yayanos, 1986; Yano et al., 1998; Kato & Nogi, 2001).

Umgekehrt werden bei hohen Temperaturen (und niedrigem Druck wie Atmosphärendruck) Phospholipide mit hohen Schmelztemperaturen und hohen Packungsdichten zur Stabilisierung der Membranfluidität synthetisiert, um zu verhindern, dass die Membran zu fluid wird (Rilfors et al., 1978). Untersuchungen zur thermalen Anpassung der Membranlipide von grampositiven Bakterien und thermophilen Bakterien haben gezeigt, dass die Membranfluidität hauptsächlich über die hohen Anteile verzweigter Fettsäuren reguliert wird (Russell & Fukunaga, 1990; Koga, 2012). Mit Hilfe massenspektrometrischer Analysetechniken werden auch homöostase Änderungen

in den polaren Kopfgruppen der Phospholipide infolge von Temperatur- oder Druckänderungen nachgewiesen (Pluschke & Overath, 1981; Fang et al., 2000; Mangelsdorf et al., 2005; Zhang & Rock, 2008). Phospholipide beeinflussen die Durchlässigkeit der Zellmembran und sind für die Anwesenheit und Aktivität membrangebundener Enzyme entscheidend (Sinensky, 1974). Es wird daher vermutet, dass dieser Mechanismus dazu dient, die lebenswichtigen Zellfunktionen aufrecht zu erhalten.

Mittlerweile existieren verschiedene Systeme, um kombinierte Effekte von Druck und Temperatur auf physiologische Eigenschaften von Mikroorganismen zu untersuchen (z.B. Kallmeyer et al., 2003). In der vorliegenden Arbeit wurden für die Wachstumsexperimente unter Druck relativ einfach zu handhabende Stahlzylinder, sogenannte „high pressure steel vessels“ benutzt (Abb. 3). Pro Zylinder können in einem Experiment mehrere Proben oder für die Lipidanalyse nötig, größere Volumen inkubiert werden. Der hydrostatische Druck wurde manuell mit Hilfe eines Druckgenerators und destilliertem Wasser als hydraulisches Fluid erzeugt und über das Septum auf die Kulturen übertragen (Kapitel 4).

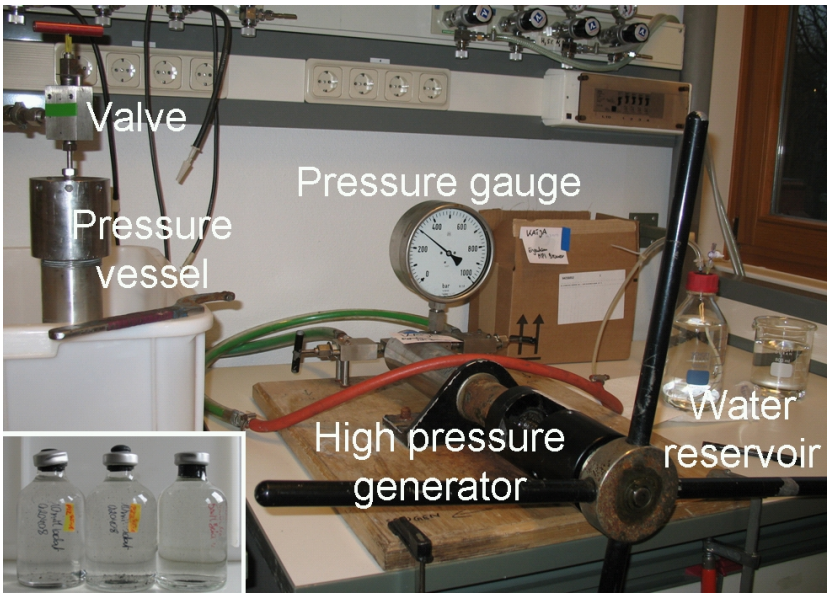

Abb. 3: Technische Ausstattung zur Durchführung von Wachstumsexperimenten unter hydrostatischem Druck und erhöhten Temperaturen. Kleines Bild: Als Kulturgefäße wurden vollständig mit Medium gefüllte Serumfläschchen verwendet.

1.10 Zielsetzung der vorliegenden Arbeit

Die vorliegende Arbeit hatte zum Ziel, mit molekular- und mikrobiologischen Methoden den Einfluss hydrothermaler Fluide der Ozeankruste auf die Tiefe Biosphäre in marinen Sedimenten zu untersuchen. Hierfür wurden Sedimente analysiert, die die östliche Flanke des hydrogeologisch gut untersuchten Juan-de-Fuca-Rückens überdecken (Abb. 1; Abschnitt 1.7). Die Sedimentproben wurden 2004 während der IODP Exp. 301 unter Mitwirkung von Herrn Dr. Bert Engelen gewonnen. Voruntersuchungen haben ergeben, dass der Untersuchungsstandort durch einen steilen Temperaturgradienten von 0,23 °C/m charakterisiert war (Expedition 301 Scientists, 2005b). In Krustennähe wurden bereits Temperaturen um 65 °C festgestellt. Diese Bedingungen lassen die Anwesenheit thermophiler Mikroorganismen vermuten. Geochemische Profile zeigten an, dass Sulfat sowohl aus der Wassersäule als auch aus dem basaltischen Untergrund in die methanhaltige Zone der 265 m dicken Sedimentsäule hineindiffundiert (Expedition 301 Scientists, 2005b), wodurch es zur Ausbildung von zwei Sulfat-Methan-Übergangszonen kommt, möglichen *hot spots* hoher mikrobieller Aktivität.

Hauptziel der ersten Studie war es, die Hypothese von DeLong (2004) zu überprüfen (Kapitel 2; Engelen et al., 2008). Dessen Einschätzung zufolge könnte der basaltische Aquifer aufgrund seiner chemischen Zusammensetzung das Potenzial haben, mikrobielles Leben in darüberliegenden Sedimenten durch die Bereitstellung reduzierbarer Verbindungen anzutreiben. Eine der wichtigsten Fragen war deshalb, welchen Einfluss Fluide der Ozeankruste auf die Abundanz und metabolische Aktivität indigener Mikroorganismen in bis zu 3,6 Millionen Jahre alten Sedimenten haben. Hierfür wurde mittels voneinander unabhängiger Methoden (Direktzählung und qPCR) die Verteilung allgemeiner und domänenspezifischer mikrobieller Zellzahlen entlang der Sedimentsäule bestimmt. Des Weiteren wurden über das kultivierungsabhängige MPN (*most probable number*)-Verfahren Lebendzellzahlen aerober und anaerober Mikroorganismen ermittelt. Potenzielle Sulfatreduktions- und AOM-Raten wurden mit Hilfe von radioaktiv markierten Substraten (*radiotracer*) bestimmt. Da die Produktion von Exoenzymen in substratlimitierten Umgebungen eine wichtige Überlebensstrategie von Prokaryoten ist, wurden mit Hilfe von Substratanaloga potenzielle Exoenzymaktivitäten als allgemeines Maß für mikrobielle Aktivität ermittelt.

Erhöhte Zellzahlen, metabolische Umsatzraten und Exoenzymaktivitäten in den tiefen, durch Fluide beeinflusste Sedimentschichten deuteten darauf hin, dass sich dort potenziell lebensfähige Anaerobier und aktiv sulfatreduzierende bzw. methankonsumierende mikrobielle Populationen befinden. Die zweite Veröffentlichung befasste sich daher mit dem Einfluss der Fluide auf die mikrobielle Diversität am Untersuchungsstandort (Kapitel 3; Fichtel et al., 2012). Im Mittelpunkt der Untersuchungen standen die Kultivierung, Isolierung, Identifizierung und Charakterisierung indigener, insbesondere sulfatreduzierender Mikroorganismen. Eine bewährte Methode zur Isolierung abundanter Sedimentbakterien ist die Flüssigverdünnungsreihe „*dilution-to-extinction*". Weniger abundante und weniger gut ans Medium angepasste, langsam wachsende Bakterien werden bei diesem Vorgehen ausverdünnt und damit nicht identifiziert (Ziegelmüller, 2005). Neben den MPN-Anreicherungen wurden deshalb weitere Kultivierungsmethoden eingesetzt und der Isolierungsprozess mit molekularbiologischen Methoden (PCR, DGGE, Sequenzierung) verfolgt. Auf diese Weise sollten gezielt Sulfatreduzierer in Reinkultur gebracht sowie Verschiebungen in der Diversität potenziell wachstumsfähiger Bakterien entlang der Sedimentsäule festgestellt werden. Die physiologische Untersuchung der Isolate sollte einen Einblick geben, welchen Einfluss Fluide auf den mikrobiellen Stoffwechsel in Umgebungen haben, in denen Substrate limitiert, aber Elektronenakzeptoren verfügbar sind. Isolate wurden insbesondere auf autotrophes Wachstum getestet, da Wasserstoff der möglicherweise wichtigste Elektronendonator in tiefen Sedimentschichten und basaltischen Umgebungen ist (Stevens & McKinley, 1995).

Über einen dieser Kultivierungswege wurde ein Sulfatreduzierer isoliert, dessen Wachstumsverhalten unter *in situ*-Temperatur und -Druck untersucht wurde (drittes Manuskript, Kapitel 4). Interessanterweise wurde das Isolat im Labor bei 20 °C gewonnen, obwohl es ursprünglich aus einer ca. 60 °C warmen, krustennahen Schicht stammt. Unter Atmosphärendruck lag die maximale Wachstumstemperatur des Stammes bei nur 48 °C (Kapitel 3; Fichtel et al., 2012). Es stellte sich deshalb die Frage, ob der vorliegende hydrostatische Druck einen Einfluss auf die maximale Wachstumstemperatur hat. Des Weiteren bot das Isolat die Gelegenheit herauszufinden, welche Mechanismen eine piezothermophile Bakterienart nutzt, um sich an die extremen Bedingungen im Habitat anzupassen. Die Analyse zellulärer Lipide sollte hierüber Aufschluss geben.

2 Fluids from the oceanic crust support microbial activities within the deep biosphere

Dieses Kapitel wurde veröffentlicht: Engelen, B.*, Ziegelmüller, K., Wolf, L., Köpke, B., Gittel, A., Cypionka, H., Treude, T., Nakagawa, S., Inagaki, F., Lever, M. A., Steinsbu, B. O., 2008. Fluids from the oceanic crust support microbial activities within the deep biosphere. *Geomicrobiol. J.* 25, 56-66. doi:10.1080/01490450701829006. Das Manuskript wurde am 3. Juli 2007 eingereicht, am 26. November 2007 akzeptiert und am 01. Januar 2008 online publiziert. Die Autoren Engelen und Ziegelmüller trugen gleichermaßen zu dieser Arbeit bei.

Geomicrobiology Journal, 25:56–66, 2008

ISSN: 0149-0451 print / 1521-0529 online
DOI: 10.1080/01490450701829006

Taylor & Francis
Taylor & Francis Group

Fluids from the Oceanic Crust Support Microbial Activities within the Deep Biosphere

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Keywords: Integrated Ocean Drilling Program, JOIDES Resolution, Expedition 301, deep biosphere, subsurface microbiology, oceanic crust, Juan de Fuca hydrogeology, hydrothermal fluids, microbial activity, AOM, sulfate reduction, methanogenesis, rates, MPN, exoenzymes

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2.1 Abstract

The importance of crustal fluid chemical composition in driving the marine deep subseafloor biosphere was examined in northeast Pacific ridge-flank sediments. At IODP Site U1301, sulfate from crustal fluids diffuses into overlying sediments, forming a transition zone where sulfate meets *in situ*-produced methane. Enhanced cell counts and metabolic activity suggest that sulfate stimulates microbial respiration, specifically anaerobic methane oxidation coupled to sulfate reduction. Cell counts and activity are also elevated in basement-near layers. Owing to the worldwide expansion of the crustal aquifer, we postulate that crustal fluids may fuel the marine deep subseafloor biosphere on a global scale.

2.2 Introduction

Microbiological studies on sediment cores collected during cruises of the Deep Sea Drilling Program (DSDP) and the Ocean Drilling Program (ODP) have demonstrated the presence of microbial communities in deeply buried marine sediments down to several hundred meters below seafloor (Parkes et al., 1994; Whelan et al., 1986). Prokaryotes in the marine subseafloor biosphere have been estimated to comprise one-tenth to one-third of the world's living biomass (Whitman et al., 1998) and therefore probably play an important role in global biogeochemical processes. Conditions for the deep biosphere vary tremendously across the world's oceans as a result of sediment type, sediment age, sedimentation rate, organic matter availability, temperature, etc. Not surprisingly, activity, metabolism, abundance, and composition of indigenous microbial communities will vary accordingly. Key questions of deep-subsurface microbiology include: (1) what proportion of the community is active (2) what are the carbon sources or (3) which mechanism of energy conservation is dominant? Some of these aspects are now being studied with increasing intensity, in particular by the application of molecular biological approaches (Inagaki et al., 2003, 2006; Marchesi et al., 2001; Kormas et al., 2003; Newberry et al., 2004; Schippers et al., 2005; Schippers & Neretin, 2006).

Recent investigations indicated that the deep biosphere extends into the upper layers of the oceanic crust (Fisk et al., 1998; Cowen, 2004; Ehrhardt et al., 2007; Huber et al., 2006; Nakagawa et al., 2006; Summit & Baross, 2001; Lysnes et al., 2004a,b; Thorseth et al., 2001). These porous upper

layers of igneous basements are characterized by circulation of seawater and harbor the largest aquifer on Earth (Johnson & Pruis, 2003). One of the most intensively studied areas in terms of hydrogeology is the eastern flank of the Juan de Fuca Ridge in the northeastern Pacific (Johnson et al., 2006). The hydrological model (Fisher et al., 2003; Hutnak et al., 2006) postulates a heat-driven fluid flow within the upper crust following geological formations (Fig. 4). While bottom seawater is recharged and discharged at rocky outcrops like "Grizzly Bare seamount" and "Baby Bare seamount", the compact sediment layer between the seamounts serves as a barrier to direct fluid-flow exchange with the sediment column. During circulation, fluids are altered by chemical reactions with the basaltic rock, and presumably microbial activity. In contrast to the porewater of deeply buried sediments, crustal fluids are not fully reduced and contain suitable electron acceptors for microbial respiration (Wheat et al., 2000).

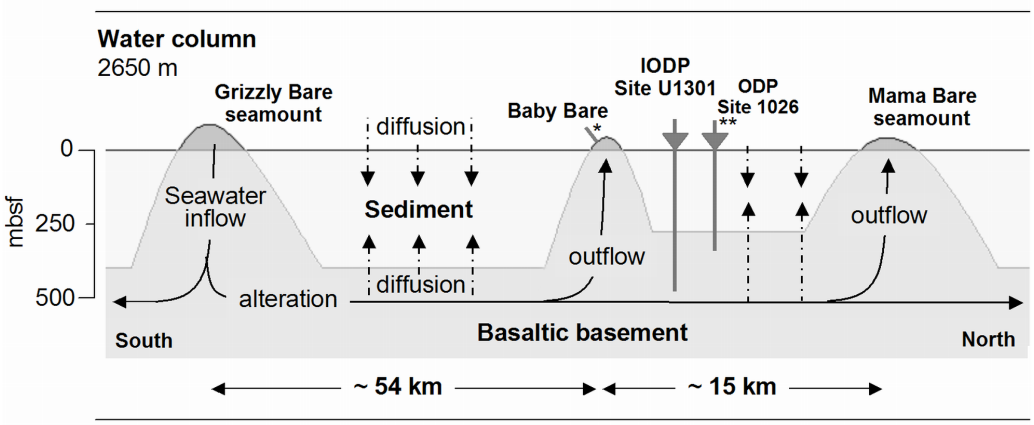


Fig. 4: Scheme of the hydrogeological regime at the eastern flank of the Juan de Fuca Ridge. While bottom seawater is recharged at "Grizzly bare seamount", it is discharged at "Baby bare" and "Mama bare seamount". Circulating fluids are altered by rock-interactions within the basaltic basement and diffuse into the overlaying sediments. Boreholes of IODP Site U1301 and the adjacent ODP Site 1026, both sealed by a CORK observatory, are indicated. *Sampling site for microbiological investigations by Huber et al. (2006). **Microbiological surveys performed by Cowen et al. (2003) and Nakagawa et al. (2006).

In a previous microbiological experiment conducted by Cowen et al. (2003), a filtration device, called BioColumn, was deployed at the CORK (Circulation-Obviation-Retrofit-Kit) borehole sealing of ODP Site 1026B on the Juan de Fuca Ridge flank. A molecular survey indicated the presence of relatives of known nitrate reducers, sulfate reducers and thermophilic fermentative heterotrophs in crustal fluids emanating from the borehole. These microbes probably originated from the porous and permeable aquifer zone of

the basalt. In another sampling campaign at the same location, Huber et al. (2006) punctured the fluid-discharging top of "Baby Bare seamount" by a steel tube. The microbiological analyses of the collected fluids confirmed the presence of diverse (hyper)thermophiles within the oceanic crust. The chemical composition of crustal fluids might not only fuel the deep biosphere within the crust, but also that of overlying sediments by introducing electron acceptors, such as sulfate, metal oxides, nitrate, or even oxygen (DeLong, 2004).

We tested this hypothesis during an expedition to the eastern flank of the Juan de Fuca Ridge (IODP Exp. 301) by analyzing an entire sediment column including samples taken only 4.6 meters above the sediment-basement interface. Sampling was performed at IODP Site U1301, located 1-2 km north of the above mentioned ODP Site 1026B. At this ridge flank site, the diffusion of sulfate into the sediment column from both directions, from underlying basement and overlying seawater, results in the formation of two sulfate-methane transition zones. The formation of a lower transition zone by fluid diffusion from the basaltic aquifer is supposed to be the typical setting at oceanic ridge flanks. A similar transition zone resulting from an unusual deep brine incursion was previously found at the continental margin off Peru and was shown to be an important habitat for the subseafloor microbial populations (D'Hondt et al., 2004; Parkes et al., 2005).

In our investigation, we have determined microbial abundance by independent methods and metabolic activities in terms of sulfate reduction, anaerobic methane oxidation, and exoenzyme activities throughout the sediment column. The result of this assessment indicates that indigenous microbial populations of deeply buried sediments are stimulated by electron acceptors introduced from underlying basalt. A local elevation of microbial abundance and activity correlates to the overall geochemical setting.

2.3 Methods

2.3.1 Sediment sampling and site characteristics

Sediment samples were retrieved by the drill ship "JOIDES Resolution" during IODP Expedition 301 in August 2004. The sampling site (IODP Site U1301) was located at the eastern flank of the Juan de Fuca Ridge in the northeast Pacific. Two boreholes were drilled for sediment recovery by advanced piston coring (APC). Hole C (position: 47°45.280´N, 127°45.800´W,

water depth 2656 m) was discontinuously sampled from the sediment surface down to 130 mbsf and from 178 to 263 mbsf, app. two meters above the sediment-basement interface. *In situ* temperature increased linearly with depth, from ~2 °C in surface sediment to ~65 °C at the basement. Due to the expedition schedule, another hole (Hole D; 47°45.276´N, 127°45.780´W) was drilled 26 meters apart from Hole C to sample the layers between 120 and 175 mbsf. Hole D is considered to be one interval of Hole C as seen by the continuous lithological, geochemical and physical characteristics (Expedition 301 Scientists, 2005b). Furthermore, these characteristics are nearly identical to those of ODP Site 1026, located even 1.2 km north on the same ridge flank (Shipboard Scientific Party, 2004).

2.3.2 Subsampling and contamination tests

Sediment cores (length: 9.5 m) were cut onboard into 1.5-meter sections. Subsamples used for geochemical analyses were stored under nitrogen atmosphere to prevent oxidation processes. For microbiological analyses, the innermost parts of whole-round cores from 17 different depths were sampled (Table 1). Sediments to be used for DNA extraction were collected in 50-ml sterile cut-off syringes which were sealed by rubber stoppers and stored at −20 °C. Subsamples for enzyme-activity measurements and metabolic turnover-rates were taken similarly with 5-ml sterile cut-off syringes and stored under N_2 at 4 °C. For Acridine orange direct counts (AODC) that were directly determined onboard, 1-cm^3-samples were fixed in 50 % ethanol. For cultivation experiments, including serial dilutions for most probable number (MPN) counts, sediment slurries were prepared in sterile flasks containing artificial seawater (Süß et al., 2004), flushed with N_2 and stored at 4 °C until further processing.

Perfluorocarbon tracer (PFT) experiments (Lever et al., 2006) were performed during coring operations to assess sample contamination with drilling fluid-derived microbial cells. The majority of whole-round cores were nearly free of PFT in the center part corresponding to the presence of less than 0.09 exterior cells·g^{-1} sediment. In only a few cases a possible contamination of 0.1 to 1.7 cells g^{-1} sediment was found, indicating that the contamination of drilling fluids was almost negligible (Lever et al., 2006).

2.3.3 Porewater and gas analyses

Interstitial water and gas analyses were performed immediately after subsampling by shipboard scientists using IODP standard procedures (Expedition 301 Scientists, 2005a). Concentrations of dissolved sulfate were determined via standard ion chromatography (Dionex method, Gieskes et al., 1991). Methane concentrations were calculated from headspace gas measurements (Kvenvolden & McDonald, 1986) using the onboard gas chromatograph. Dissolved phosphate concentrations were determined spectrophotometrically using the phosphomolybdate blue method after Presley (1971).

2.3.4 Determination of total cell counts by Acridine orange and SybrGreenI

Total cell counts from Hole C were determined onboard using the Acridine orange direct count (AODC) method according to Mather & Parkes (2000) with minor modifications. Aliquots of fixed sediment samples were ultrasonicated for 2 min in an ethanolic sodium chloride (saline) solution (50 % v/v ethanol in 3 % NaCl w/v). After 40 min of sedimentation, up to 1 ml supernatant was transferred to 5 ml saline solution, and stained with Acridine orange (final concentration 5 mg l^{-1}). After 30 min, the solution was filtered through a 0.2 µm black polycarbonate membrane (Poretics, 25 mm diameter, Osmonics, Inc., USA). The filter was rinsed with 10 ml sterile distilled water, dipped in ethanol, dried and mounted in a small amount of paraffin oil under a cover slip. For each sample, a total of sixteen fields of view were counted independently by two scientists.

Samples of Hole 1301D could not be counted onboard and did not show any signals after long-term fixation. Repeated AODC counting with anoxically stored samples was hampered by unspecific background fluorescence. This issue was overcome by applying a newly developed technique. Total cell counts were repeated with sediment samples from Hole C and D by using SybrGreenI as fluorescent dye according to Lunau et al. (2005), adapted to sediment samples. 1.22 ml TAE buffer (40 mmol l^{-1} Tris, 1 mmol l^{-1} EDTA, pH 7.4) and glutaraldehyde (final concentration 0.5 %) were added to 0.2 g sediment sample for fixation of living cells, and incubated for 30 min at 4 °C. For detaching cells from particles, 540 µl methanol were added, and the tube content was gently homogenized. The suspension was incubated for 15 min at 35 °C in an ultrasonic bath (Bandelin, Sonorex RK 103 H,

35 kHz, 2 x 320 W per period). Aliquots of homogenized suspensions (2.5 µl to 5 µl) were resuspended in 1 ml TAE buffer, added to 7 ml TAE buffer, and filtered through a polycarbonate membrane as described here. The membrane filter was washed with 5 ml of TAE buffer, transferred to a microscope slide, stained and mounted with 7 µl of SybrGreenI staining and mounting medium prepared according to Lunau et al. (2005). After 15 min of incubation, 70 randomly selected fields of view were counted for each layer by epifluorescence microscopy.

2.3.5 Most probable number analysis

The most probable number (MPN) method applied here was described in detail by Süß et al. (2004). The growth medium used in our study has been successfully applied to other subsurface samples, exhibiting a sound cultivation efficiency (Batzke et al., 2007; Süß et al., 2004; Köpke et al., 2005). This medium was composed of artificial seawater that was amended with a defined substrate mixture of glycerol, glucose, lactate, fumarate, malate, succinate, methanol, ethanol, 1-propanol, 1-butanol, formate, acetate, propionate, butyrate, valerate, caproate, and all the 20 L-amino acids as electron and carbon source (final concentration of each compound: 0.1 mmol l^{-1}). The medium was transferred to 96-deep-well plates (Beckman, Fullerton, CA), and inoculated with samples from seventeen different depths. Both, oxic and anoxic dilution series were used with three replicates each. Every MPN-plate contained additional rows of control wells without inoculum. For a better comparison between sediment layers exhibiting different ambient temperatures, incubation was performed at 20 °C, the optimum of mesophilic activity. The enrichments were analyzed for growth fluorometrically and microscopically after 14 weeks of incubation in the dark (Süß et al., 2004). MPN viable counts were calculated as described by de Man (1977). MPN series that showed irregular growth or cross-contaminated controls were not included in the calculations.

2.3.6 Nucleic acid extraction

Total genomic DNA was extracted from 10 g (wet weight) of each sediment sample using the UltraClean Soil DNA Kit Mega Prep (MO BIO Laboratories, Inc., Carlsbad, CA), according to the manufacturer's instructions. The DNA was concentrated by standard ethanol precipitation, and dissolved in

100 µl of sterile distilled water. DNA yields were fluorometrically quantified using PicoGreen stain (Molecular Probes, Eugene, OR), as described in detail by Wilms et al. (2007).

2.3.7 Preparation of DNA standards for quantitative PCR (qPCR)

DNA standards for qPCR (Higuchi et al., 1993) were prepared according to Wilms et al. (2007) and the references therein. In short, for *Bacteria* 16S rRNA genes of *Desulfovibrio vulgaris*^{T} (DSM 644) were amplified using the bacterial primer set 8f and 1492r (Lane, 1991). For *Archaea*, 16S rRNA genes of *Methanosarcina barkeri*^{T} (DSM 800) were amplified using the domain-specific primer pair S-D-Arch-0025-a-S-17 and S-*-Univ-1517-a-A-21 (Vetriani et al., 1999). The PCR products were purified by using the QIAquick PCR Purification Kit (Qiagen GmbH, Hilden, Germany) and DNA concentrations were quantified by using PicoGreen stain. The numbers of bacterial and archaeal 16S rRNA gene targets were calculated from the DNA content, the length of the amplified fragment (1484 bp and 1492 bp, respectively) and the mean weight of a base pair ($1.1 \cdot 10^{-21}$ g) according to Süß et al. (2006).

2.3.8 Quantitative PCR of *Bacteria* and *Archaea* based on 16S rRNA gene copy numbers

Quantitative PCR was used to estimate the vertical distribution of bacterial and archaeal cells within the sediment column. Bacterial and archaeal targets were measured in at least three different dilutions of environmental DNA extracts (1:500 to 1:1500) and in threefold parallels. To check for contamination, two PCR amplifications without DNA were used as a non-target control in each qPCR.

The qPCR mixtures contained 12.5 µl of the premix solution of a DyNAmo HS SYBR Green qPCR Kit (New England Biolabs, Inc., Hitchin, UK), 1 µl of each primer (5 µmol l^{-1}) and 10 µl standard or DNA extract as template in a final reaction volume of 25 µl. For the quantification of bacterial 16S rRNA gene-targets, the universal primers 519f (Lane, 1991) and 907r (Muyzer et al., 1995) were used. Archaeal targets were amplified with Arch-0025-a-S-17 and S-D-Arch-0344-a-20 (Vetriani et al., 1999). The PCR was carried out in a Rotor-Gene-3000 cycler (Corbett Research, Sydney, Australia).

After initial denaturation and activation of the hot start polymerase at 95 °C for 15 min, 50 cycles followed. Each cycle consisted of denaturation for 10 s at 94 °C, annealing for 20 s at 54 °C for *Bacteria* and 48 °C for *Archaea*, elongation for 30 s at 72 °C, and fluorescence measurement at 72 °C. To check amplification specificity, fluorescence was measured additionally at the end of each cycle for 20 s at 82 °C for *Bacteria* and at 80 °C for archaeal targets. After the last cycle, a melting curve was recorded subsequently by increasing the temperature from 50 °C to 99 °C (1 °C every 10 s).

For the calculation of gene copy numbers, standard curves were generated twice for each qPCR and each domain by 10-fold dilutions of the DNA standards, ranging from 1 to $1{\cdot}10^6$ target molecules. Data analysis was performed using the software package RotorGene 4.6. Calculated template concentrations were corrected by subtraction of the average number of 16S rRNA genes from non-target controls. Cell numbers of *Bacteria* were estimated using an average 16S rRNA copy number of 4.1 (http://rrndb.cme.msu.edu/). For the conversion of archaeal gene targets into cell numbers, an average of 1.5 16S rRNA gene copies per cell was assumed. Cell numbers were expressed as cells per cm^3 sediment.

2.3.9 Potential rates of anaerobic oxidation of methane (AOM)

For potential AOM rate measurements, sediment slurries (1:1 v/v) from 17 depths were prepared in three separate replicates with artificial seawater (Süß et al., 2004) in serum vials. The vials were flushed with methane, sealed by a rubber stopper and were preincubated for 2 weeks at near *in situ* temperatures (Table 1; samples C1-C4 at 10 °C, C6-C13 at 25 °C, D1-C15 at 35 °C and C17-C19 at 60 °C).

The slurries were transferred into 5-ml Hungate tubes injected with ^{14}C-labeled methane (cleaned from $^{14}CO_2$ and ^{14}CO, dissolved in water, injection volume 30 µl, specific activity 2.28 GBq $mmol^{-1}$) and incubated for 96 days at the above described temperatures. To determine the abiotic conversion of the methane tracer to $^{14}CO_2$ over time, at total of three sediment-free controls were incubated at 10 °C, 35 °C, and 60 °C, respectively. To stop microbial activity, samples were transferred into glass bottles filled with 25 ml of NaOH solution (2.5 % w/w) and closed immediately with rubber stoppers. Further processing of AOM samples, gas measurements and calculation of turnover rates were performed according to Treude et al. (2003) and references therein.

2.3.10 Potential sulfate reduction (SR) rates

For potential sulfate reduction rate measurements, water dissolved ^{35}S-labeled carrier-free sulfate was injected (volume 20 µl, activity ~170 kBq) into the triplicate cut-off syringes containing subsamples from the respective sediment layers. After 28 days of incubation, bacterial activity was stopped by the addition of 10 ml of zinc acetate solution (20 % w/v) and freezing. The total amount of reduced inorganic ^{35}S (TRIS), i.e. the end products of sulfate reduction was quantified by a singlestep chromium reduction and distillation method as described by Kallmeyer et al. (2004a). The radioactivity of $^{35}SO_4^{2-}$ and TRIS were measured using a Lumasafe®Plus scintillation cocktail (Biomol, Hamburg, Germany) and a liquid scintillation counter (1415 Wallace, Turku, Finland). Potential sulfate reduction rates were calculated by comparing the activity of the radioisotope-labeled TRIS to that of the total amount of sulfate radiotracer, and by taking account of porewater sulfate-concentration and the porosity of the sediment.

2.3.11 Potential phosphatase activities

Potential activity of hydrolytic extracellular alkaline phosphatase was determined using the fluorescent labeled substrate analogue 4-methylumbelliferyl-phosphate (MUF-P). Alkaline phosphatase activity was estimated from the conversion of MUF-P to the free fluorophor MUF. All measurements and calculations were performed according to Coolen & Overmann (2000). Two 1.4 ml aliquots of sediment slurries (1:2 suspensions with anoxic artificial seawater) were transferred to sterile 15-ml vials containing stirring bars. The enzymatic reaction was started by the addition of 100 µl MUF-P solution (final concentration 550 µM). After substrate addition, all serum flasks were flushed with N_2, sealed with butyl rubber stoppers and incubated for 18 h on a magnetic stirrer (250 rpm) at near *in situ* temperatures. After incubation, 100 µl NaOH (final concentration 40 mM) and 100 µl Na_4EDTA (final concentration 100 mM) were added.

Samples were transferred to reaction tubes, and centrifuged for 5 min at 10,000 g (Heraeus Biofuge 15 R, Hanau, Germany). To estimate the enzymatic conversion of the substrate analogue, 200 µl supernatant was transferred into a 96-well-microtiter plate (Nunc GmbH, Wiesbaden, Germany). The concentration of free fluorophores was measured against standard dilution series of MUF-P (0.025 µM to 2.5 µM) using a fluorometer (FLUOstar

Optima, BMG Labtechnologies, Offenburg, Germany) at an excitation wavelength of 360 nm and emission at 450 nm.

The resulting values were corrected for sediment autofluorescence, abiotic hydrolysis and the adsorption of free dissolved fluorophores. Four control experiments were performed for each sediment: (1) autofluorescence control of untreated sediments containing 1.4 ml sediment slurry and 100 µl of artificial seawater, (2) autofluorescence control of inactivated sediments: same as above but boiled for 30 min at 95 °C, (3) abiotic hydrolysis control: heat inactivated sediment slurry amended with 100 µl substrate-analogue solution to quantify a nonenzymatic substrate conversion, and (4) quenching control: sediments spiked with MUF-P to a final concentration of 37 µM.

2.4 Results

2.4.1 Prokaryotic cell counts along the sediment column

Microbial populations generally decreased with sediment depth. Exceptions to this trend were local peaks below the sulfate-methane transition zones (Figure 5a) and close to the basement. This result was obtained by epifluorescence counting as well as by quantitative PCR and cultivation experiments (Figure 5b, Table 1).

Total counts of Acridine orange and SybrGreenI stained cells

Quantification of cells from Hole U1301C using Acridine orange direct counts (AODC) revealed a similar trend as for other ODP sites (Parkes et al., 1994, 2000). Our counts were slightly higher than ones conducted with samples from nearby ODP Site 1026 (Mather & Parkes, 2000). Cell densities decreased from $7.5 \cdot 10^8$ cells cm^{-3} at the surface to a minimum of $1.8 \cdot 10^7$ at 248 mbsf, but almost reached near-surface values of $2.3 \cdot 10^8$ above the basement at 262 mbsf. The newly developed cell-counting method using SybrGreenI (SGI; Lunau et al., 2005) gave slightly lower cell densities than the conventional AODC method. In general, SGI counts decreased with depth from $5.2 \cdot 10^8$ in the uppermost sediment sample to the same minimum value as found with AODC, exhibiting a near-basement maximum of $8.0 \cdot 10^7$ cells cm^{-3} at 251.9 mbsf.

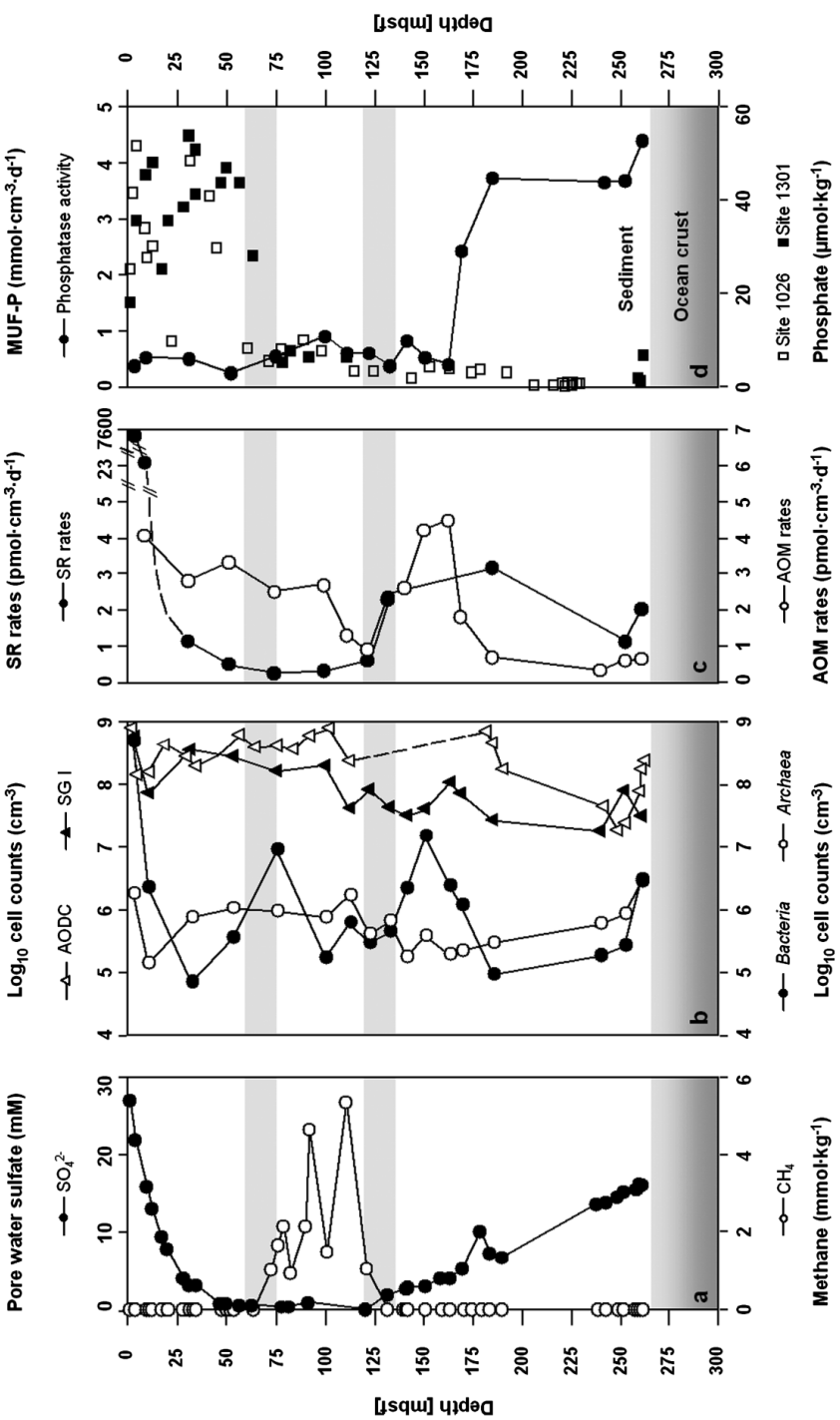


Fig. 5: Depth profiles of geochemical parameters, microbial abundance and metabolic activities along the sediment column of IODP Site U1301. (a) Porewater sulfate and methane concentrations. Both sulfate-methane transition zones are indicated. (b) Acridine orange direct counts (AODC), SybrGreenI counts (SGI) and specific counting of *Archaea* and *Bacteria* via quantitative PCR. (c) Potential sulfate-reduction (SR) rates and potential anaerobic methane oxidation (AOM) rates. (d) Potential phosphatase activities using the substrate analogue 4-methylumbelliferyl-phosphate (MUF-P) and phosphate concentrations of IODP Site U1301 and the nearby ODP Site 1026.

Increasing cell numbers towards the sediment-basement interface were found with both methods but was less pronounced for the SGI counts. In general, due to the absence of background fluorescence and the much brighter, long-lasting fluorescent signal of stained cells, we are more confident with results of the SybrGreenI technique. However, the higher resolution for AODC in this zone elucidates the trend of increasing cell numbers towards the basement. The higher abundance in these deep layers is probably caused by upward flux of crustal-fluid compounds that stimulates microbial growth or maintenance.

Specific enumeration of *Bacteria* and *Archaea* by quantitative PCR

While *Bacteria* dominated near-surface sediments and two layers below the sulfate-methane transition zones, the number of *Archaea* appeared constant throughout the sediment column (Figure 5b). Apart from the surface and the two local peaks, bacterial numbers were in the same order of magnitude as those of the *Archaea*, and in some cases even lower. The more or less even distribution of *Archaea* was unanticipated, as one might expect an increase of archaeal cells within the methanogenesis zone between ~60 and ~125 mbsf, or in the sulfate-methane transition zones.

While biological methanogenesis and AOM have only been observed in *Archaea*, an increase in archaeal biomass could be expected in zones where rates of methanogenesis and AOM increased. Due to low DNA-recovery ($<$1 ng cm^{-3} sediment), bacterial and archaeal cell numbers estimated from quantitative PCR were lower than numbers of directly counted cells, but nonetheless agreed with the other quantification methods applied here. For instance, the increase in cell numbers in near-basement layers was confirmed by quantitative PCR.

Most probable number viable counts of anaerobic and aerobic respiring cells

Serial dilutions in 96-deep-well microtiter plates, inoculated immediately after core recovery, showed microbial growth in all investigated sediment layers (Table 1). The highest MPN viable counts of up to 10^6 cells cm^{-3} sediment were obtained from anoxic enrichments using sediment layers at 31.1 and 140.9 mbsf as well as close to the basement at 251.9 mbsf. By comparing MPN counts with total cell counts obtained by SGI-staining, we estimated

cultivation efficiencies of up to 3.6 %. Similar MPN counts were obtained from oxic enrichments using sediments from 31.1, 99.4, and 111.9 mbsf, showing cultivation efficiencies of up to 2.8 %. In general, MPN counts from oxic enrichments fluctuated from 150 to $1.1 \cdot 10^6$ cells cm^{-3} sediment. However, no aerobes were found in layers from 239.5 mbsf and deeper. By contrast, cell numbers and resulting cultivation efficiencies of anaerobes were elevated above the basement in layers between 239.5-260.4 mbsf.

2.4.2 Activity measurements

The measured microbial activities indicated that not only the numerical abundance of the microbial community was influenced by crustal fluids but also their overall metabolic activity.

Rate measurements of potential sulfate reduction and potential anaerobic oxidation of methane

Potential sulfate and anaerobic methane oxidation rates were elevated below the lower sulfate-methane transition zone indicating a stimulation of sulfate reduction (SR) and AOM by the diffusion of sulfate from the oceanic crust into the methane-containing zone (Figure 5c).

SR rates near the surface followed the sulfate profile and were in the same range of previously analyzed sites (Parkes et al., 2000; Treude et al., 2005). Rates decreased from 8 nmol cm^{-3} d^{-1} at 1.3 mbsf by a factor of 450 in the upper 10 meters and dropped strongly to the detection limit of about 0.2-0.5 pmol cm^{-3} d^{-1} below. SR rates increased by one order of magnitude at the lower sulfate-methane transition zone and remained at this level towards the basement.

Potential AOM rates indicate the presence of an indigenous microbial community, capable of anaerobic methane oxidation and therefore stimulated by incubation with methane and sulfate. In our study AOM rates were low compared to methane-rich sites investigated by Treude et al. (2005), but yet measurable throughout the sediment column. All measured values exceeded the mean standard deviation of controls by a factor of 3, indicating biological AOM. While 2.5 pmol methane cm^{-3} d^{-1} were oxidized anaerobically in the upper 120 meters, an increase was observed below the lower sulfate-methane transition zone. Here a maximum turnover rate of 4.5 pmol cm^{-3} d^{-1} was determined at 162.6 mbsf. In deeper layers, AOM rates were lower than 1 pmol cm^{-3} d^{-1}.

Table 1: Cultivation efficiency in %, estimated from anoxic and oxic MPN viable counts in correlation to SybrGreenI total cell counts (cm^{-3} sediment) in sediment layers of IODP Site U1301, holes C and D.

Sample origin						Anoxic MPN series		Oxic MPN series	
Site	Core	Section	Depth (mbsf)	Temperature (°C)	Total cell counts (cm^{-3} sediment)	MPN counts (cm^{-3} sediment)	Cultivation efficiency (%)	MPN counts (cm^{-3} sediment)	Cultivation efficiency (%)
U1301C	1	1	1.3	2.2	$5.2 \cdot 10^8 \pm 3.1 \cdot 10^6$	n.a.	n.a.	n.a.	n.a.
	2	3	9.1	4.0	$6.8 \cdot 10^7 \pm 1.0 \cdot 10^6$	40 (5–200)	<1%	n.a.	n.a.
	4	5	31.1	9.0	$3.5 \cdot 10^8 \pm 1.1 \cdot 10^7$	$1.1 \cdot 10^6 (1.5–48 \cdot 10^5)$	0.311	$1.1 \cdot 10^6 (1.5–48 \cdot 10^5)$	0.311
	6	6	52.1	13.7	$2.7 \cdot 10^8 \pm 5.4 \cdot 10^6$	$7.5 \cdot 10^4$ ($1.4–23 \cdot 10^4$)	0.027	$2.8 \cdot 10^4$ ($1.0–15 \cdot 10^4$)	0.010
	9	2	74.6	18.9	$1.6 \cdot 10^8 \pm 2.7 \cdot 10^6$	210(40–470)	<1%	n.a.	n.a.
	12	1	99.4	24.5	$1.9 \cdot 10^8 \pm 3.3 \cdot 10^6$	n.a.	n.a.	$1.1 \cdot 10^6 (1.5–48 \cdot 10^5)$	0.571
	13	2	111.9	27.3	$4.0 \cdot 10^7 \pm 1.1 \cdot 10^6$	90(10–360)	<1%	$1.1 \cdot 10^6 (1.5–48 \cdot 10^5)$	2.757
U1301D	1	2	121.9	29.6	$7.9 \cdot 10^7 \pm 1.6 \cdot 10^6$	n.a.	n.a.	$9.3 \cdot 10^2 (1.5–38 \cdot 10^2)$	0.001
	2	3	132.4	32.0	$4.1 \cdot 10^7 \pm 1.1 \cdot 10^6$	g.n.d.	0	$9.3 \cdot 10^2 (1.5–38 \cdot 10^2)$	0.002
	3	2	140.9	34.0	$3.1 \cdot 10^7 \pm 6.3 \cdot 10^5$	$1.1 \cdot 10^6 (1.5–48 \cdot 10^5)$	3.569	$9.3 \cdot 10^2 (1.5–38 \cdot 10^2)$	0.003
	4	2	150.1	36.1	$3.9 \cdot 10^7 \pm 1.1 \cdot 10^6$	$1.5 \cdot 10^5$ ($3.0–44 \cdot 10^4$)	0.388	$1.1 \cdot 10^4 (1.5–48 \cdot 10^3)$	0.028
	5	4	162.6	38.9	$1.0 \cdot 10^8 \pm 2.3 \cdot 10^6$	$4.6 \cdot 10^3$ ($0.7–24 \cdot 10^3$)	0.004	150(30–440)	<1%
	6	2	169.0	40.4	$6.8 \cdot 10^7 \pm 6.6 \cdot 10^5$	110(30–360)	<1%	$1.5 \cdot 10^3$ ($3.0–44 \cdot 10^2$)	0.002
U1301C	15	5	185.0	44.0	$2.6 \cdot 10^7 \pm 9.7 \cdot 10^5$	150(30–440)	<1%	n.a.	n.a.
	17	4	239.5	56.4	$1.7 \cdot 10^7 \pm 7.2 \cdot 10^5$	$4.6 \cdot 10^5$ ($0.7–24 \cdot 10^5$)	2.682	g.n.d.	0
	18	4	251.9	59.2	$8.0 \cdot 10^7 \pm 2.4 \cdot 10^6$	$1.1 \cdot 10^6 (1.5–48 \cdot 10^5)$	1.376	g.n.d.	0
	19	4	260.4	61.2	$2.9 \cdot 10^7 \pm 1.2 \cdot 10^6$	$2.1 \cdot 10^5$ ($3.5–47 \cdot 10^4$)	0.713	g.n.d.	

Abbreviations: mbsf, meters below seafloor; n.a., not analysable; g.n.d., growth not detected 95% confidence levels of MPN counts are given in brackets.

Potential exoenzyme activities

Exoenzyme turnover rates measured after applying an excess of substrates are an indicator of potential microbial activity (Coolen & Overmann, 2000). In general, the first step in utilizing organic matter is the extracellular hydrolysis of polymeric compounds. The production of exoenzymes is a fundamental survival strategy of prokaryotes in substrate limiting environments (Boetius & Lochte, 1996). As the deeper layers of IODP Site U1301 are characterized by low phosphate concentrations, we have chosen phosphatase activity measurements as a general measure for microbial activities. At the investigated site, a dramatic increase in potential phosphatase rates was observed in the lower sediment column (Figure 5d). The enzymatic conversion of the phosphate analogue MUF-P increased 7-fold from an average of 0.56 mmol cm^{-3} d^{-1} in layers between 52.1 mbsf and 162.6 mbsf within only 20 meters and reached its maximum directly above the basement at 260.4 mbsf of 4.39 mmol MUF-P cm^{-3} d^{-1}.

No activities of glycosidase or aminopeptidase were measured in any sample after 72 hrs of incubation. Hence, the measured phosphatase activity increase might merely reflect phosphate deficiency in the lower 100 meters of sediment (Figure 5d). Relative to available phosphate, the lowest phosphatase activities were found in the phosphate-rich upper fifty meters of the sediment column (average: 0.46 mmol MUF-P cm^{-3} d^{-1}). Rates increased slightly in the phosphate-poor layers below, and were highest in the phosphate-depletion zone near the basement.

2.5 Discussion

Our study supports the hypothesis that crustal fluids can foster microbial life in deep subsurface sediments. Both, microbial cell numbers and metabolic activity were enhanced around the lower sulfate-methane transition zone and near the basement.

2.5.1 Site characteristics and sample quality

IODP expedition 301 offered an opportunity to study possible stimulating effects of crustal fluid-derived compounds on microbial communities in deeply buried sediments. The predicted model of electron acceptor diffusion from the oceanic crust into overlying sediments (DeLong, 2004) is displayed in the geochemical profile of IODP Site U1301. Our results support the find-

ings of Parkes et al. (2005) who investigated a geochemically comparable site on the Peru margin (ODP Site 1229), and found a similar increase in microbial biomass and activity. Furthermore, semi-quantitative cultivation methods applied in our study indicate that some of these microorganisms can be stimulated to grow *ex situ*, giving hope to successful isolations and hence characterizations of indigenous strains in the future.

We are confident that we studied activities of indigenous microorganisms, not contaminants. Samples were recovered by the least invasive technique, advanced piston coring (Graber et al., 2002). Contamination tests performed all the way down to the basement indicated minimal if any microbial contamination during the drilling procedure (Lever et al., 2006).

2.5.2 Diffusion of electron acceptors into electron donor-poor sediments

Our study supports the hypothesis (D'Hondt et al., 2004) that diffusion of electron acceptors from crustal fluids into electron acceptor-depleted sediment layers stimulates microbial activity far below the seafloor.

The "lower" sulfate concentration profile has a different shape from the "upper" sulfate profile. The virtually straight slope of the "lower" sulfate gradient indicates that only diminished sulfate reduction occurs between the basement and the methanogenic zone. Basement-derived sulfate is almost entirely consumed in the lower sulfate-methane transition zone. This suggests that AOM is one predominant sink for sulfate due to availability of methane as an electron donor (Boetius et al., 2000; Borowski et al., 2000; Treude et al., 2003). The near-absence of sulfate reduction in deeper sediment horizons with no methane is probably due to low availability or quality of organic matter. Even though organic carbon is present (TOC:~0.4 wt %; mean DOC: 0.8 mM), TOC may be unavailable, e.g., partly adsorbed to mineral surfaces (Keil et al., 1994) and the DOC may be recalcitrant, e.g., of kerogenic origin (Wakeham & Canuel, 2006). Fluid-derived organic carbon with an average of 11 µM DOC (Lang et al., 2006) might also be highly degraded after long-term circulation in the basaltic aquifer, and hence be of little use to microbes.

The "concave-up" shape of the upper sulfate profile indicates sulfate reduction throughout the upper 50 m of sediment, until sulfate is depleted. This was supported by high SR rates measured in the upper tens of meters. Here, the amount of organic carbon is slightly higher (TOC: 0.2 to 0.9 wt %;

mean DOC: 13 mM), and, perhaps more importantly, organic carbon might be less degraded than in deeper layers. It has only been altered in the water column and in geologically "young" surface sediments.

2.5.3 Activity measurements on subseafloor biosphere samples

So far, the number of metabolic activity measurements on the deep biosphere is limited to few sampling sites. Coolen & Overmann (2000), for example, have determined exoenzyme activities in up to 124,000-year-old subsurface Mediterranean sapropels. Sulfate reduction rates were determined on a few more subseafloor biosphere sites sampled during ODP Legs 112, 128, 146 and 164 (Parkes et al., 2000), whereas AOM rates have mostly been measured in shelf sediments (e. g. Bussmann et al., 1999; Iversen & Blackburn, 1981) or highly active sites such as methane seeps and gas hydrate locations (Joye et al., 2004; Michaelis et al., 2002; Treude et al., 2003). Many estimates of sulfate and methane turnover rates in deep-sea sediments have been made by modeling of geochemical profiles only (e. g. Borowski et al., 2000; D'Hondt et al., 2004; Hinrichs & Boetius, 2003; Jørgensen et al., 2001). Despite the overall low methane production at IODP Site U1301, we were able to measure AOM rates throughout the sediment column. And, we were able to demonstrate experimentally that sulfate derived from crustal fluids fosters microbial metabolism in deep subseafloor sediments.

2.5.4 Distribution of prokaryotes along the sediment column

The question whether *Bacteria* or *Archaea* are dominant in the deep biosphere is still open. The investigation of sediment samples from ODP Leg 201 provided controversial results. While Schippers et al. (2005) determined bacterial dominance via fluorescent probes (CARD-FISH) and qPCR, Biddle et al. (2006) showed the opposite by intact phospholipid analyses of the same sample set. In our investigations, the abundance of *Bacteria* was elevated at the sediment surface and below both sulfate-methane transition zones but we did not find a general dominance of one domain over the other. Surprisingly, archaeal numbers did not even show a peak in the methane-rich horizon as expected from qPCR quantifications on other subsurface sediments using the same experimental approach (Wilms et al., 2007). However, the chosen PCR-primers might not target methanogenic *Archaea* within the particular sediments of IODP Site U1301. Another explanation is that many *Archaea* are not involved in methanogenesis or AOM. An increasing abundance

would not be detected if methanogens only accounted for a small portion of the archaeal community. Low yields of DNA during the extraction procedure, as found by Newberry et al. (2004), are a further possible explanation, and could be caused by a high resistance of cells to enzymatic, chemical and mechanical disruption or the adsorption of DNA to sediment particles. However, the relative values should reflect a general trend in prokaryotic distribution.

2.5.5 Facultative aerobes within the deep biosphere

The application of the MPN technique or "dilution to extinction method" was not only used to obtain enrichments of indigenous microorganisms for further isolation procedures. The cultivation efficiency as calculated in correlation to SGI total cell counts is also an indirect measure for the fraction of the microbial communities that can be stimulated to grow by supply of electron donors and acceptors.

Due to the high redox-potential in the oxidized but oxygen-free zone directly above the oceanic crust, we expected this to be a favorable habitat for facultative aerobes. Surprisingly, no microorganisms were enriched from these layers in aerobically incubated MPN series. In aerobic enrichments of near-surface sediments with a similar redox-potential, we stimulated growth of large numbers of aerobes. This could indicate the differences in the effect of crustal-fluid and bottom-seawater chemical composition on microbial communities. Furthermore, microorganisms from the deepest layers have been buried over a longer time period and might have lost their adaptation to oxygenated conditions. However, the presence of facultative aerobes within certain horizons is consistent with previous cultivation studies on deep-subsurface sediments from the Peru margin (Batzke et al., 2007) and eastern Mediterranean sapropels (Süß et al., 2004). In both investigations, the vast majority of strains isolated under anoxic conditions turned out to be facultative aerobes. One of the most frequently isolated strains, *Rhizobium radiobacter*, is a generalist capable of a wide range of metabolisms, including aerobic respiration and fermentation (Süß et al., 2006), which might be characteristic of subseafloor biosphere microorganisms.

2.5.6 Impact of fluids on deeply buried sediments

The volume of water passing through ridge flanks is probably much greater than in seafloor spreading centers (Fisher et al., 2005). Sediment thickness systematically grows with crustal age and causes an increase in fluid residence-time, when recharging and discharging sites are buried (Johnson & Pruis, 2003). Due to the progressive fluid alteration, the diffusion of potential electron acceptors into the overlying parts of the sediments is probably much lower in such seafloor spreading centers.

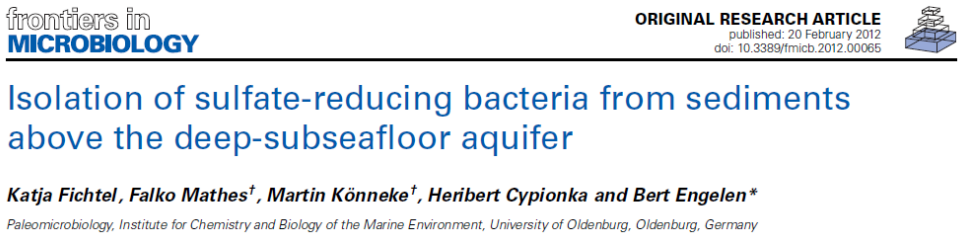
Within the young on-axis crust, the input of magmatic volatiles, e.g., H_2 and CO_2, supports chemolithoautotrophic microbial activity (Holland & Baross, 2003). In comparison, fluids of the older crust, such as the eastern flank of the Juan de Fuca ridge, are depleted in those compounds and instead rich in electron acceptors; hence, the presence of heterotrophic microorganisms in the crust (Cowen et al., 2003; Huber et al., 2006; Nakagawa et al., 2006). In our study, we showed that crustal fluid-derived electron acceptors stimulate microbial activity in overlying sediments. The supply of sulfate from crustal fluids is high enough to cause deep sediments to be electron donor rather than electron acceptor-limited. The response of microbial communities to crustal fluid-derived compounds is hence more complex than previously thought. Nonetheless, our results, put into context with the worldwide range of the oceanic crustal aquifer, demonstrate the potential for chemical exchanges between oceanic crust and overlying sediments to support deep subsurface microbial populations on a global scale.

2.6 Acknowledgements

We thank the crew, the shipboard scientific party of IODP Exp. 301 and the chief scientists for valuable support during the cruise. Special thanks go to Helge Niemann for his help with the AOM data analysis. This research used samples and data provided by the Integrated Ocean Drilling Program (IODP). Funding for this research was provided by a grant of the German Research Foundation (DFG).

3 Isolation of sulfate-reducing bacteria from sediments above the deep-subseafloor aquifer

Dieses Kapitel wurde veröffentlicht: Fichtel, K., Mathes, F., Könneke, M., Cypionka, H., Engelen, B.*, 2012. Isolation of sulfate-reducing bacteria from sediments above the deep-subseafloor aquifer. *Front. Microbiol.* 3, Artikel 65. doi: 10.3389/fmicb.2012.00065. Das Manuskript wurde am 15. November 2011 für die Veröffentlichung in der Spezialausgabe *Deep Subsurface Microbiology* bei *Frontiers in Extreme Microbiology* eingereicht, am 06. Februar 2012 akzeptiert und am 20. Februar 2012 online publiziert.



Keywords: *Desulfovibrio*, *Desulfotignum*, diversity, deep biosphere, Juan de Fuca Ridge, hydrogen, chemolithoautotrophy, IODP

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3.1 Abstract

On a global scale, crustal fluids fuel a large part of the deep-subseafloor biosphere by providing electron acceptors for microbial respiration. In this study, we examined bacterial cultures from sediments of the Juan de Fuca Ridge, Northeast Pacific (IODP Site U1301). The sediments comprise three distinctive compartments: An upper sulfate-containing zone, formed by bottom-seawater diffusion, a sulfate-depleted zone, and a second (~140 m thick) sulfate-containing zone influenced by fluid diffusion from the basaltic aquifer. In order to identify and characterize sulfate-reducing bacteria, enrichment cultures from different sediment layers were set up, analyzed by molecular screening, and used for isolating pure cultures. The initial enrichments harbored specific communities of heterotrophic microorganisms. Strains affiliated to *Desulfosporosinus lacus*, *Desulfotomaculum sp.*, and *Desulfovibrio aespoeensis* were isolated only from the top layers (1.3-9.1 meters below seafloor, mbsf), while several strains of *Desulfovibrio indonesiensis* and a relative of *Desulfotignum balticum* were obtained from near-basement sediments (240–262 mbsf). Physiological tests on three selected strains affiliated to *Dv. aespoeensis*, *Dv. indonesiensis*, and *Desulfotignum balticum* indicated that all reduce sulfate with a limited number of short-chain *n*-alcohols or fatty acids and were able to ferment either ethanol, pyruvate, or betaine. All three isolates shared the capacity of growing chemolithotrophically with H_2 as sole electron donor. Strain P23, affiliating with *Dv. indonesiensis*, even grew autotrophically in the absence of any organic compounds. Thus, H_2 might be an essential electron donor in the deep subseafloor where the availability of organic substrates is limited. The isolation of non-sporeforming sulfate reducers from fluid-influenced layers indicates that they have survived the long-term burial as active populations even after the separation from the seafloor hundreds of meters above.

3.2 Introduction

The subseafloor biosphere is probably the largest reservoir for prokaryotic life on Earth (Whitman et al., 1998; Heberling et al., 2010). It extends several hundred meters into deeply buried sediments (Parkes et al., 1994; Roussel et al., 2008) and even further down into the upper layers of the oceanic crust (Thorseth et al., 1995; Furnes & Staudigel, 1999; Ehrhardt et al., 2007). Recently, it was estimated that the ocean crust contains a

similar amount of microorganisms as the entire volume of the world's oceans (Heberling et al., 2010). The continuous circulation of seawater within the upper crust turns these voluminous, porous, and permeable basalts into the largest globally connected aquifer (Johnson & Pruis, 2003; Johnson et al., 2006).

Intense fluid circulation is a consequence of specific geological settings evolved during crust formation at ocean-spreading centers. It is especially pronounced at ocean ridges such as the Juan de Fuca Ridge in the Northeast Pacific (Johnson et al., 2006). This area is one of the most intensively studied locations in terms of heat-driven fluid flow (Fisher et al., 2003; Hutnak et al., 2006). While cold bottom-seawater is recharged at seamounts, it warms up within the oceanic crust beneath the sediments before being discharged again at other rocky outcrops exposed at the seafloor. The chemical composition of these low-temperature hydrothermal fluids ($<$150 °C, Cowen, 2004) is altered during long-term circulation through the basalt due to continuous abiotic water-rock interaction (Edwards et al., 2003) especially with increasing basement temperature (Wheat & Mottl, 1994; Wheat et al., 2000), or as a response to volcanic eruption (Butterfield et al., 1997). Additionally, microbial activity of crust-hosted communities contributes to changes in fluid composition by removing seawater constituents such as sulfate as indicated by sulfur-isotope measurements (Rouxel et al., 2008). However, due to a limitation in electron donors, crustal fluids are not fully reduced and still contain suitable electron acceptors, such as sulfate, for anaerobic respiration (Wheat & Mottl, 1994; Wheat et al., 2000; Cowen et al., 2003; Edwards et al., 2005).

It was postulated that basement fluids not only supply electron donors and acceptors to microbial life within the crust, but also to the microbial communities in the overlying sediments by diffusion from below (Cowen et al., 2003; DeLong, 2004; D'Hondt et al., 2004). We tested this hypothesis during an expedition to the eastern flank of the Juan de Fuca Ridge (IODP Exp. 301) by analyzing a 265-m-long sediment column of IODP site U1301. Sampling included material taken only two meters above the sediment-basement interface (Expedition 301 Scientists, 2005b). At this site, sulfate diffuses into the sediments from both the seafloor (~27 mM) and the underlying basement (~16 mM). As a precondition for a sound microbiological and geochemical analysis, contamination controls were performed

directly onboard the drillship JOIDES Resolution and proved the pristine character of the sediment samples (Lever et al., 2006).

Our previous work has shown that fluids from the oceanic crust do support microbial life in the overlying sediments (Engelen et al., 2008). Exoenzyme activities and sulfate reduction rates were not only elevated near the seafloor but also at the bottom of the sediment column, which correlated well with the overall geochemical settings. We detected enhanced microbial abundance in sediment layers above the basement by direct counting and the cultivation-based most probable number (MPN) technique. Microbial growth in anoxic MPN dilution series from sediment layers near the oceanic crust indicated considerable amounts of viable microbial populations. Thus, the detection of a deep sulfate reduction zone and the successful enrichment of anaerobic microorganisms was the motivation for isolating sulfate-reducing bacteria (SRB) especially from fluid-influenced sediment layers. Identifying defined physiological adaptations of indigenous microorganisms to environmental conditions can be achieved best when pure cultures are available.

Even though sulfate reduction is supposed to be an important process in deeply buried sediments, only few isolates are available in strain collections. The type strain of *Desulfovibrio profundus* was isolated from 500 m depth in sediments of the Japan Sea (Parkes et al., 1995; Bale et al., 1997). Other piezophilic isolates closely related to *Dv. profundus* were cultivated from 222 m deep sediments of the Cascadia margin of the Pacific Ocean (Barnes et al., 1998). However, cultivation-based studies on the marine deep biosphere are still limited to a few sampling sites representing pinpricks in the ocean floor. So far, isolates from the marine subsurface were obtained from sediment samples retrieved from Mediterranean sediments (Süß et al., 2004) and from various sites in the Pacific Ocean: The Sea of Okhotsk, north of Japan (Inagaki et al., 2003), the Nankai Trough south-east of Japan (Mikucki et al., 2003; Toffin et al., 2004a,b, 2005; Kendall et al., 2006), the Equatorial Pacific, and the Peru Margin (D'Hondt et al., 2004; Biddle et al., 2005; Lee et al., 2005; Batzke et al., 2007). Recently, several heterotrophic bacteria and methanogenic *Archaea* were isolated from up to 106 mbsf deep sediments off Shimokita Peninsula, Japan using a continuous-flow bioreactor (Imachi et al., 2011).

In this study, we extended our previous investigations on IODP Site U1301 to determine the microbial diversity within different sediment layers

of the deep subsurface. We hypothesize, that zones with different sulfate concentrations harbor different populations of SRB due to varying substrate availabilities. A cultivation-based approach in combination with molecular screening tools was chosen to isolate and compare SRB from fluid-influenced sediments and near-surface layers. The metabolic properties of the isolates might provide new insights on the impact of crustal fluids on microbial metabolism in the deep-subseafloor biosphere where substrates are recalcitrant but electron acceptors are still available.

3.3 Material and Methods

3.3.1 Sample material

Sediment samples were recovered from the eastern flank of the Juan de Fuca Ridge by the drill ship "JOIDES Resolution" during IODP Expedition 301 in 2004. Characteristics of IODP Site U1301 were described in the expedition report (Expedition 301 Scientists, 2005b). Sediment sampling, contamination tests, and subsampling for further analyses were described in detail by Engelen et al. (2008). All samples proved to be free of contamination as previously described by Lever et al. (2006).

3.3.2 Initial enrichments of deep-biosphere bacteria

To elucidate the diversity of cultured bacteria, a total of 736 initial enrichment cultures were set up directly onboard. Sediment slurries from 17 representative depth intervals (Engelen et al., 2008) were prepared immediately after sample recovery with anoxic artificial seawater medium (Süß et al., 2004). MPN series for anoxic and oxic microorganisms from these slurries were performed in 10-fold steps within 96-deep-well microtiter plates as previously described (Engelen et al., 2008). In addition, liquid dilution series in 20 ml-glass tubes were inoculated, flushed with N_2 and sealed with butyl rubber stoppers. Anoxic substrate gradient tubes were prepared with undisturbed 1-cm^3-sediment subcores from hole U1301C, only by embedding them within agar-solidified artificial seawater media (Köpke et al., 2005). In general, a mixture of the following substrates were supplied to stimulate microbial growth: Glycerol, glucose, lactate, fumarate, malate, succinate, methanol, ethanol, 1-propanol, 1-butanol, formate, acetate, propionate, butyrate, valerate, caproate, and all the 20 L-amino acids (final concentration

of each compound: 0.1 mM). For a better comparison of all enrichments, incubation was performed at 20 °C.

Anoxic and oxic MPN viable counts were determined after 14 weeks of incubation to quantify the cultured part of the microbial communities within the sampled sediment layers. Procedure and results have already been published by Engelen et al. (2008). For the present cultivation study, all dilution cultures showing growth were transferred into 20 ml-glass tubes containing freshly prepared media and further incubated for at least five months at 20 °C. Since cell densities were generally low, growth was determined several times during incubation by epifluorescence microscopy using Sybr®GreenI as a fluorescent dye. Growth of sulfate reducers was monitored by measuring the formation of sulfide (Cord-Ruwisch, 1985). Gradient cultures were incubated for approximately one year without interruption. Stimulation of growth within the sediment subcore was analyzed by microscopy and molecular methods. Finally, a total of 116 positive cultures were analyzed by means of molecular biological methods as described below to identify the cultivated microorganisms and to select enrichments for further isolation processes.

3.3.3 Isolation of pure cultures

Pure cultures from SRB and other anaerobes were isolated and maintained in a slightly different artificial seawater media. One liter of this basal medium contained 24.32 g NaCl, 10.0 g $MgCl_2 \cdot 6\,H_2O$, 1.5 g $CaCl_2 \cdot 2\,H_2O$, 4.0 g Na_2SO_4, 0.66 g KCl, and 0.09 g KBr. Resazurin (1 mg/l) was added as redox-indicator. The media was autoclaved, cooled under a nitrogen atmosphere, and supplemented with the following sterile solutions: NH_4Cl (2 mM), KH_2PO_4 (1 mM), CO_2-saturated sodium bicarbonate (30 mM), and from sterile stocks: 1 ml/l of trace element solution SL10 (Widdel & Bak, 1992), 0.2 ml/l of selenite-tungsten solution (Widdel & Bak, 1992), and 2 ml/l of a solution of 10 vitamins (Balch et al., 1979). The anoxic medium was reduced by addition of Na_2S (final concentration:~1 mM) and few crystals of sodium dithionite. The pH was adjusted to 7.2-7.5 with 4 M NaOH. To increase cell density of all subcultures, a 10-fold higher concentrated substrate mix was provided (i.e., final concentration of each compound: 1 mM).

Repeated application of the deep-agar dilution method (Widdel & Bak, 1992) or dilution-to-extinction was performed to isolate deep-biosphere bacteria from liquid enrichments. Sediment subcores from gradient cultures

were homogenized and slurried with 4 ml anoxic artificial seawater to further establish subcultures as gradient dilution series (up to 10^{-6}). Aerobic microorganisms were subcultured for isolation by the liquid dilution-to-extinction method with subsequent purification on agar plates using a HEPES/bicarbonate-buffered oxic seawater medium. The purity of all isolates was checked by microscopy and molecular analysis as described below. Furthermore, the cultures were transferred to a complex HEPES-buffered oxic seawater medium containing yeast extract (0.03 g/l), glucose (1 mM), lactate (5 mM), and peptone (0.06 g/l) as substrates to check for contamination.

3.3.4 Molecular screening of enrichment cultures

The above described enrichment and isolation procedure was monitored and directed by molecular screening to identify unique phylotypes. Positive dilutions or growing colonies were analyzed by using polymerase chain reaction (PCR) of 16S rRNA gene-fragments, denaturing gradient gel electrophoresis (DGGE), and subsequent sequencing of re-amplified DGGE bands. DNA from liquid cultures was extracted using a protocol combining bead-beating with phenol/chloroform/isoamyl alcohol treatment and isopropanol/sodium acetate precipitation (Stevens et al., 2005). Nucleic acid extraction from substrate gradient cultures was performed by using the UltraClean™ Soil DNA Isolation Kit (MoBio Laboratories, Inc., Carlsbad, CA, USA) according to the manufacturers' instructions.

Polymerase chain reaction-amplification of bacterial 16S rRNA genes was conducted in 50-µl volumes containing the following components: 1.2 µl of DNA-template, 10 pmol of each primer, 0.2 mM of each dNTP, 0.5-2 µl of bovine serum albumin (BSA, 10 mg/ml), 5 µl of 10x-ThermoPol reaction buffer and 1U/µl of Taq Polymerase (New England Biolabs, Inc., Ipswich, MA, USA) and nuclease-free water. For DGGE analysis, the almost complete 16S rRNA genes were amplified with the primer set 8f/1492r (Overmann & Tuschak, 1997). The samples were incubated in a thermal cycler (Mastercycler, Eppendorf, Hamburg, Germany) under the following conditions: Initial denaturation at 95 °C for 5 min, 28 cycles of amplification by denaturation at 95 °C for 30 s, annealing at 40 °C for 60 s, and elongation at 72 °C for 3 min. Terminal elongation was performed at 72 °C for 10 min. The resulting amplicons were used as templates for a nested PCR. Shorter 16S rRNA gene-fragments were amplified (Wilms et al., 2006a) using

the universal bacterial primer set GC-341f and 907r (Overmann & Tuschak, 1997). All PCR products were always visualized by agarose-gel electrophoresis (Wilms et al., 2006a). DGGE was performed with a gradient from 40 to 70 % (Süß et al., 2004). PCR products were mixed with loading buffer before loading onto the gel (Wilms et al., 2006a).

3.3.5 Sequencing of DGGE bands and pure cultures

For sequence analysis of DGGE bands, distinctive bands were excised, eluted in 50 µl nuclease-free water, re-amplified in a 25-µl PCR (primers 341f/907r, Wilms et al., 2006b), and purified (Wilms et al., 2006a) using the QIAquick PCR purification Kit (Qiagen GmbH, Hilden, Germany) or the PCR-Purifying-Kit (SeqLab, Göttingen, Germany) and sequenced with a IRDyeTM800 labeled 907r-primer (Süß et al., 2004). For phylogenetic identification of pure cultures, genomic DNA was extracted from the isolated strains using a freeze and thaw procedure. From picked colonies or 2 ml of liquid cultures, 1 µl of a cell pellet was resuspended with 100 µl of filter-sterilized Tris-buffer (50 mM, pH 7.4). The suspension was frozen at −80 °C for 3 min and heated at 85 °C for 3 min. This procedure was repeated five times, and 2 µl of the final extract were added to 48 µl of PCR mixture. Partial or nearly full-length bacterial 16S rRNA gene sequences were amplified using the bacteria-specific primer set 341f/907r and 8f/1492r, respectively, and sequenced as described above. In case of sulfate-reducing strains, DNA was sequenced in both directions using the respective PCR primers and the service of GATC Biotech AG (Konstanz, Germany). Consensus sequences were constructed after alignment by using the BioEdit software tool version 7.0.9[1]. All 16S rRNA gene sequences obtained in this study were compared for their affiliation to the closest relatives using the BLASTN program 2.2.26+ (Altschul et al., 1990; Morgulis et al., 2008)[2]. The partial 16S rRNA gene sequences of all 40 isolates are deposited in GenBank database under the accession numbers JQ411257-JQ411296.

3.3.6 Physiological characterization of sulfate-reducing isolates

Physiological tests were generally performed in sealed glass tubes containing 10 ml of artificial seawater medium. Sulfidogenic growth was tested with 18 different substrates at final concentrations between 1 and 5 mM in the

[1] http://www.mbio.ncsu.edu/BioEdit/bioedit.html
[2] http://blast.ncbi.nlm.nih.gov/Blast.cgi

presence of sulfate. Fermentative growth with betaine, ethanol, malate, or pyruvate (2.5 mM, each) was tested in medium without additional electron acceptors. The cultures were incubated for at least 4 weeks at 20 °C in the dark. Chemolithotrophic growth with H_2 as electron donor was tested with a headspace (2/3 of the culture volume) filled with a mixture of H_2/CO_2 (80/20 v/v, 1 kPa). Those cultures were incubated horizontally at 20 °C. Growth was checked by visual inspection of turbidity, by phase contrast microscopy, and by sulfide formation (Cord-Ruwisch, 1985). Substrate utilization was defined to be positive after the third successful transfer into fresh media.

The capability of anaerobic respiration was tested in sulfate-free medium with ethanol or lactate (5 mM, each) as electron donor in combination with six different electron acceptors. Reduction of Fe(III) was indicated by the formation of black precipitates under the expense of the reddish ferric hydroxide. Mn(IV) utilization was shown by the disappearance of brown manganese carbonates and the occurrence of white precipitates. The production of sulfide as a result of the reduction of thiosulfate or sulfite was measured at 480nm using a Shimadzu UV-1202 photometer (Cord-Ruwisch, 1985). In addition, cultures were checked microscopically for the presence of bacterial cells.

Growth experiments for autotrophic growth were performed at 35 °C, the optimum temperature for growth of our test strain P23. Growth rates were calculated from linear regression of produced sulfide (Cord-Ruwisch, 1985) and formed cell protein (Bradford, 1976) as function of time.

The temperature range for growth of SRB was tested from 4 to 55 °C with lactate (10 mM) as electron donor. Growth was followed at OD_{436} via sulfide production and by photometrical determination of protein concentrations (Bradford, 1976).

For phase contrast microscopy, agarose-coated slides were used. To prepare those, slides were thoroughly cleaned and preheated by infrared light in order to get a smooth agarose film. Then, 1 ml of the hot agarose solution (2 % w/v) was dispensed on the warm slides. Before usage, the agarose slides were air dried. Upon placing a drop of a bacterial culture to a coated slide, the liquid diffuses into the dry agarose, while the cells are gently squeezed under the cover slip and get fixed in the same plane. Cell dimensions were determined using a Leitz DMRB microscope (Wetzlar, Germany).

Transmission-electron microscopy was performed as follows: A 400-mesh Formvar copper grid (Plano) was placed on a drop of cell suspension for

10 min. Cells adsorbed to the grid were stained with 0.5 % aqueous uranyl acetate for 1 min, washed twice in a drop of water for a few seconds and examined with a transmissionelectron microscope (EM 902A, Zeiss). A Proscan High Speed SSCCD camera system with iTEMfive software was used for images acquisition.

3.4 Results

3.4.1 Geochemical profiles divide the sediment column into three distinctive zones

The geomorphological structure of the eastern flank of the Juan de Fuca Ridge leads to a hydrological situation where sulfate-containing fluids from the oceanic crust diffuse ~140 m into overlying sediment layers. The effect of this heat-driven fluid circulation was reflected by the temperature gradient within the sediments of 2 °C at the seafloor to approximately 62 °C above the basement (Expedition 301 Scientists, 2005b). Using the porewater profile of sulfate, the sediment column can be separated into three zones (Figure 6). The upper sulfate-containing zone was formed by bottom-seawater diffusion showing decreasing concentrations from 27 mM at the top to 3 mM in 35 mbsf. Below, a sulfate-depleted zone was located between 47 and 121 mbsf (<1 mM). The lower sulfate-containing zone was characterized by increasing sulfate concentrations from 2 to 16 mM toward the basement at ~265 mbsf due to sulfate diffusion from crustal fluid flow into the overlying sediments.

3.4.2 Shifts in microbial diversity between the initial enrichment cultures from the different zones

Anoxic and oxic MPN series, liquid dilution series in tubes and the substrate gradient technique were used to enrich and further isolate deep-biosphere bacteria. The cultivation progress was monitored by microscopy and PCR-DGGE. Unique DGGE bands were subsequently sequenced to identify the community composition within the enrichments. A total of 135 partial 16S rRNA gene sequences were obtained after DGGE analysis of growing cultures. The technique was not only chosen to prevent multiple isolation of one strain and to check the purity of cultures, but also to identify community members that could not be isolated.

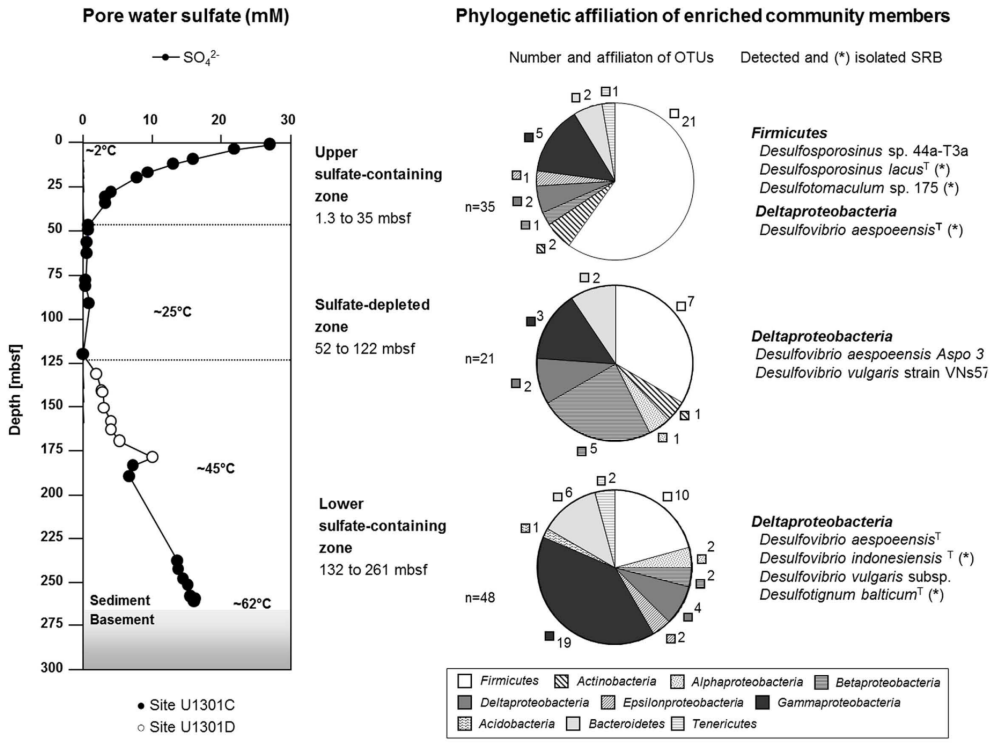

Fig. 6: Zonation of the 265-m-long sediment column of the eastern flank of the Juan de Fuca Ridge, Northeast Pacific (IODP Site U1301), and phylogenetic affiliation of enriched and isolated marine subsurface bacteria with special emphasis on sulfate-reducing bacteria. Operational taxonomic units (OTUs) detected via PCR-DGGE are defined at 97 % sequence similarity.

This molecular-directed cultivation indicated the presence of diverse viable microbial populations within the different zones of the investigated sediment column.

The phylogenetic screening of the initial enrichments identified different bacterial populations among the growing cultures obtained from the three sediment zones (Figure 6). A typical decrease in cultivation success with respect to the conditions set in our growth media was observed for the two upper zones, which correlates with the general depletion of electron donors and acceptors. Within the top 30 m of the sediment column, 35 different operational taxonomic units (OTUs, defined at 97 % sequence similarity) were detected via PCR-DGGE in enrichments from the respective sediment layers. From the sulfate-depleted zone, 21 OTUs were retrieved. For samples from the deep, fluid-influenced sediment zone, the cultivation success increased again with 48 identified OTUs.

In general, the number of OTUs belonging to the *Firmicutes* decreased with sediment depth from 60 % in enrichments from the upper sulfate-containing zone to 21 % in the lower sulfate zone. In addition, *Gammapro-*

teobacteria accounted for 40 % of all OTUs retrieved from enrichments of the lower sulfate zone. Bacteria belonging to the phylum *Acidobacteria*, *Bacteroidetes*, and the classes *Beta-* and *Epsilonproteobacteria* were enriched as identified by molecular methods but could not be isolated or were lost during purification procedures. The majority of the enriched but not isolated organisms were phylogenetically affiliated to uncultured bacteria from different terrestrial and marine environments (data not shown in detail).

3.4.3 Diversity of isolated pure cultures

From the 116 initial enrichments that were tested positively for growth, 40 strains could be isolated (14 from the upper sulfate-containing zone, 8 from the sulfate-depleted zone, and 18 from the lower sulfate-containing zone). Based on 16S rRNA gene sequences, the 40 pure cultures could be affiliated to the phyla *Actinobacteria*, *Firmicutes*, and *Tenericutes* or the classes *Alphaproteobacteria*, *Gammaproteobacteria*, *Deltaproteobacteria* (Table 2). The majority of isolates (32 of 40) were obtained from liquid dilution series that were initially inoculated with hundred to million fold diluted sediment (10^{-2} to 10^{-6}), indicating a significant number of cells *in situ*. Nearly all isolates were closely related to cultivated species from sediments or soils, fluids, or other aquatic environments. Among them, 13 were strict anaerobes. With exception of the sporeforming *Firmicutes* all other pure cultures including those obtained from oxic media were considered to be facultatively anaerobic, since they originated from anoxic sediment horizons. While some isolates seem to be ubiquitous within the sediment column (e.g., *Shewanella frigidimarina*, 98-99 % sequence similarity or *Bacillus* spp., 96-100 %), others were retrieved from single sediment layers, only (e.g., *Anaerovirgula multivorans* or *Marinobacter flavimaris*, both 99 % sequence similarity).

3.4.4 Sulfate-reducing bacteria were isolated from both sulfate-containing zones

The sulfate reducers isolated from the upper 10 m predominantly belonged to the *Firmicutes* (Figures 6 and 7; Table 2). Three strains were identified as members of the genera *Desulfotomaculum* and *Desulfosporosinus*. The latter shared 97 % sequence similarity with its closest described relative *Desulfosporosinus lacus*, firstly isolated from freshwater lake sediments (Ramamoorthy et al., 2006).

Table 2: Origin and phylogenetic affiliation of isolated strains from IODP Site U1301, a 265 m-long sediment column of the eastern flank of the Juan de Fuca Ridge, Northeast Pacific.

Phylogenetic group, closest relative* in GenBank (accession no.)	Similarity (%)	Sediment depth (mbsf)	No. of isolates	Habitat of closest relatives***
ACTINOBACTERIA				
Bacterium Ellin5115 (AY234532)	99	112	1	Soil, Australia
[*Actinomycetospora chibensis*^{T} (AB514517)]	99			Paddy soil, Japan
Iron-reducing enrichment clone CI-A3 (DQ676995)	99	31	1**	Estuary sediment, Europe
[*Propionicimonas paludicola*^{T} (FR733712)]	99			Rice-field soil, Japan
FIRMICUTES				
Anaerovirgula multivorans^T (NR_041291)	99	1.3	1**	Owens Lake, USA
Bacillus siralis^T (NR_028709)	98, 96	112, 132	2	General study
Bacillus sp. AS7 HS-2008 (AM950301)	98, 99	99, 112	2	Brine Lake Sediment, Mediterranean
Bacillus sp. Hs56 (JF803865)	99	1.3, 169	2	Marine sponge, Bay in Ireland
Desulfosporosinus lacus^T (NR_042202)	97	1.3	1**	Sediments of Lake Stechlin, Germany
Desulfotomaculum sp. 175 (AF295656)	98, 99	1.3, 9.1	2**	Aquifer/lignite seam, Germany
Marinilactibacillus sp. A5 (DQ344853)	98, 99	75, 99	2**	Deep-sea sediment, Pacific
Paenibacillus sp. UXO5-11 (DQ522106)	99	31	1	Marine sediments, Hawaii
Bacillus circulans USC24 (HQ441221)	99	9.1	1	Fish tank sediment, Spain
Uncult. bacterium clone LCKS880B24 (EF201766)	98	9.1	1**	Lake Chaka, China
[*Desulfonispora thiosulfatigenes*^{T} (NR_026497)]	90			Sewage plant, Germany
ALPHAPROTEOBACTERIA				
Pelagibacterium halotolerans^T (EU709017)	99	132	1	Ocean water, China
GAMMAPROTEOBACTERIA				
Alteromonas sp. USC168 (HQ441215)	99	31, 52	2	Mediterranean surface water, Spain
Halomonas sp. 1B.4 (HQ427421)	99	141	1	Ocean crust, JdF Ridge. Pacific
Halomonas axialensis^T (NR_027219)	100	163	1	Hydrothermal fluid, JdF Ridge, Pacific
Halomonas sp. PEB09 (GU213166)	99	163	1	Estuarine microbial mat, Spain
Marinobacter flavimaris^T (NR_025799)	99	141	1	Sea water, Yellow Sea in Korea
Pseudoalteromonas sp. D20 (AY582936)	99	150	1	Deep-sea sediment, Pacific
Pseudomonas sp. G12a-1 (FN397994)	99	141, 150	2	Deep-sea sediment, Indian Ocean
Shewanella frigidimarina ACAM 584 (U85902)	98–99	1.3, 31, 52, 260	4	Southern Ocean waters
Vibrio diazotrophicus^T (NR_026123)	98	150	1	General study
Vibrio pelagius^T (X74722)	98	31, 150	2	General study
DELTAPROTEOBACTERIA				
Desulfotignum balticum^T (NR_041852)	99	260	1**	Marine mud, Denmark
Desulfovibrio aespoeensis^T (NR_029307)	98	1.3	1**	Aespoe hard rock borehole, Sweden
Desulfovibrio indonesiensis^T (NR_044916)	99	240, 252, 260	3**	Corroding ship, Indonesia
TENERICUTES				
Anaerobic bacterium MO-XQ (AB598274)	99	260	1	Subseafloor sediments, Japan
[*Acholeplasma palmae*^{T} (NR_029152)]	93			Plant surface

*In case of environmental clones the next cultivated organism is indicated in square brackets.

**Strictly anaerobic isolates.

***Based upon the results of the megaBLAST search (NCBI).

The *Desulfotomaculum* strains were phylogenetically related to isolates originally obtained from a terrestrial aquifer system (Detmers et al., 2001). Another isolate from a near-surface layer (strain P20) was closely affiliated to *Desulfovibrio aespoeensis*. This *Deltaproteobacterium* was also enriched in co-culture with strains related to *Desulfovibrio indonesiensis* (culture P34 and P19) from 240 to 260 mbsf, respectively, as identified by DGGE and subsequent sequencing of the bands. Two sequences affiliated to sulfate reducers were also detected in enrichment cultures from sediments of the sulfate-depleted zone (Figure 6). However, no isolates could be retrieved. SRB isolated from the deepest sediments above the basement solely belonged to the *Deltaproteobacteria*, namely *Desulfotignum balticum* (strain P18; 260 mbsf)

and *Dv. indonesiensis* (strains P12, P19-1, P23, P33, and P34). The latter phylotype was highly abundant in the lower sulfate-containing zone as it was frequently retrieved from different fluid-influenced layers (240, 252, 260 mbsf). Furthermore, strains P18, P23, and P34 were isolated from million fold diluted MPN-cultures, allowing the assumption, that they must be present in higher numbers within the respective sediment layer, where they probably play an active role.

3.4.5 Morphological and physiological characteristics of three representative sulfate-reducing isolates

Strains affiliated to *Dv. aespoeensis* (strain P20), *Dv. indonesiensis* (strain P23), and *Desulfotignum balticum* (strain P18) were morphologically and physiologically investigated in more detail (Figure 7; Table 3). Strain P20 was used for further analysis since it was the only available pure culture related to *Dv. aespoeensis* that was obtained in this study. Other relatives of this species were enriched from near-basement layers, but only in co-culture with strains affiliated to *Dv. indonesiensis*. Various efforts to separate the two species failed. Strain P23, obtained from the deepest sediment layer (260 mbsf), was chosen as a representative for strains related to *Dv. indonesiensis*, since the other closely related isolates showed nearly identical characteristics under the growth conditions tested. For all investigated strains, colonies formed in deep-agar dilution series exhibited yellowish to brownish colors. The *Desulfovibrio* affiliated strains showed curved, motile cells (Figures 7A,C) with single polar flagella as identified by electron microscopy of negatively stained cells (Figures 7B,D). The relative of the non-motile *Desulfotignum balticum* formed ~2-3µm short thick rods with rounded ends (Figures 7E,F).

Desulfovibrio aespoeensis strain P20 grew within a temperature range of 20-35 °C with an optimum at 25 °C. *Desulfotignum* strain P18 and *Dv. indonesiensis* strain P23 instead exhibited growth within a broad temperature range from 4 to 48 °C and 10 to 48 °C, respectively, with the optimal growth temperature lying between 25 and 35 °C.

All strains were capable of using sulfite or thiosulfate as alternative electron acceptor other than sulfate. Slow growth by iron or manganese reduction on lactate was observed for *Desulfotignum balticum* strain P18 and *Dv. indonesiensis* strain P23. Growth was not as fast as with sulfate as electron acceptor and high cell densities were not achieved.

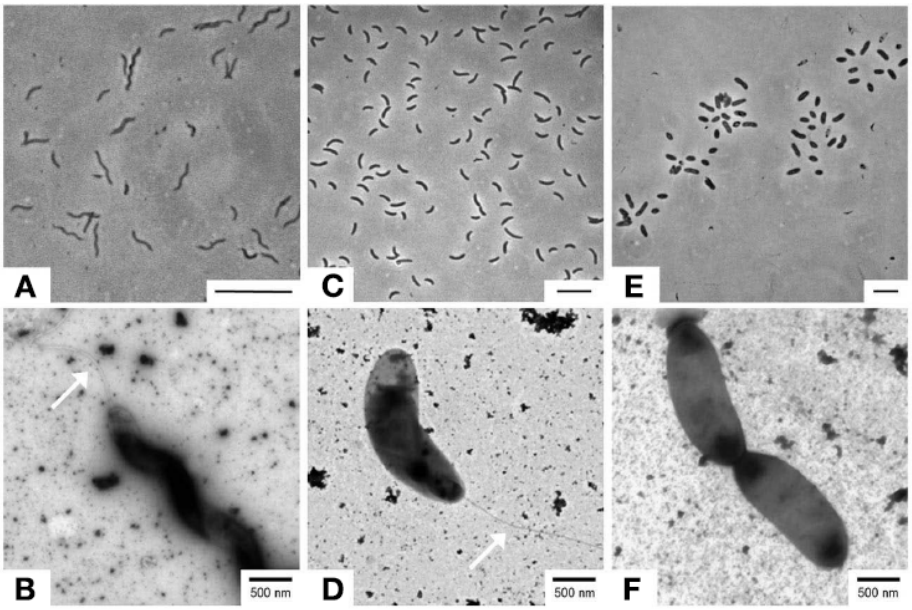

Fig. 7: Microscopic images from three sulfate-reducing isolates obtained from sediments of IODP Site U1301. (A,B) *Desulfovibrio aespoeensis* strain P20 (1.3 mbsf); (C,D) *Desulfovibrio indonesiensis* strain P23 (260.4 mbsf); (E,F) *Desulfotignum balticum* strain P18 (260.4 mbsf). Upper images: Phase contrast (bar=5 µm); Lower images: Transmission-electron microscopy, TEM (bar=500 nm). Arrows in (B,D) indicate flagella.

However, growth on metal oxides occurred even after the third transfer. None of the strains used nitrate as electron acceptor for anaerobic respiration.

Of all substrates provided, *Dv. aespoeensis* strain P20 only utilized lactate and formate for growth in the presence of sulfate. In contrast, the type strain of *Dv. aespoeensis* only grew on lactate as sole substrate (Motamedi & Pedersen, 1998). The two other strains tested (P18 and P23) showed a slightly broader substrate spectrum. Strain P23, for instance, grew on different *n*-alcohols (C2-C4), formate, fumarate, lactate, and pyruvate. Only *Desulfotignum* strain P18 grew on acetate, benzoate, betaine, butyrate, and succinate, whereas fast growth and high cell densities were achieved with betaine, which was also fermented. Fermentative growth with pyruvate occurred in *Desulfovibrio* strains, only.

All strains used hydrogen as electron donor. *Desulfotignum balticum* strain P18 grew autotrophically but only in the presence of vitamins. This was already known for the type strain of *Desulfotignum balticum*, which was described to grow on H_2 and CO_2 (Kuever et al., 2001).

Table 3: Comparison of characteristics of sulfate-reducing isolates from IODP Site U1301: Temperature range of growth, morphology, substrate utilization, and alternative electron acceptors.

Isolated strain	**P20**	**P23**	**P18**
Closest relative in GenBank	***Desulfovibrio aespoeensis*^{T}**	***Desulfovibrio indonesiensis*^{T}**	***Desulfotignum balticum*^{T}**
Sediment depth (mbsf)	1.30	260.43	260.43
T_{range}	20–35°C	10–48°C	4–48°C
T_{opt}	25°C	25–35°C	25–35°C
Morphology	Highly motile, thin, *vibrio* like, spirilloid cells, 3.8 μm (±0.9 μm) long, 0.4 μm (±0.1 μm) thick	Motile, *vibrio* shaped cells, 2.7 μm (±0.4 μm) long, 0.7 μm (±0.1 μm) thick	Non-motile, short thick rods with rounded ends, 2.3 μm (±0.4 μm) long, 1.0 μm (±0.1 μm) thick
ELECTRON DONORS AND SUBSTRATES IN THE PRESENCE OF SULFATE			
H_2, CO_2 + acetate (1 mM)	+	+	+**
H_2, CO_2 (excess)	–	+*	+**
Acetate (5 mM)	–	–	(+)
Benzoate (2.5 mM)	–	–	+
Betaine (2 mM)	n.t.	–	+
Butanol (5 mM)	–	+	+ (No H_2S)
Butyrate (5 mM)	–	–	(+)
Ethanol (5 mM)	–	+	–
Formate (5 mM)	+	+	(+) Slow
Fumarate (5 mM)	–	+	+
Lactate (5 mM)	+	+	+
Propanol (5 mM)	–	(+)	–
Pyruvate (5 mM)	–	+	+
Succinate (5 mM)	–	–	(+) Slow
FERMENTATION			
Ethanol (5 mM)	–	(+) Slow	(+) Slow
Betaine (2 mM)	n.t.	n.t.	+
Pyruvate (5 mM)	(+)	+	–
ELECTRON ACCEPTORS* **			
Sulfate (28 mM)	+	+	+
Sulfite (10 mM)	+	+	+
Thiosulfate (10 mM)	+	+	+
Fe(III) hydroxide (~40 mM)	–	(+)	(+)
Mn(IV) (20 mM)	–	(+)	(+)

+, Substrate used for growth as indicated by turbidity increase and production of H_2S in the presence of sulfate, sulfite, or thiosulfate; (+), poor growth, no turbidity increase, but significant production of H_2S, in the presence of sulfate; -, no growth; n.t., not tested.
*Even in absence of vitamins and resazurin as redox-indicator.
**In presence of vitamins only.
***In presence of N_2/CO_2 or H_2/CO_2 and lactate or ethanol.
The culture medium contained 28 mM sulfate as electron acceptor. For fermentation tests and utilization of alternative electron acceptors, a sulfate-free culture medium was used. No strain grew on amino acid mix (1 mM), glucose (5 mM), malate (5 mM), methanol (5 mM), propionate (2 mM), or yeast extract (0.005 % v/v). None of the strains fermented malate (5 mM) or used nitrate (10 mM) as alternative electron acceptor.

Surprisingly, autotrophic growth for *Dv. indonesiensis* strain P23 was observed in media that did not contain any organic additives such as vitamins, resazurin, or yeast extract and after at least 10 transfers to eliminate carbon sources from initial cultures (Figures 8). Growth rates (based on protein production) for strain P23 were 0.12 d^{-1} under autotrophic conditions, and approximately three times higher (0.30 d^{-1}) when 1 mM of acetate was added.

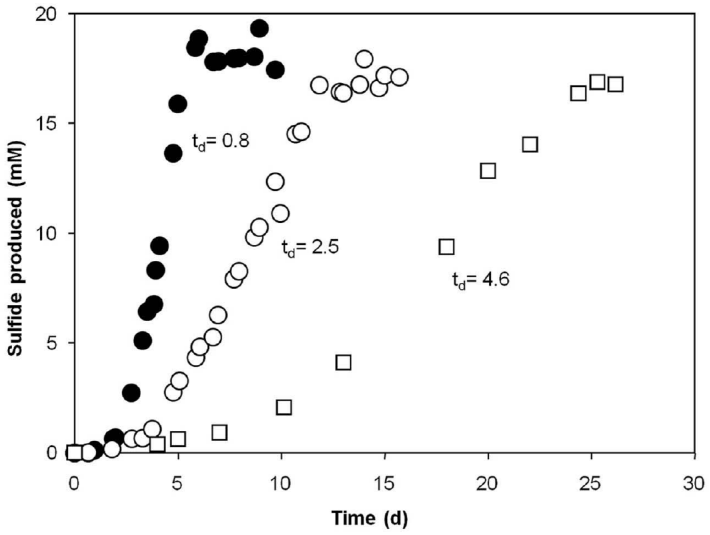

Fig. 8: Comparison of heterotrophic and autotrophic growth of *Desulfovibrio indonesiensis* strain P23 at 35°C (○) under autotrophic conditions using hydrogen, CO_2 and sulfate (28 mM); (●) after addition of 1 mM acetate, both in the presence of resazurin and vitamins; (□) under autotrophic conditions in media without vitamins and resazurin. Doubling times t_d in days are indicated.

3.5 Discussion

3.5.1 Organic matter and sulfate availability generate the three different zones of the sediment column

The stratification of the different sediment compartments has an imprint on the life conditions. In both, the seawater- and fluid-influenced layers, the availability of electron acceptors stimulates microbial growth and activity of indigenous microorganisms (Engelen et al., 2008). In terms of electron donors, bacteria that thrive in the upper 30 m of the sediments are supported by burial of relatively young organic carbon (Fisher et al., 2003; Johnson et al., 2006). Therefore, they are used to a higher supply of electron donors and adapt much better to the given cultivation conditions. In deeper sediment horizons, indigenous bacteria have to survive long-term burial by adapting to a minimum supply of substrates and electron acceptors. Their limited availability strongly influences the metabolic activities in the deep marine subsurface. Indeed, based on geochemical porewater profiles, it has been concluded that the metabolic activities of subseafloor prokaryotes are very low (D'Hondt et al., 2002, 2004). They probably have developed different life strategies such as slow growth or survival as spores. The latter were presumably stimulated to germinate during our cultivation experiments since a major part of 16S rRNA gene sequences detected in all enrichment cultures affiliated to sporeforming *Firmicutes* (Figure 6). How-

ever, the decreasing number of *Firmicutes* with depth indicates that not all of them survive the long-term burial as spores as they might have germinated stochastically over geological time scales (Epstein, 2009).

Other subsurface organisms that are adapted to low organic carbon concentrations might not be able to grow under the given laboratory conditions. Even though the composition of our culture media was designed to provide organic substrates in submillimolar concentrations, a substrate shock (Straskrabová, 1983) might not have been circumvented. For instance, we were not able to grow any *Archaea* (data not shown) even though they are proposed to represent a substantial part of the deep biosphere as indicated by intact-lipid analysis (Lipp et al., 2008).

The supply of electron acceptors into the sediment column by crustal fluid diffusion dramatically changes the situation for microbial life within these deeply buried layers. The large numbers of non-sporeforming *Gammaproteobacteria* that were enriched from near-basement layers indicate the presence of viable cells. Many *Gammaproteobacteria* are adapted to elevated substrate concentrations (Lauro et al., 2009) and are therefore readily cultivated using our media. Some of them might even be typical for oceanic ridge systems. *Halomonas* and *Marinobacter* species were found to be present in hydrothermal fluids collected at the Juan de Fuca Ridge (Kaye et al., 2011). They were enriched during *in situ* colonization experiments on basaltic crust (Smith et al., 2011) and have also been detected in basaltic seafloor lavas and overlying seawater at the East Pacific Rise (Santelli et al., 2008).

3.5.2 The upper and lower sulfate-containing zones harbor different sulfate-reducing bacteria

The majority of sequences obtained from upper sediment horizons that were affiliated to SRB have *Desulfosporosinus* and *Desulfotomaculum* species as closest relatives, both sporeforming *Firmicutes*. However, it is unclear if they contribute to the high sulfate reduction rates of up to 8 nmol cm^{-3} d^{-1} determined for the upper sulfate-containing zone of IODP Site U1301 (Engelen et al., 2008). This would only be the case if these SRB are present as viable cells. It cannot be specified if they are metabolically active or if they only survive as spores within these layers.

In contrast, fluid-influenced sediments exclusively harbor sulfate reducers that are members of the *Deltaproteobacteria*, which are not known to form any resting stages. These viable populations contribute to sulfate reduction

rates of up to 3 pmol cm^{-3} d^{-1} within the lower sulfate reduction zone (Engelen et al., 2008). Due to their high abundance, this activity might derive from sulfate reducers affiliated to *Dv. indonesiensis*. This is quite surprising since the *in situ* temperature is around 60 °C and most *Desulfovibrio* species are not active above 40 °C (Widdel & Bak, 1992). However, a broad temperature range of growth was not only found for our isolates, but also for the Japan Sea isolates of *Dv. profundus* (Bale et al., 1997) and might represent an adaptation to the conditions in the deep biosphere.

Thus, one reason for the divergence in the SRB communities detected in both sulfate-containing zones might be the different temperature and pressure regimes present at the top and bottom of the sediment column. Surprisingly, the isolates from the deepest fluid-influenced layers did not grow at *in situ* temperatures of approximately 60 °C. This might be due to the chosen initial incubation conditions at 20 °C and ambient hydrostatic pressure instead of the *in situ* pressure of ~30 MPa. As temperature and pressure counteract on the cell membrane composition (Mangelsdorf et al., 2005), an insufficient combination of both parameters might result in membrane disintegration. This assumption is supported by the fact that no isolates were obtained from enrichment cultures that were incubated under *in situ* temperatures (data not shown). In future experiments, pressure incubations might help to overcome such problems in cultivation efficiencies.

3.5.3 Sulfate-reducing bacteria from the lower zone have relatives in deep terrestrial aquifers

Previous microbiological investigations on crustal fluids from the Juan de Fuca Ridge have identified several isolates (Nakagawa et al., 2006) and 16S rRNA clones (Cowen et al., 2003; Huber et al., 2006) that were affiliated to SRB. In general, the overlap between these studies compared with our culture collection from fluid-influenced sediments is quite low. Only relatives of *Desulfotomaculum* and *Desulfonatronovibrio* species were detected in two studies on the adjacent ODP Site 1026. One 16S rRNA gene sequence that is affiliated to *Desulfobacterium* species was found in fluids that discharge at "Baby bare seamount". A possible explanation for this discrepancy might be that most of our isolates represent typical sediment inhabitants, which do not necessarily occur in the upper oceanic crust. However, our *Deltaproteobacteria* that were isolated from the lower sulfate-containing

zone are facing similar physicochemical conditions in the highly compacted sediments above the basement as in the crustal aquifer.

A close relation of deep marine with terrestrial aquifers is indicated by the cultivation of *Dv. aespoeensis* strains from the fluid-influenced layers. *Dv. aespoeensis* is the most abundant sulfate reducer within formation waters of deep terrestrial boreholes at the Aespoe hard rock laboratory in Sweden (Motamedi & Pedersen, 1998). Those aquifers are also inhabited by complex microbial communities that are comparable to those thriving within the ocean crust (Pedersen, 2000). The energetical constrains are similar and select for, e.g., iron-reducing bacteria, acetogens, methanogens, and sulfate reducers (Pedersen, 1997).

Our most frequently isolated strains from up to 260 m deep fluid-influenced sediments that are affiliated to *Dv. indonesiensis* also have close relatives within the deep terrestrial biosphere. Even though the type strain was originally isolated from a biofilm on a corroded ship off the Indonesian coast (Feio et al., 1998, 2000), relatives were obtained from porewater brines of a deep terrestrial gas–reservoir (Sass & Cypionka, 2004b). Furthermore, these organisms are supposedly involved in iron corrosion as determined during a study on hydrogen-consuming microorganisms in oil facilities from Japan (Mori et al., 2010). Biocorrosive capabilities (Feio et al., 1998) of *Dv. indonesiensis* might be an indication for a crustal origin of this species as this process plays an important role in the weathering of basalts (Edwards et al., 2005). Under anoxic conditions, SRB, and especially *Desulfovibrio* species are responsible for the corrosion of metal surfaces in consuming cathodic hydrogen (Pankhania, 1988; Dinh et al., 2004). This process might occur in the habitat as well as in our metabolic tests. As all isolates deriving from the fluid-influenced zone were capable of using hydrogen as electron donor, they might even exhibit a chemolithoautotrophic life-mode *in situ*.

3.5.4 Chemolithoautotrophy within the deep biosphere

Autotrophic, hydrogen-consuming microorganisms were repeatedly detected in deep continental aquifers and can even outnumber heterotrophs (Stevens & McKinley, 1995). The assumption that autotrophy is also a common metabolic attribute within the crust at IODP Site U1301, is supported by the isolation of a novel member of the genus *Archaeoglobus* from a fluid-influenced sample of ODP Site 1226 (Steinsbu et al., 2010). *Archaeoglobus sulfaticallidus* sp. nov., is a thermophilic and facultatively lithoautotrophic

sulfate reducer and was isolated from black rust formations on top of a leaking borehole seal.

Although there is no clear evidence available for lithoautotrophy within the subseafloor (Stevens, 1997), there are numerous studies that deal with hydrogen as suitable source for deep subsurface life. In these habitats, hydrogen can originate from many sources (Nealson et al., 2005) such as the fermentation of organic matter or mechanochemical processes due to the tectonic action of the Earth (Parkes et al., 2011), degassing from the Earth's mantle during serpentinization of ultramafic rocks (McCollom & Bach, 2009), or even by radiolysis of water (Blair et al., 2007; D'Hondt et al., 2009). Furthermore, in the presence of sulfate, the oxidation of hydrogen is thermodynamically favored at high temperatures (Orcutt et al., 2010).

Thus, in many deep subsurface habitats, hydrogen might become apparently the biochemically most important electron donor and carbon dioxide is a ubiquitous carbon source. For example, both gases were found in micro-molar concentrations in deep igneous-rock aquifers (Pedersen, 1997) and deep aquifers of the Columbia river basalt which is located close to our investigated site (Stevens & McKinley, 1995). For both sites, the authors have proposed a model for a hydrogen-driven biosphere. They assume autotrophic acetogens to form acetate from hydrogen and carbon dioxide. Acetoclastic methanogens can utilize acetate to produce methane or hydrogenotrophic methanogens might directly use hydrogen and CO_2. At relatively young ridge-flank systems, hydrogen-utilizing sulfate reducers will outcompete methanogens as sulfate is still available within the fluids.

3.6 Conclusion

Even though cultivation might not cover the whole microbial diversity of a given habitat, we were able to isolate and physiologically characterize indigenous microorganisms that are numerically and metabolically important for the marine deep subsurface. Thus, cultivation-based studies offer the opportunity to complement molecular techniques. In our study, the isolation of SRB from deep sediment layers was the precondition to answer questions concerning specific metabolic adaptations to the conditions at the sediment-basement interface.

The isolation of facultatively autotrophic sulfate reducers from near-basement layers strongly suggests that these organisms survive due to their

capability of consuming hydrogen after organic compounds have been depleted or become too recalcitrant for microbial degradation. The continuous supply of sulfate from the aquifer below supports their viability within their respective sediment layers even after the separation from organic matter input at the seafloor due to sediment accumulation. When organic substrate availability from the ocean becomes a limiting factor, hydrogen becomes the most important electron donor.

3.7 Acknowledgements

Katharina Schmidt, Heiner Hartwich, Dewi Nasima, and Martin Mierzejewski assisted in molecular analyses and cultivation studies. Andrea Schlingloff is thanked for her help in DNA sequencing and Erhard Rhiel is acknowledged for preparing TEM-photos. This study used samples and data provided by the Integrated Ocean Drilling Program from IODP Exp. 301. The work was financially supported by the German Research Foundation (DFG).

4 Temperature and pressure adaptations of a piezothermophilic sulfate reducer from the deep marine subsurface

Dieses Kapitel ist in Vorbereitung zur Einreichung bei *The ISME Journal*. Das Manuskript liegt den Koautoren J. Logemann, J. Fichtel, J. Rullkötter, H. Cypionka und B. Engelen* zur Überarbeitung vor. Die Autoren K. Fichtel und J. Logemann trugen gleichermaßen zu dieser Arbeit bei.

Keywords: *Desulfovibrio*, Juan de Fuca Ridge, piezophiles, fatty acids, intact polar lipids, ornithine

4.1 Abstract

Microbial life in deep marine subsurface is facing increasing temperatures and hydrostatic pressure with depths. In this study, we have examined growth characteristics and temperature-related adaptations of the *Desulfovibrio indonesiensis*-affiliated strain P23 to the *in situ* pressure of 30 MPa. The strain originates from 61 °C-warm deep subsurface sediments influenced by crustal fluids but has been isolated under standard laboratory conditions at 20 °C and atmospheric pressure. Faster growth was recorded at *in situ* pressure and high temperature, while cell filamentation was induced by further compression. Complementary cellular lipid analyses revealed a two-step response of membrane viscosity to increasing temperatures with an exchange of unsaturated to saturated fatty acids and subsequent changes from branched to unbranched moieties. While temperature had a stronger effect on the degree of fatty acid saturation and restructuring of main phospholipids, pressure mainly affected branching and length of side chains. The detection of phosphate-free ornithine lipids points towards an adaptation to the low *in situ* values of phosphate. Incorporation of ornithine lipids into the cell membrane was found to be temperature- rather than pressure-dependent. However, the simultaneous decrease of both factors to ambient laboratory conditions allowed the cultivation of our moderately thermophilic strain, which in turn might be one key to a successful isolation of high-temperature and -pressure adapted microorganisms from the subsurface.

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4.2 Introduction

The volume of world's oceans 200 m below sea level constitutes more than 95 % of all aquatic habitats (Michiels et al., 2008). Additionally, the subseafloor represents a large reservoir for prokaryotic life (Whitman et al., 1998; Kallmeyer et al., 2012) and even extends into the upper oceanic crust (Heberling et al., 2010; Orcutt et al., 2011). Taking the deep ocean and the marine subsurface into account, the majority of all prokaryotic cells on earth are facing high-pressure conditions. Indigenous microorganisms share the ability to thrive even under extreme pressure.

Previous investigations on pressure adaptation of marine microorganisms were performed mainly on psychrophilic deep-sea bacteria (DeLong & Yayanos, 1985; Wirsen et al., 1986). While some data exist for thermophilic bacteria and hyperthermophilic archaea from hydrothermal vents (Jannasch et al., 1992; Alain et al., 2002), physiological data on mesophilic piezophiles are limited to a few isolates (Kaneko et al., 2000; Alazard et al., 2003; Khelaifia et al., 2011). Moreover, pressure studies on isolates from marine subsurface sediments are rare (Bale et al., 1997).

In our previous studies, we have isolated several sulfate reducers from up to 260 m deep subsurface sediments of the Juan de Fuca Ridge exhibiting a water depth of 2656 m, corresponding to an *in situ* pressure of ~30 MPa (Fichtel et al., 2012). At this site, low-temperature hydrothermal fluids are diffusing from the underlying oceanic crust into the sediment column, resulting in a steep temperature gradient from 2 °C to 62 °C. Crustal fluids are introducing energy sources like sulfate (16 mM) from below, thus stimulating sulfate-reducing communities to thrive within this habitat (Engelen et al., 2008). Pure cultures were obtained under standard laboratory conditions, i.e. at atmospheric pressure and 20 °C. The sulfate-reducing bacteria isolated from the deepest sediments above the basement solely belonged to the *Deltaproteobacteria*, namely one *Desulfotignum balticum*-affiliated strain from 260 meters below seafloor (mbsf) and five strains related to *Desulfovibrio indonesiensis* from 240 to 260 mbsf. As members of the *Deltaproteobacteria* are not known to form any resting stages, they are supposed to belong to active microbial populations of the deep subsurface. Physiological characterization of the isolates revealed that *D. indonesiensis*-affiliated strains turned out to be moderately thermophilic, growing from 10 °C to 48 °C, exhibiting chemoheterotrophic and -lithoautotrophic life-mode (Fichtel et al., 2012). Interestingly, the temperature range for growth did not

reach *in situ* temperatures of 56 °C to 61 °C. As temperature and pressure can have opposing influences on the cell membrane, an insufficient combination of both parameters might result in an inhibition of cross-membrane processes or even the disintegration of cells (Mangelsdorf et al., 2005). Thus, the question arose wether incubation under *in situ* pressure would induce a shift in their temperature range of growth.

This adaptation capacity is expected due to the ability of microorganisms to regulate structure and organization of their cell membrane as a response to changes in temperature and pressure in order to maintain the membrane fluidity necessary for sustaining biological functions ('homeoviscous adaptation', Sinensky, 1974; Macdonald, 1988; Somero, 1992; Kaye & Baross, 2004). The reorganization influences the membrane lipid composition, the degree of saturation of membrane-bound fatty acids, as well as their chain length and branching (DeLong & Yayanos, 1985, 1986; Wirsen et al., 1986; Yano et al., 1998).

In this study, several sulfate-reducing isolates from the deep marine subsurface are examined for growth under high pressure and various temperatures. We have chosen the *D. indonesiensis*-affiliated strain P23 to be analyzed representatively as the strain has been isolated from the deepest sediment sample, and exhibited a relatively fast growth both, under high hydrostatic pressure and high temperatures. Microbial investigations such as the recording of specific growth curves were complemented by detailed lipid analysis to identify a cellular response to changing incubation conditions.

4.3 Material and Methods

4.3.1 Bacterial strains, their origin and growth conditions

Pure cultures of strictly anaerobic, sulfate-reducing bacteria used in this study were obtained from up to 260 m deep subseafloor sediments. Samples were collected in the northeast Pacific at the Eastern Flank of the Juan de Fuca Ridge, Site U1301C (47°45.28'N, 127°45.80'W; water depth: 2656 m) during IODP Expedition 301 in 2004. Details of environmental conditions, sampling, contamination tests, subsampling and isolation procedures have been reported previously (Expedition 301 Scientists, 2005b; Lever et al., 2006; Engelen et al., 2008; Fichtel et al., 2012). Enrichment and isolation of pure cultures were performed at ambient conditions, i.e. atmospheric pressure of ~0.1 MPa and 20 °C. Culture media and cultivation procedures to obtain axenic cultures and the phylogenetic analysis have been described in

detail by Fichtel et al. (2012). Strain P23, affiliated to *Desulfovibrio indonesiensis* (99 % 16S rRNA sequence similarity), was analyzed representatively for pressure and temperature adaptation in more detail.

For comparison, five additional isolates from the same sampling site, affiliated to *D. indonesiensis* (strains P12, P34), *D. aespoeensis* (P20), *Desulfotignum balticum* (P18), *Desulfosporosinus orientis* (P26), and the type strain of *D. indonesiensis* (Ind1^{T}, DSM 1512) were taken as references. All strains were pre-cultured to early stationary phase at atmospheric pressure and 25 °C to 35 °C in sulfate-containing (28 mM) artificial-seawater media that has originally been used for isolation (Fichtel et al., 2012). Lactate (10 mM) or betaine (5 mM) was used as carbon source. Growth was routinely followed by photometrical determination of sulfide in form of colloidal CuS at 480 nm (Cord-Ruwisch, 1985) and of cell protein concentrations at 595 nm (Bradford, 1976) as well as by visual inspection of the cells using phase-contrast microscopy. Transmission-electron microscopy (TEM) of strain P12 was performed as described by Fichtel et al. (2012).

4.3.2 Pressure incubations

All pure cultures were generally examined whether they are able to grow under pressure (10 and 40 MPa). Bacterial growth experiments were performed in 'high-pressure steel vessels' (High Pressure Equipment (HiP) Company, Linden, PA, USA). Inoculations were done in 60 or 70 ml serum bottles containing freshly prepared culture media and sealed with rubber stopper and crimp caps. Pre-cultures (4 % of final volume) were injected and bottles were completely filled with the respective media, while gas bubbles were carefully eliminated by the usage of a second syringe. Three serum bottles were placed inside a pre-heated pressure vessel filled with distilled water. Samples were set under hydrostatic pressure by means of a hand operated 'high-pressure generator' (model 81-5.75-10, HiP) using distilled water as hydraulic fluid. For subsampling, the vessel was carefully depressurized (~1 min) through a valve. The triplicate set of bottles was subsampled for growth analyses as quickly as possible (15-30 min), refilled with media and again compressed within a few minutes. Pressurized samples were incubated between one and sixteen days depending on growth behaviour. Growth at hydrostatic pressure was defined to be positive after two independent successful experiments. Growth at 0.1 MPa was assessed by using the same general protocol except pressurization. In general, all assays were carried out in triplicates and repeated at least twice.

4.3.3 Hydrostatic pressure effects on growth of *Desulfovibrio* strain P23

Growth behavior of strain P23 in response to different hydrostatic pressures was assessed by comparing the amount of sulfide and protein formed during defined times of incubation at constant low and high temperature. Assays at 20 °C were incubated for five days, whereas assays at 45 °C were stopped after 36 hours. Duration of the experiments was set to the time the strain was in late exponential growth phase at atmospheric pressure at 20 °C and 45 °C, respectively, as determined in duplicate in preliminary tests.

Growth rates were calculated from 3 to 5 data points along the logarithmic slope of the exponential portion of sulfide and protein curves using linear regression analysis. For this experiment, substrate-free media was used to refill serum bottles.

The upper temperature limit for growth of strain P23 under high pressure was determined as described as follows. In preliminary experiments, growth was tested in the range of 45 °C to 62 °C at 20, 26, and 30 MPa. Growth curves were finally recorded in parallels with slowly increasing temperatures from 45 °C to 52 °C at both, 0.1 and 20 MPa. Cultures grown at 45 °C were allowed to adapt to higher temperatures for nine hours before incubation at 48 °C. Temperature was raised again to 50 °C after 12 h and to 52 °C after 36 hours of incubation. Pressure vessels were decompressed for growth analyses as described above at the end of each temperature-step. After subsampling, serum bottles were refilled with fresh medium to circumvent substrate limitation.

4.3.4 Cultivation and extraction for lipid analysis

For determination of whole cellular fatty acids and intact polar lipids (IPL) strain P23 was grown as described above at 20, 35 and 45 °C at both, atmospheric and high pressure, in total culture volumes of 1.5 to 2.2 liters. To obtain enough cell material, all pressure incubations were performed in parallels of up to 30 serum bottles using several pressure cylinders. To compensate for growth phase differences (Hamamoto et al., 1994; Allen et al., 1999), cells of each experiment were immediately harvested at late exponential growth phase, combined by centrifugation at 4 °C, and stored at −20 °C until further analyses. Total lipids were obtained by ultrasonic extraction from each washed cell pellet following a modified Bligh&Dyer procedure

(Sturt et al., 2004) as described by Logemann et al. (2011). The lipid extracts were combined and evaporated to dryness under nitrogen at room temperature, stored at −20 °C and analyzed by combined gas chromatography and mass spectrometry (GC-MS).

4.3.5 Cellular fatty acids

The following described technical procedures were adopted from Rütters et al. (2002). Aliquots of the total lipid extracts were transesterified with trimethylsulfonium-hydroxide as described by Müller et al. (1990). Fatty acid-methyl esters obtained were quantified by using a gaschromatograph (7890A GC-System Agilent Technologies, Santa Clara, CA, USA) equipped with a flame-ionization detector (FID) and a capillary column (DB-5HT, length 30 m, ID 0.25 mm, 0.1-µm film thickness; J&W Scientific, Folsom, CA, USA). Identification was performed on a GC-MS using a HP 5890 Series II gas chromatograph (Hewlett Packard, Waldbronn, Germany) also equipped with a DB-5HT-column and coupled to a Finnigan MAT SSQ 710B mass spectrometer (Finnigan-Thermoquest, San Jose, CA, USA). Helium with a constant pressure of 12 psi was used for both systems. Oven temperature was raised from 60 °C (isothermal for 2 min) to 360 °C at a rate of 3 °C min^{-1} and held for 5 min. Mass spectra were collected in full scan mode (m/z 50-650, ionization energy 70 eV and 230 °C source temperature). Mass spectrometric investigations were used to confirm the results obtained with GC-FID. Fatty acids were identified by comparison of the retention times with those of known standards (Bacterial Acid Methyl Esters CP Mix; Supelco, Bellefonte, PA, USA).

4.3.6 Intact polar lipids

Intact polar lipids (IPLs) were analyzed from an aliquot of each cell extract using HPLC-electrospray ionization (ESI)-MS in the negative ion mode as described by Logemann et al. (2011). MS/MS spectra and full scan mass spectra (m/z 100-2000) were used for identification of head groups, diacylglycerols (DAG) or acyl/ether glycerol (AEG) core lipids as well as fatty acyl side chains. Quantification was achieved by using an external multipoint calibration via compound mass trace areas. Phosphatidic acid (PA), phosphatidylglycerol (PG), phosphatidylethanolamine (PE), and phosphatidylinositol (PI) (all from Avanti Polar Lipids, Alabaster, USA; Matreya, Pleasant Gap, USA; Sigma Aldrich, München, Germany, or Lipid Products, Red-

hill, UK) were used as standard compounds representative for different IPL classes. Due to the lack of commercially available standards for ornithine lipids (OL) or unknown polar lipids, OL were quantified via the calibration curve for PG, while for unknown lipids the average signal response of all standards at every concentration was used.

4.4 Results

4.4.1 Growth of sulfate-reducing strains under high hydrostatic pressure

All sulfate-reducing pure cultures, which we previously have isolated at atmospheric pressure from a subseafloor habitat (Fichtel et al., 2012), exhibited growth under elevated hydrostatic pressure of up to 30 MPa. This was also true for the type strain of *Desulfovibrio indonesiensis* ($Ind1^T$), which was originally isolated from a corroding ship at the sea-surface (Feio et al., 1998). The combination of the highest pressure and temperature applied (40 MPa / 45 °C) severely affected the shape of *D. indonesiensis*-like strains P12 and P23. Cells grew as long filaments (Figures 9A,B) and cell motility was completely reduced. Cell division otherwise producing motile, vibrio-shaped cells appeared to be incomplete. Indeed, highly elongated cells of straight or twisted shape were observed by transmission electron microscopy (Figure 9C). Phase-contrast micrographs of cultures grown at 45 °C revealed that the cell length of strain P23 varied from 1-1.7 µm at atmospheric pressure and increased to an average of 14.7 µm at 40 MPa. Consequently, growth in further experiments was determined via sulfide formation and by measuring of protein production rather than cell counting.

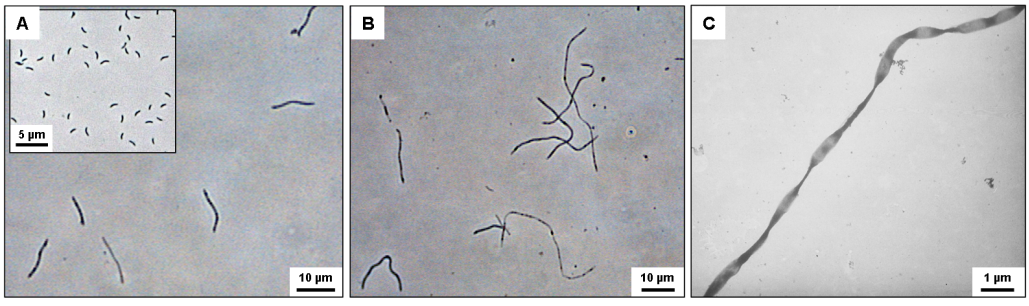

Fig. 9: Microscopic images of *Desulfovibrio indonesiensis*-affiliated strains grown at 45 °C and 40 MPa obtained by phase-contrast (A, B) and transmission-electron microscopy (C): A) strain P23 (small picture: at 0.1 MPa), B and C) strain P12.

The only *Firmicute* tested in this study, the *Desulfosporosinus orientis*-affiliated strain P26 did not form vegetative cells under elevated pressure of up to 20 MPa. Instead, hydrostatic pressure appeared to induce spore formation since only spores or sporulating cells were observed ten days after incubation after inoculation of freshly grown active cells, pre-cultured using the same media. This finding might indicate that the isolate originally has derived from a spore that has germinated during the isolation procedure from relatively young sediments located ~1 m depth below seafloor.

4.4.2 Specific growth rates of *Desulfovibrio* strain P23

Preliminary experiments were performed to identify the effect of various hydrostatic pressures of up to 40 MPa at constant low or high temperatures on the growth behaviour of strain P23 (Figure 10). While at 20 °C increasing pressure diminished biomass formation, growth was accelerated at 45 °C, and pressures between 10 and 30 MPa, as indicated by reaching higher protein contents at a given time-point.

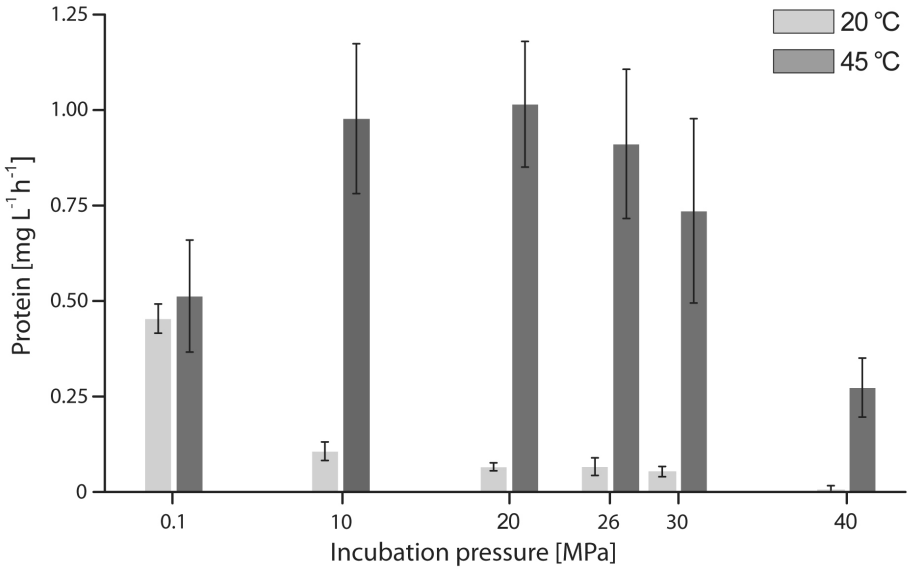

Fig. 10: Growth of *Desulfovibrio indonesiensis*-affiliated strain P23 at 20 and 45 °C under pressures of 0.1 to 40 MPa. Growth is shown as protein gain per hour, after 120 hours of pressure-incubation at 20 °C and 36 h at 45 °C, respectively. Assays at 0.1 MPa represent outgrown cultures. Error bars indicate the standard deviation of five cultivation assays.

To confirm these findings, detailed specific growth rates were recorded. Therefore, strain P23 was grown to early stationary phase at 20 °C and 45 °C both at atmospheric conditions and 30 MPa (Figure 11). Based on protein production, growth at 20 °C was two times faster under 0.1 MPa than under

high-pressure conditions. The exponential growth rate μ was calculated as 0.74 d^{-1} at atmospheric pressure and 0.38 d^{-1} at high pressure. In contrast, growth rates obtained at 45 °C under high-pressure conditions (2.45 d^{-1}) were nearly comparable with those at atmospheric pressure (2.38 d^{-1}). A similar trend was determined via sulfide measurements. Comparing all rates, fastest growth was found at 45 °C and 30 MPa, indicating the stimulation of growth by both, high temperature and *in situ* pressure.

Highest protein yields were obtained at combinations of low temperature and atmospheric pressure (62 mg l^{-1}) as well as high temperature and *in situ* pressure (53 mg l^{-1}). Interestingly, the opposite combination of low temperature and high pressure revealed with ~40 mg l^{-1} a similarly diminished yield as high temperature and low pressure.

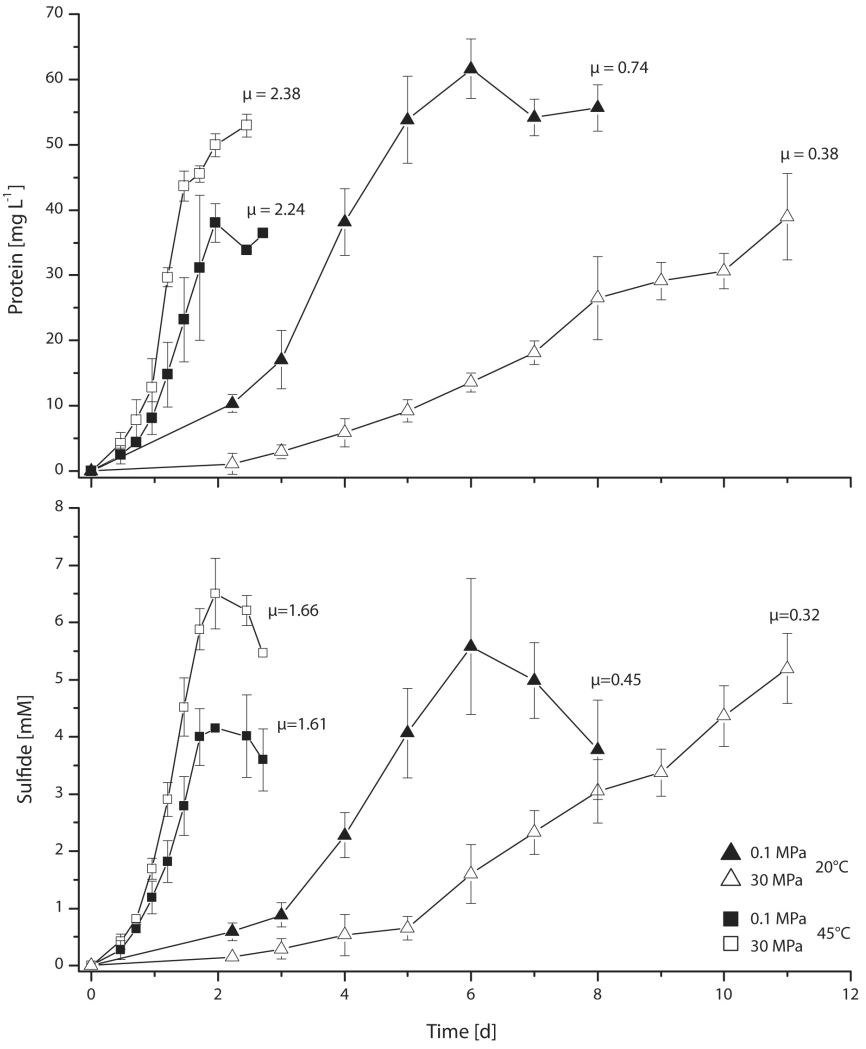

Fig. 11: Specific growth curves and rates μ [d^{-1}] of strain P23 grown at atmospheric and high hydrostatic pressure both at 20 and 45 °C. Values were calculated from photometrical measurements of protein and sulfide. Error bars indicate the standard deviation of three cultivation assays.

4.4.3 Pressure-induced shift of the maximum growth temperature

During a cultivation experiment to determine the upper temperature of growth at elevated hydrostatic pressure (20 MPa), cells were allowed to adapt to slowly increasing temperatures. Under these conditions, the previously determined maximum growth temperature of 48 °C at atmospheric pressure could be shifted to 50 °C. At temperatures higher than 50 °C, neither an ongoing protein production nor increasing sulfide formation was observed. This was proven in further test series that were inoculated with cells freshly grown at 50 °C and 20 MPa. The upper temperature limit for growth was again indicated by the observation of highly elongated, non-motile or deformed cells in comparison to the respective assays from 0.1 MPa.

4.4.4 Changes in whole cell fatty acid composition as a response to increasing temperatures and elevated pressure

The majority of whole cell fatty acids (FAs) of strain P23 were branched. They accounted for up to 79 % under atmospheric pressure (Table S1). Regardless of growth temperatures and pressures, major components were *iso*- and *anteiso*-branched 15:0 and *n*-18:0 FAs, which was already known for the type strain (Feio et al., 1998). Concerning the degree of unsaturation, only monounsaturated fatty acids were detected.

During incubations at high pressure, strain P23 showed strongly elevated levels of *n*-saturated FAs in comparison to cells grown at atmospheric conditions (Figure 12 and Table S1).

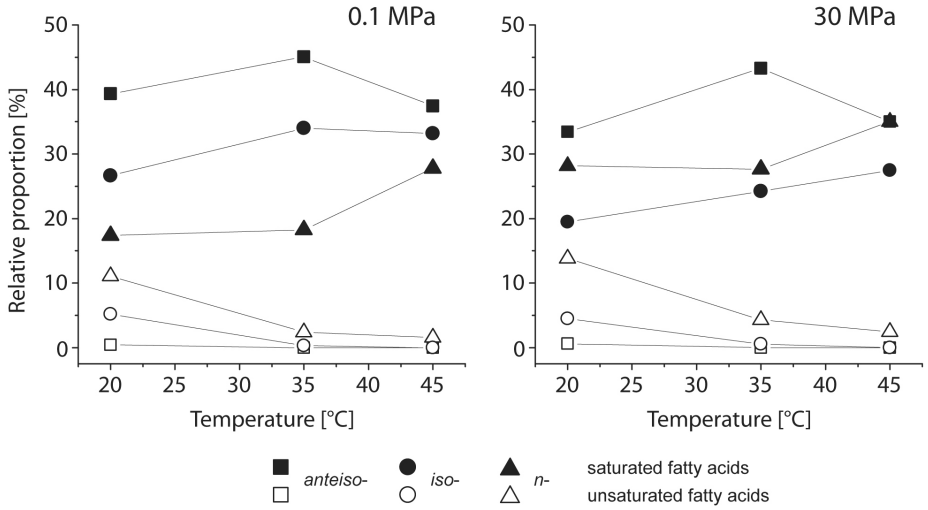

Fig. 12: Changes in relative amounts of whole cell fatty acids of *D. indonesiensis*-affiliated strain P23 grown at different temperatures and pressures.

Under both pressure regimes, a two-step response of strain P23 was detected for increasing incubation temperatures. First, the relative amount of unsaturated FAs decreased strongly. Second, at higher temperatures, levels of *n*-saturated FAs increased mainly at the expense of *ai*-branched-saturated FAs. Comparing temperature-dependent incubations under atmospheric and under *in situ* pressure, pressure did not substantially increase the ratio of unsaturated to saturated FAs, but led to a higher *n*-saturation and concomitant decreased branching of FAs. Additionally, relative proportions of longer-chained FAs were substantially elevated under high-pressure conditions, only (Table S1).

4.4.5 Relative distribution of main IPLs depending on temperature and pressure

Under all conditions tested, strain P23 possessed two classes of intact polar lipids: Phospholipids (phosphatidylglycerol, PG; phosphatidylethanolamine, PE; phosphatidic acid, PA) and the phosphorus- and glycerol-free ornithine-containing lipids, OL (Figure S1). Phospholipids mainly contained a diacylglycerol (DAG) core lipid with ester-bound fatty acid moieties as identified by MS-MS experiments. Additionally, PG was also detected as acyl/ether glycerol (AEG) with mixed ether/ester-bound side chains. Four further IPLs with unidentified head groups were found (Un1-4). It appears likely that they represent yet unknown phospholipids as they were also detected as DAG or AEG (Figures S2 and S3; Table S2).

Comparing the IPL compositions in all assays, the sum of phospholipids mostly dominated over ornithine lipids which was most pronounced under high pressure (Table S3). However, with increasing incubation temperatures the amount of OL increased, while relative proportions of phospholipids decreased (Figure 13). Major shifts with increasing temperatures were found for diacyl-phosphatidylglycerol (PG-DAG) and acyl-ether-phosphatidylglycerol (PG-AEG). While levels of PG-DAG dominated over PG-AEG at low temperature, the opposite ratio was found at higher temperatures. The values of total unknown IPLs ($\sum$ Un 1-4) showed a similar response to temperature changes like the phospholipids. In high pressure cultures, the effect of increasing temperature on the IPL composition was most pronounced between 20 °C and 35 °C.

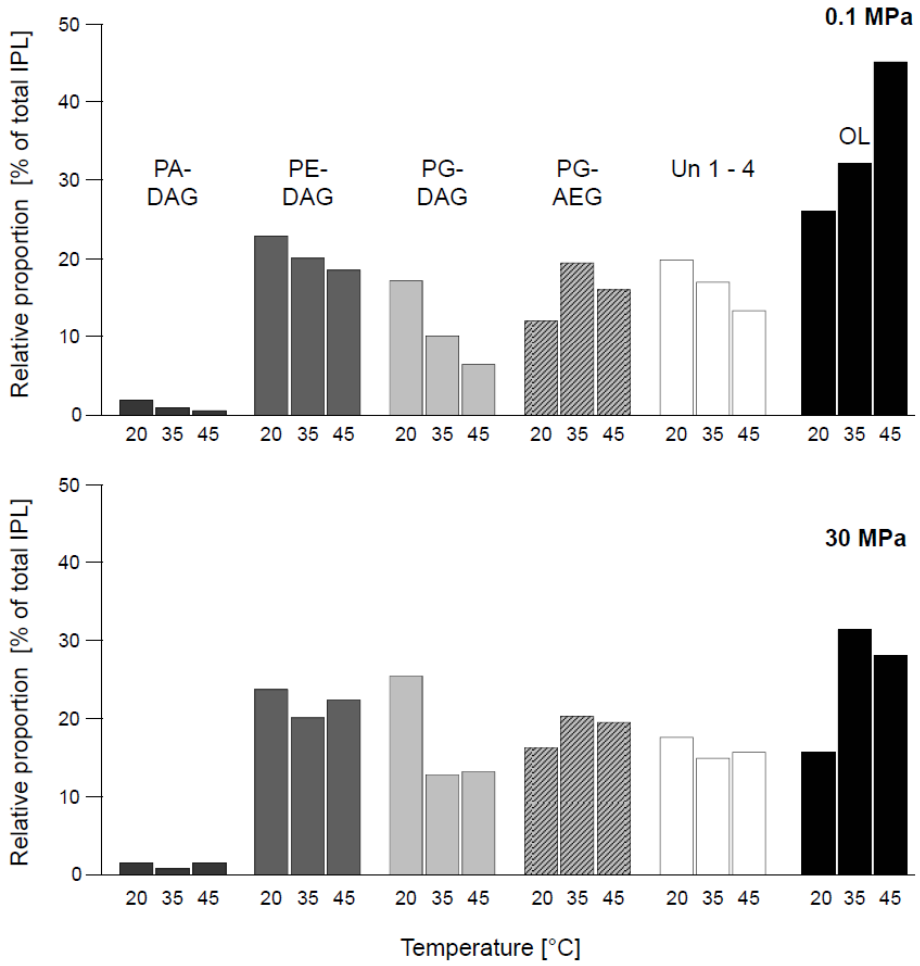

Fig. 13: Changes in relative amounts of major intact polar lipid species of *D. indonesiensis*-affiliated strain P23 depending on growth temperature and pressure.

4.4.6 Variations of IPLs side chains according to changes in cultivation pressure and temperature

The majority of analyzed IPLs contained a fatty acid with 15 carbon atoms (Table S2). Moreover, the C_{15}-FA was the only fatty acid component in IPLs with an AEG core. Polyunsaturated fatty acids were never detected. Ratios of unsaturated to saturated IPLs decreased with raising incubation temperatures and were slightly higher in high pressure assays. These findings are in good accordance with the whole cell fatty acid analysis (Table S1).

Side-chain combinations of PE and PG were quite similar with either C_{14}- or C_{15}-FA together with a C_{15}-C_{20}-moiety at *sn*-2 position. Interestingly, two unknown IPLs (Un-1 and Un-2) contained either a fairly long fatty acyl chain or an ether-bound alkyl moiety of 21-23 carbon atoms. Ornithine lipids possessed either 14:0 or 15:0 FAs together with a 3-hydroxy C_{16}-C_{20} fatty acid.

Cell response of strain P23 to pressure and temperature effects was either reflected in changes of abundance, saturation and carbon-number distribution of fatty acyl side chains in the three major polar lipids PE, PG, and OL. However, focusing on a single mass of an intact polar lipid without MS/MS-experiments, several possibilities for the combination of ester- or ether-linked moieties arise. To simplify our data we have used the radyl value, comprising the total carbon number of both side chains. As a result, PG generally possessed the greatest diversity of side chains which is reflected in the broad range of radyl values from 28 to 37. Radyl values in PE varied between 30-36, and 29-35 in OL. The radyl-value pattern for PE and PG was dominated by 33 carbon atoms, resulting from high proportions of C_{15}- and C_{18}-FAs, while that of OL was dominated by 32, resulting from C_{15}- and 3-OH-C_{17}-FAs (Figure 14).

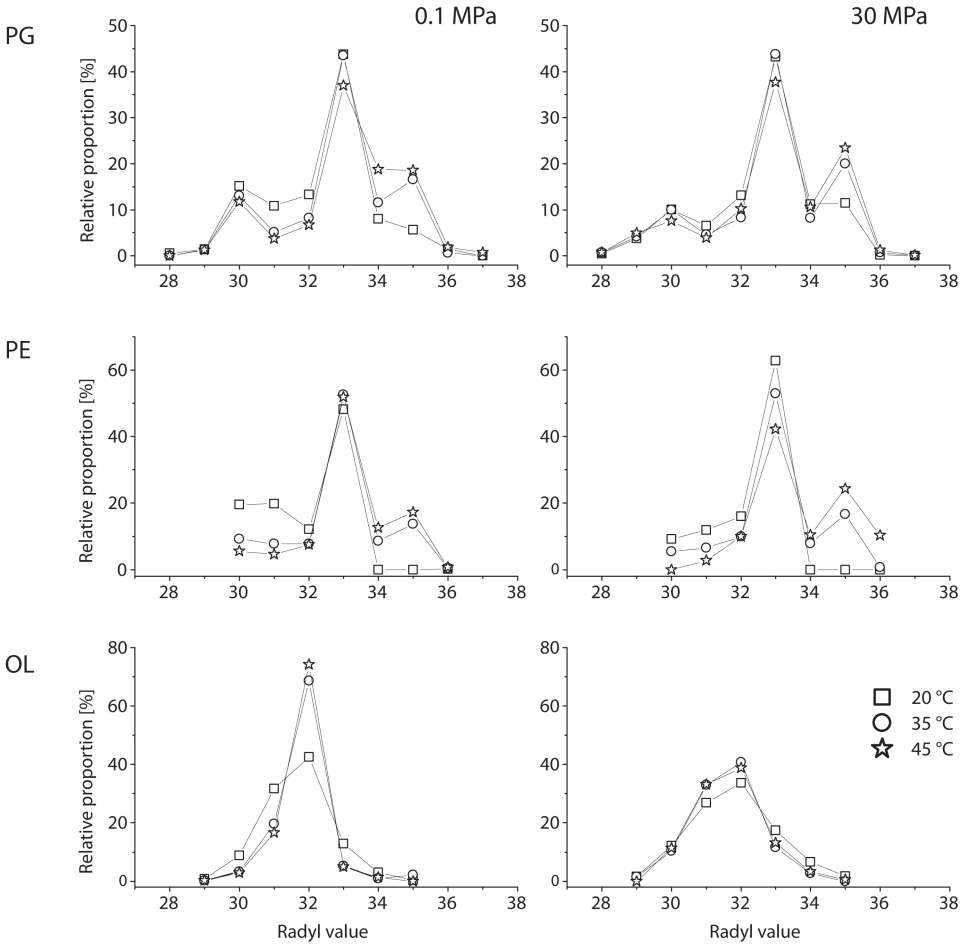

Fig. 14: IPLs inventory of strain P23 depending on different incubation conditions. Changes in fatty acyl side-chain length are expressed by radyl values, the combined number of carbon atoms of the two fatty acid substituents of one IPL type. PG, phosphatidylglycerol; PE, phosphatidylethanolamine, and OL, ornithine lipid. PG data is based on the sum of PG-DAG and -AEG.

For PE and PG, higher incubation temperatures induced an increase of longer side chains on the expense of shorter ones. Additionally, at high-pressure incubations, the most abundant PE molecular species with a radyl value of 33 systematically decreased with temperature.

Most strikingly, the radyl-value pattern of OL was not affected by temperature during high-pressure incubations. Here, the distribution patterns were nearly identical, and were similar to that obtained at 20 °C and 0.1 MPa. In contrast, major structural changes were found at atmospheric pressure and high temperatures with a dramatically increase of the relative proportion of the most dominant OL-species with a radyl value of 32 carbon atoms.

In general, levels of unsaturated side chains in all major IPLs were highest at 20 °C and decreased strongly with raising incubation temperatures. While PE-DAG lipids contained the largest proportion of unsaturated moieties, PG lipids possessed the greatest diversity of side chains. As the PG pool even showed a temperature-induced restructuring with an internal shift from PG-DAG to PG-AEG, findings indicate, that in strain P23 restructuring of PE and PG molecules was relevant for bilayer stabilization, as a result of both, temperature and pressure changes.

4.5 Discussion

Reflecting the *in situ* conditions, our isolate P23 grew best at combined high pressure and high temperature. The cell response to a temperature rise was much more pronounced than to elevated pressure. By decreasing pressure and temperature simultaneously, it was even possible to cultivate our moderately piezothermophilic strain from the warm deep subsurface at ambient conditions.

4.5.1 High-pressure experiments reveal the piezothermophilic nature of *Desulfovibrio* strain P23

A high pressure tolerance should demonstrate that microorganisms of the deep biosphere are well-adapted to their pressurized subsurface habitat, and that they do belong to the active part of deeply buried microbial communities (Bale et al., 1997). In this study, we could demonstrate that the sulfate-reducing *Desulfovibrio* strain P23 was able to grow under hydrostatic pressure of up to 40 MPa (*in situ* pressure ~30 MPa), even after cultivation

at atmospheric pressure for more than three years. Strain P23 obviously has not lost its piezophilic properties, which might also be a common feature of pressurized marine deep-sea organisms, that were sampled and isolated under decompressed conditions (Zobell & Johnson, 1949).

The degree of piezophily was strongly dependent on the incubation temperature (Zobell & Johnson, 1949; Kato et al., 1995). In our previous study, strain P23 was found to have an untypical upper temperature for growth of 48 °C (Fichtel et al., 2012). Strain P23 showed a shift from piezosensitive to piezophilic behaviour when grown at higher temperatures. At 20 °C, strain P23 is piezosensitive, growing at both, atmospheric and elevated hydrostatic pressure with fastest growth rates at 0.1 MPa (Yayanos, 1995; Molina-Höppner et al., 2003). In contrast, at higher temperatures, growth was even accelerated by high hydrostatic pressure in the broad range from 10 to 30 MPa, and the maximum temperature of growth could be elevated to 50 °C only under pressure. Under these conditions, strain P23 is considered to be moderately piezothermophilic (Yayanos, 1995; Kato & Bartlett, 1997), reflecting its adaptation to the *in situ* conditions present in its original warm subsurface habitat.

However, an increase of growth temperature does not necessarily improve the piezotolerance of microorganisms. For instance, in a study on typically atmosphere-adapted lactic acid bacteria, higher temperatures did not stimulate microbial growth under elevated pressure (Molina-Höppner et al., 2003). The authors speculated that unlike many pressure-adapted species lactic acid bacteria might be unable to mount a specific pressure response in order to maintain membrane fluidity.

During growth at conditions above *in situ* pressure, the cell morphology of our isolates changed. With increasing pressure and temperatures, cells became more elongated and cell division was inhibited, indicating a typical stress response. Cell filamentation seems to be a characteristic pressure-related phenomenon in mesophilic bacteria (Zobell & Cobet, 1962, 1964; Lauro & Bartlett, 2008). Pressure is believed to have a direct inhibitory effect on FtsZ ring formation, which is a prerequisite for membrane construction during cell division (Molina-Höppner et al., 2003; Ishii et al., 2004). Filament formation might also be mediated via a pressure-triggered SOS response involving the RecD protein, which is essential for DNA recombination and repair (Bidle & Bartlett, 1999; Aertsen et al., 2004).

4.5.2 Whole cell fatty acid data are confirmed and complemented by IPL analysis

While the whole cell fatty acid analysis provides a fast overview on all cellular fatty acids, the analysis of intact polar lipids directly targets the membrane building blocks. The results of both methods can be combined as fatty acids extracted from the membrane fraction are very similar to those extracted from whole cells (Pluschke & Overath, 1981; Allen et al., 1999; Kaneko et al., 2000).

Due to the theory of 'homeoviscous adaptation of membrane lipids' (Sinensky, 1974; Somero, 1992), it was expected that increasing growth temperature mainly results in a higher degree of saturation of membrane lipids to keep them appropriate fluid for integrity, and cell function. For strain P23, this adaptation was detected with both analytical methods confirming previous studies on a variety of organisms (DeLong & Yayanos, 1985). Moreover, the cell response of strain P23 to changes in temperature apparently occurred stepwise. After changing the saturation level, as a subsequent response to higher incubation temperatures strain P23 decreased its membrane fluidity by exchanging branched FAs with straight-chained FAs. This was most pronounced for *anteiso*-branched FAs, as they have lower melting points compared to *iso*-branched FAs, which have similar effects like saturation (Zhang & Rock, 2008). For strain P23, elevated initial proportions of straight-chained FAs under *in situ* pressure and 20 °C were independent from the degree of saturation and indicated that pressure mostly diminished the branching of FAs. These findings are consistent with results obtained in previous studies about thermal adaptation of bacterial membrane lipids (Rilfors et al., 1978; Nordström & Laakso, 1992; Koga, 2012).

Another factor for membrane fluidity is the number of double bonds of unsaturated fatty acids. While polyunsaturated fatty acids have been found in many piezophilic deep-sea bacteria, this regulatory capacity seems to be limited to psychrophilic microorganisms (DeLong & Yayanos, 1986; Wirsen et al., 1986; Kamimura et al., 1993; Yano et al., 1998). As strain P23 derives from a warm deep-sea habitat, production of polyunsaturated fatty acids might only be relevant when growth temperatures fall below 20 °C.

4.5.3 High proportions of ornithine-containing lipids might reflect phosphate-limitation within the original habitat

Phosphorous-free ornithine-containing lipids are major membrane constituents in strain P23, as already described for other *Desulfovibrio* species (Makula & Finnerty, 1975; Seidel et al., 2013). The presence of ornithine lipids was found to be negatively correlated with available amounts of phosphate present in the medium used for cultivation (Geiger et al., 1999; Weissenmayer et al., 2002). Therefore, the assumption was made that in case of phosphate limitation bacteria can replace phosphate-containing membrane lipids by phosphorus-free lipids such as OL, sulphoquinovosyl diacylglycerol (SQDG) or diacylglycerol trimethylhomoserine (DGTS). Although phosphate was not a limiting nutrient in our cultivation medium, strain P23 was isolated from phosphate-depleted sediment layers (Engelen et al., 2008). In this environment, the ability to produce phosphate-free membrane building blocks gives an advantage over other microorganisms that are not capable of this feature. Thus, it is possible that this adaptation has originally developed in the deep biosphere and was not lost when strain P23 was cultivated in phosphate-rich media.

Furthermore, our study indicates that OL might not only be a substitute for phosphate-containing membrane lipids. Cells grown at atmospheric pressure responded to increasing temperatures with higher relative amounts of OL within the membrane, and chain-length variations. This temperature dependence proofs previous findings by Seidel et al. (2013) who either found elevated proportions of OL with increasing incubation temperatures for several *D. acrylicus* strains. The authors suggested that changes in lipid composition of the cytoplasmic membrane may rather be important for the presence and activity of membrane-bound enzymes and do not necessarily influence its viscosity. This assumption is supported by our results, as we could find neither the expected decrease of OL levels with temperature in high-pressure incubations nor any obvious structural changes of OL moieties as an adaptation of membrane fluidity to high pressure.

4.5.4 Simultaneous decrease of pressure and temperature favours the cultivation of piezomesophiles from deep subsurface sediments

To mimic environmental conditions, most enrichment cultures are incubated at *in situ* temperatures. Even for the cultivation of deep-sea microorganisms, pressure is normally not taken into account. This might be due to the high technical expenditure during sampling, storage and microbial analyses. While all our enrichments from IODP Site U1301 that were performed at *in situ* temperatures and atmospheric pressure did not result in pure cultures, a great variety of isolates were gained at ambient laboratory conditions (Fichtel et al., 2012). This might be explained by opposed effects of pressure and temperature on general cell functions and the capability of microorganisms in adapting to these variations. Thus, a key for a successful isolation of piezomesophilic and other high-temperature adapted prokaryotes from the subsurface under atmospheric pressure might be the decrease of the cultivation temperature below the *in situ* temperature. For the marine subsurface, this might be feasible for the isolation of mesophilic *Archaea*, which are of special interest as they were found to dominate respective clone libraries without any cultivated representative (Teske & Sorensen, 2008). For piezopsychrophiles in turn, this approach is not feasible as *in situ* conditions of e.g. 40 MPa and 2 °C, the average values at the seafloor, would require an incubation temperature of −2 °C to −6 °C at atmospheric pressure (Chong & Cossins, 1983; Bartlett, 2002; Wang et al., 2009). However, previous cultivations from cold deep-sea habitats have obtained isolates with a broad adaptation capability to changes in temperature and pressure. On the other hand, pressure above *in situ* values might be advantageous for cultivating piezopsychrophiles at elevated temperatures, which generally accelerates growth (Kato et al., 1995).

In case of strain P23, applying the optimum hydrostatic pressure resulted in growth at 50 °C but not at the respective *in situ* temperature of ~60 °C. This could possibly be due to the fact that the chemical composition of our media did not reproduce the chemical condition of the natural habitat. Or it might be explained by the effect of pressure on abiotic factors such as the solubility of gases. Strain P23 turned out to be a chemolithoautotrophic sulfate reducer as it was able to grow on hydrogen and CO_2 as sole energy and carbon sources (Fichtel et al., 2012). In the present study, pressure incubations under autotrophic conditions were not performed, as this would

have required a completely different technical equipment allowing cultivation with a gaseous headspace. On the other hand, pressure is an inevitable factor for isolating obligate piezophiles (Kato et al., 1996) or investigating the microbial utilization of gases or hydrocarbons in the subsurface, including field studies on gas and oil reservoirs or CO_2-sequestration facilities.

4.6 Acknowledgements

The authors are grateful to Friedrich Widdel and Christian Deusner for providing the high-pressure equipment and for technical advice. Bernd Kopke and Michael Seidel are appreciated for their help on lipid and data analysis. Erhard Rhiel is acknowledged for his help in preparing TEM photos. This research used samples provided by the Integrated Ocean Drilling Program (IODP) and was financially supported by the German Research Foundation (DFG).

4.7 Supplemental Data

Table S1: Whole cell fatty acid composition of *D. indonesiensis*-affiliated strain P23 grown at three different temperatures at constant atmospheric and high hydrostatic pressure. Single values represent relative amounts in % of total fatty acids, while types with all parts <1 % are not listed. Fatty acids are commonly designated by number of carbon atoms:number of double bonds. *Iso*- and *anteiso*-branching refer to the prefixes "*i*" and "*ai*", respectively, "*n*" symbolizes straight chains. $\sum$ = Sum of all measured values

Fatty acid	0.1 MPa			30 MPa		
	20 °C	35 °C	45 °C	20 °C	35 °C	45 °C
i-14:0	6.4	4.2	3.0	5.2	5.4	6.4
n-14:0	2.1	2.6	2.8	4.8	5.1	6.8
i-15:1	1.6	-	-	2.1	0.5	-
i-15:0	16.2	24.9	24.5	11.1	15.9	18.4
ai-15:0	39.3	45.1	37.0	33.5	43.3	35.0
i-16:0	2.0	1.0	0.7	1.6	1.1	1.0
n-16:0	4.2	4.1	6.7	7.2	4.3	5.9
i-17:1	1.6	-	-	0.9	-	-
i-18:0	1.3	1.1	1.0	1.0	0.8	0.6
n-18:1	8.2	1.0	0.9	7.1	1.9	0.8
n-18:0	10.3	8.5	14.7	14.3	11.9	11.7
i-19:1	2.1	0.3	-	1.5	-	-
i-19:0	0.3	1.7	3.0	0.2	0.6	0.5
n-20:1	2.9	1.1	-	6.8	2.4	1.3
n-20:0	0.5	2.9	3.2	1.3	2.9	9.2
$\sum$ Unsat. FAs	17	3	2	19	5	3
$\sum$ Sat. FAs	83	97	98	81	95	98
$\sum$ *n*-sat. FAs	17	18	28	28	28	35
$\sum$ *i*-sat. FAs	27	34	33	19	24	28
$\sum$ *ai*-sat. FAs	39	45	37	34	43	35
$\sum$ Branched FAs	72	79	71	58	68	62
Unsat. / Sat.	0.20	0.03	0.02	0.23	0.05	0.03
Anteiso / *iso*	1.48	1.33	1.13	1.73	1.78	1.27

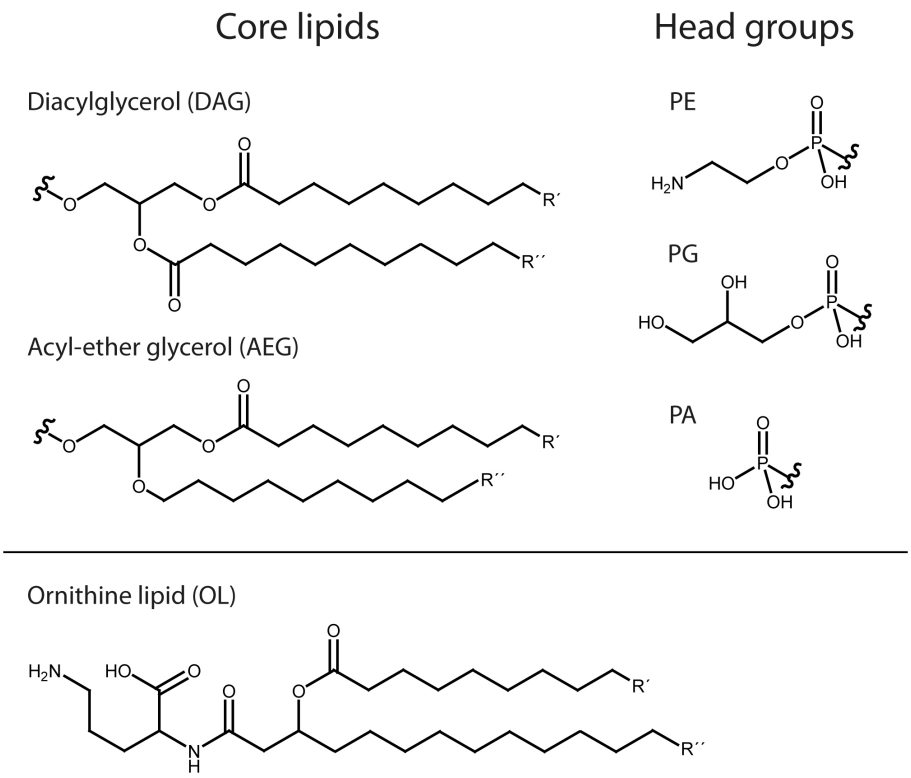

Fig. S1: Molecular structure of major intact polar lipids present in *Desulfovibrio indonesiensis*-affiliated strain P23. OL, ornithine-containing lipid; Head groups of phospholipids: PE, phosphatidylethanolamine; PG, phosphatidylglycerol; PA, phosphatidic acid; Core lipids of phospholipids: Diacylglycerol, DAG with ester-bound fatty acid moieties; Acyl-ether glycerol, AEG with mixed ether/ester bound side chains. R', R", alkyl moieties.

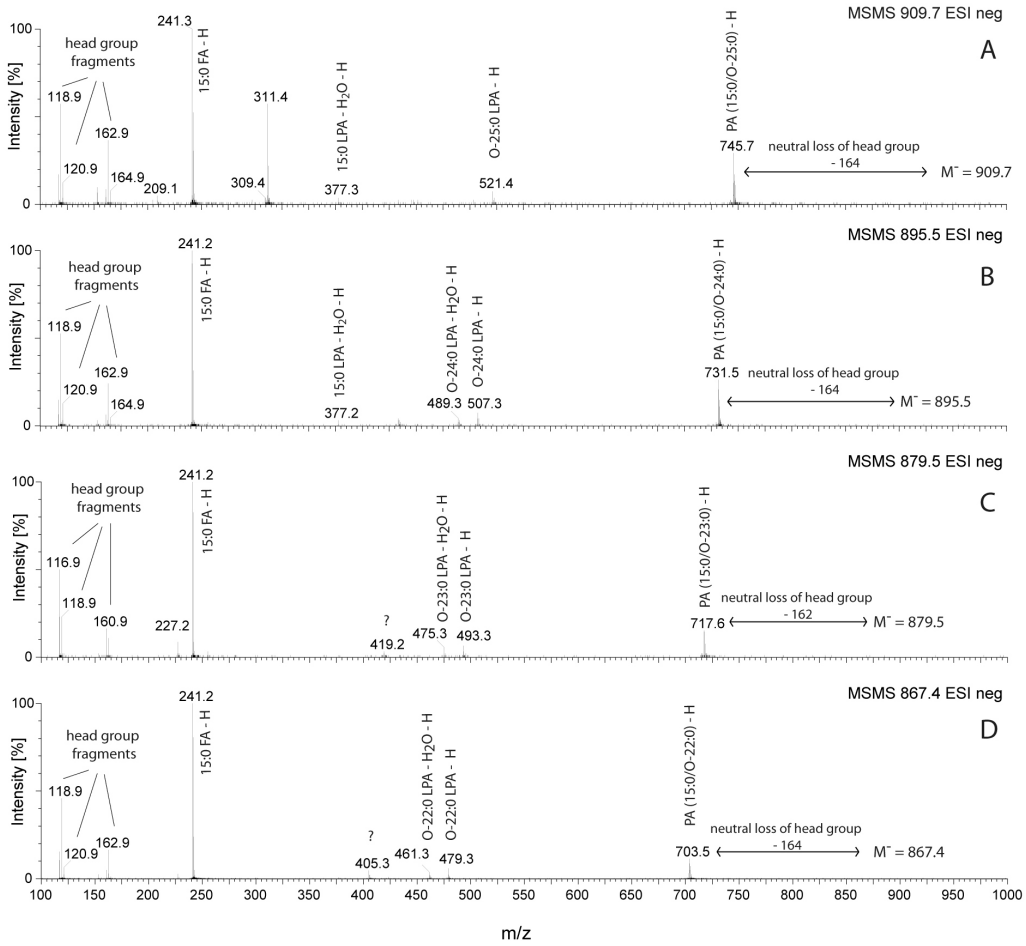

Fig. S2: MS-MS-spectra of unidentified compounds Un-1 (C) and Un-2 (A, B, D) with two unknown head groups detected in strain P23. Spectra were recorded in ESI negative ion-mode.

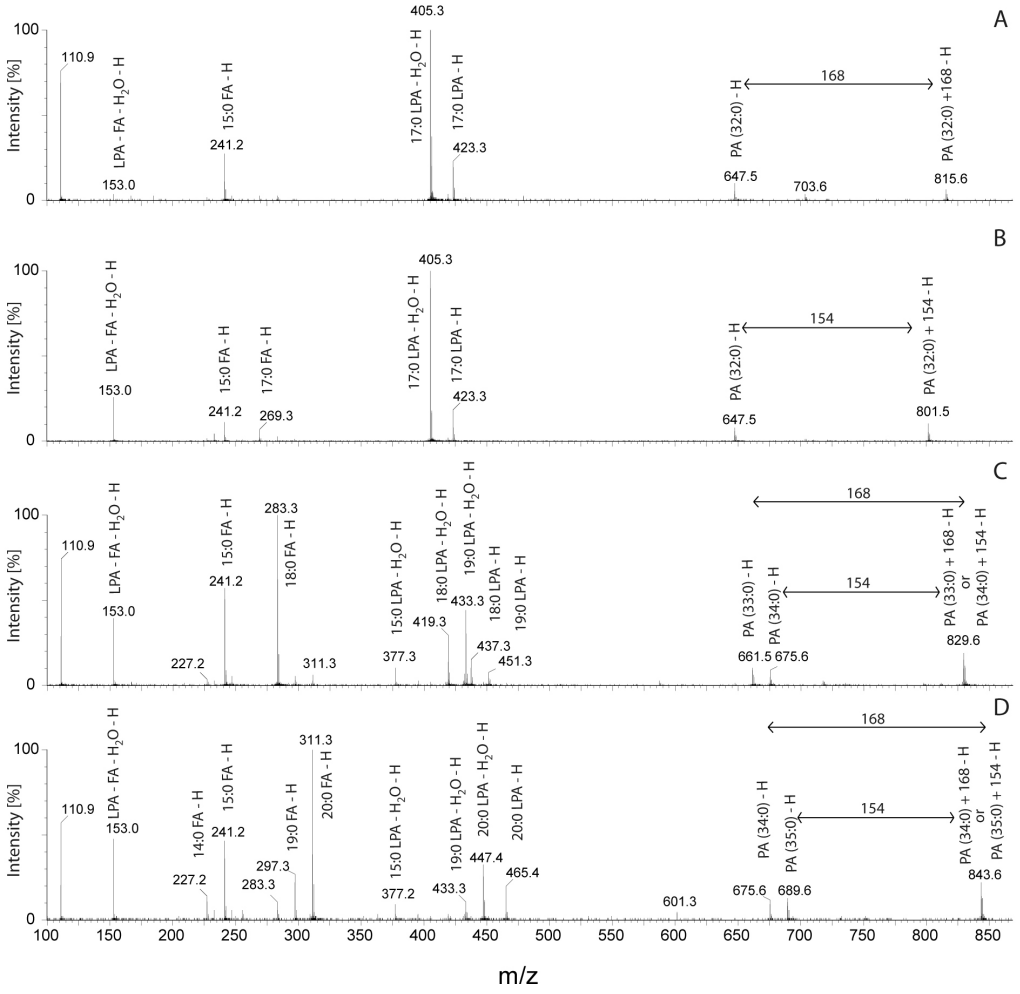

Fig. S3: MS-MS-spectra of unidentified compounds Un-3 (B, C, D) and Un-4 (A, C, D) detected in strain P23. Spectra were recorded in ESI negative ion-mode.

Table S2: IPLs inventory of strain P23. Mass-to-charge ratio (m/z), linked fatty acid combinations and retention times of major molecular species identified by HPLC-ESI-MS-MS in negative ion mode. Abbreviations: OL, ornithine lipid; PE, phosphatidylethanolamine; PG, phosphatidylglycerol; PA, phosphatidic acid; Un1-4, four unidentified lipids, probably yet unknown phospholipid species.

Lipid / Head group	m/z (neg. ion mode)	Fatty acyl side chains (MS-MS analysis)	Retention time [min]
OL	609.5	15:0/3-OH 16:0	8.8
		14:0/3-OH 17:0	
	623.5	15:0/3-OH 17:0	8.6
	635.6	15:0/3-OH 18:1	9.3
	637.6	15:0/3-OH 18:0	9.3
	651.6	15:0/3-OH 19:0	10.4
		14:0/3-OH 20:0	
PE	676.5	15:0/16:0	11.3
		14:0/17:0	
	690.5	15:0/17:0	11.3
		14:0/18:0	
	702.5	15:0/18:1	11.3
	704.6	15:0/18:0	11.1
	718.6	15:0/19:0	11.0
		14:0/20:0	
	730.6	15:0/20:1	11.3
	732.6	15:0/20:0	11.4
PG	693.6	15:0/15:0	10.7
		14:0/16:0	
	719.5	15:0/17:1	10.2
		14:0/18:1	
	721.5	15:0/17:0	9.5
		14:0/18:0	
		15:0/O-18:0	7.8
	733.6	15:0/18:1	10.8
		14:0/19:1	
		15:0/19:1	
	747.5	14:0/20:1	10.7
		16:0/18:1	
		15:0/19:0	
	749.7	14:0/20:0	10.3
		15:0/O-20:0	9.7
	763.7	15:0/20:0	10.4

Table S2: (continued)

Lipid / Head group	m/z (neg. ion mode)	Fatty acyl side chains (MS-MS analysis)	Retention time [min]
PA	661.6	15:0/18:0	
		15:0/19:0	
	675.6	14:0/20:0	7.1
		16:0/18:0	
	689.6	15:0/20:0	7.0
		14:0/21:0	
Un-1	865.5	15:0/21:0	6.4
Mass of head group:	879.5	15:0/O-23:0	4.8
162 Da	893.5	15:0/23:0	6.1
Un-2	867.4	15:0/O-22:0	5.0
Mass of head group:	881.5	15:0/22:0	6.1
164 Da	895.5	15:0/O-23:0	4.8
	909.7	15:0/23:0	6.9
Un-3	787.5	15:0/16:0	16.8
Mass of head group:	801.5	15:0/17:0	16.6
154 Da	813.6	15:0/18:1	16.8
	815.6	15:0/18:0	16.7
	829.6	15:0/19:0	15.6
		14:0/20:0	
	843.6	15:0/20:0	15.9
Un-4	787.5	14:0/15:0	15.7
Mass of head group:	801.5	15:0/16:0	14.7
168 Da	813.5	15:0/17:1	14.8
	815.6	15:0/17:0	14.7

Table S3: Major polar lipid types (in % of total IPLs, intact polar lipids) in strain P23 grown at three different temperatures at atmospheric and high hydrostatic pressure. Abbreviations: OL, ornithine lipid; PE, phosphatidylethanolamine; PG, phosphatidylglycerol; PA, phosphatidic acid; Core lipids: DAG, diacyl glycerol; AEG, Acyl-ether glycerol; Un1-4, four unidentified lipids, probably yet unknown phospholipid species.

IPLs	0.1 MPa			30 MPa		
	20 °C	35 °C	45 °C	20 °C	35 °C	45 °C
OL	26.1	32.2	45.2	15.7	31.3	28.1
∑ Phospholipids	54.1	50.7	41.5	66.8	53.9	56.5
PA-DAG	1.9	0.9	0.5	1.6	0.8	1.6
PE-DAG	22.9	20.2	18.5	23.7	20.1	22.3
PG-DAG	17.2	10.1	6.5	25.3	12.8	13.2
PG-AEG	12.1	19.5	16.0	16.2	20.2	19.4
∑ Un1-4	19.9	17.1	13.4	17.6	14.8	15.6

5 Gesamtbetrachtung

Die vorliegende Arbeit zeigt am Beispiel einer hydrogeologisch sehr gut untersuchten Flanke eines mittelozeanischen Rückens, dass niedrig temperierte, hydrothermale Fluide der Ozeankruste mikrobielles Leben in tiefen marinen Sedimenten unterstützen (Kapitel 5.1). Insgesamt gewährte die detaillierte Untersuchung des Probenahmestandortes einen einzigartigen Einblick inwieweit Umweltbedingungen mikrobielles Vorkommen, Diversität und Aktivität kontrollieren. Die Gewinnung von Reinkulturen indigener Mikroorganismen erlaubte umfassende physiologische Untersuchungen, einschließlich der in der Arbeitsgruppe Paläomikrobiologie erstmalig durchgeführten Wachstumsversuche unter Druck (Kapitel 5.2). Durch diese Analysen konnten wertvolle Informationen über die Ökophysiologie von Vertretern der Tiefen Biosphäre gewonnen werden.

5.1 Fluide der Ozeankruste unterstützen mikrobielles Leben

5.1.1 Sulfateintrag aus der Ozeankruste

Die der Arbeit zugrunde liegende Hypothese, dass Fluide der Ozeankruste durch die Bereitstellung von Elektronenakzeptoren mikrobielles Leben in tiefen, oligotrophen Meeressedimenten unterstützen, konnte in unserem umfangreichen Untersuchungsansatz aus Zellzahlbestimmung, Aktivitätsmessungen und Kultivierung bestätigt werden: Am Untersuchungsstandort wird durch die „bimodale Verteilung von Sulfat“ (Lever, 2008) Einfluss auf die geochemische Zonierung genommen (Kapitel 2) und ermöglicht, dass bis hinab zur Ozeankruste in ca. 265 m Tiefe unter dem Meeresboden eine hohe Zahl verschiedener, metabolisch aktiver Mikroorganismen lebt und Sulfatreduktion stattfindet (Kapitel 2 und 3).

In der oberen Sedimentsäule des Untersuchungsstandortes U1301 wurde ein typisch konkav verlaufendes Porenwasserprofil von Sulfat aus Meerwasser gefunden, das auf eine intensive mikrobielle Sulfatreduktion zurückzuführen ist, die im Bereich der SMTZ (~60 mbsf) die anaerobe Oxidation von Methan einschließt (Kapitel 2). Die in marinen Sedimenten typischerweise sich darunter anschließende Zone, in der hauptsächlich Methanogenese als finaler Remineralisierungsschritt abgelagerten organischen Materials stattfindet, wird durch den diffusiven Eintrag von gelöstem Sulfat aus dem ba-

saltischen Untergrund in ihrer Tiefenausdehnung begrenzt. Dadurch kommt es in ca. 125 m Tiefe zu einer zweiten Sulfat-Methan-Übergangszone, an der das aus den Fluiden stammende Sulfat fast vollständig für die anaerobe Oxidation von Methan verbraucht wird. Der nahezu lineare Sulfatgradient in der unteren Sedimentsäule deutet jedoch *nur scheinbar* auf eine Nachlieferung von gelöstem Sulfat aus der Ozeankruste in die untere SMTZ hin. Durch die Messung von Sulfatreduktionsraten wurde deutlich, dass in den tiefsten Schichten bis hin zur Kruste geringe Teile des Sulfatflusses durch mikrobielle Sulfatreduktion konsumiert werden.

Das Porenwasserprofil von Sulfat und unsere ermittelten Stoffumsatzraten spiegeln letztlich die Verfügbarkeit und Qualität des organischen Materials in den unterschiedlich alten Ablagerungen wieder. An Site U1301 sind die tiefen Sedimentschichten über dreieinhalb Millionen Jahre alter Ozeankruste im Vergleich zu den jüngeren, oberen Schichten eher elektronendonatoren-arm als elektronenakzeptor-limitiert. Aufgrund des geringen Sulfatumsatzes ist anzunehmen, dass der in der unteren Sulfatreduktionszone vorhandene und der durch diffusive Austauschprozesse aus dem Basalt eingetragene organische Kohlenstoff stark degradiert und daher nur schwer abbaubar ist.

5.1.2 AOM in warmen Tiefseesedimenten

Wir konnten zeigen, dass die anaerobe Oxidation von Methan an IODP Site U1301 eine wichtige Rolle spielt (Kapitel 2). Der hydrothermale Einfluss des Aquifers in der Ozeankruste macht sich in der Ausbildung eines Temperaturgradienten von 2 °C in Oberflächensedimenten bis ~62 °C in Krustennähe bemerkbar. Durch die Messung potenzieller Stoffumsatzraten bei *in situ*-Temperaturen stellte sich heraus, dass AOM auch in warmen sulfathaltigen Sedimentschichten mit sehr geringen Methankonzentrationen stattfindet.

Hinweise auf Methanbildner, Methanotrophe und AOM-assoziierte Sulfatreduzierer wurden in unserer Studie nicht erhalten. Die Koexistenz von Methanogenen und AOM-fähigen Konsortien in den warmen Sedimenten (T>25 °C) ist dennoch nicht ausgeschlossen. Molekularbiologische Untersuchungen von Site U1301 im Rahmen der Doktorarbeit von Lever (2008) zeigten, dass in den Schichten sowohl Methanbildner als auch Vertreter der anaeroben methanotrophen ANME-1-Gruppe vorkommen, von der vermutet wird, an AOM-Prozessen beteiligt zu sein. Dem Autor zufolge können Methanogene in sulfatreichen Schichten leben, wenn sie nichtkompetitive

Substrate nutzen, zum Beispiel Methanol, Methylamine oder Methylsulfide. AOM wurde lange nur an kalten Standorten mit hohen Methanvorkommen untersucht. Kallmeyer & Boetius (2004b) konnten jedoch bereits experimentell zeigen, dass AOM nicht auf kalte Regionen beschränkt ist, sondern auch in hydrothermalen Sedimenten, beispielsweise im Guaymas Becken, auftritt. Erst kürzlich veröffentlichte Studien zur AOM an heißen Standorten beweisen, dass der Prozess und die daran beteiligten Organismen in marinen Systemen tatsächlich ubiquitär sind (Biddle et al., 2012; Wankel et al., 2012).

5.1.3 Isolierung von nichtsporenbildenden Sulfatreduzierern

Eine weiterführende, kultivierungsabhängige Untersuchung der Sedimentsäule wurde zur Identifizierung von lebensfähigen und potenziell aktiven Bakterien angewandt. Dabei wurden durch die molekularbiologische Analyse („Screening“) der Erstanreicherungskulturen Änderungen in der Zusammensetzung der mikrobiellen Gemeinschaften aufgedeckt (Kapitel 3). Durch die Detektion und Isolierung vorwiegend nichtsporenbildender, darunter sulfatreduzierender Bakterien aus der unteren Sulfatzone werden die veränderten, stimulierenden Bedingungen für mikrobielles Leben in den tiefen Sedimentschichten durch den Einfluss der hydrothermalen Fluide widergespiegelt. Fluide der Ozeankruste unterstützen insgesamt zwar nur geringe, aber in Anbetracht des Alters der untersuchten Flanke, wahrscheinlich über lange Zeiträume andauernde mikrobielle Stoffwechselaktivitäten. Die Gewinnung der nichtsporenbildenden, sulfatreduzierenden *Deltaproteobakterien* aus ca. dreieinhalb Millionen Jahre alten, basaltnahen Sedimentschichten machte zusätzlich deutlich, dass diese die langfristige Entfernung von der Meeresbodenoberfläche als aktive Populationen überstanden haben müssen, sofern es keine anderen Besiedelungswege gibt. Dies ist wahrscheinlich a) auf ihre metabolische Vielseitigkeit zurückzuführen, die ihnen mit Hilfe der kontinuierlichen Versorgung mit Sulfat aus dem basaltischen Grundwasserleiter erlaubt, chemoheterotroph als auch -lithoautotroph Energie zu gewinnen (Kapitel 3) und b) auf die Fähigkeit, sich an hohe Temperaturen, Druck und zum Beispiel an begrenzte Phosphatkonzentrationen anzupassen (Kapitel 4 und 5.2).

5.1.4 Wasserstoff als Elektronendonator

Die in der Tiefe genutzten Elektronendonatoren sind nicht bekannt. Auch der zusätzliche Einfluss thermogener, geochemischer und anderer biogener Effek-

te auf die Verfügbarkeit potenzieller Nährstoffe am Untersuchungsstandort bleibt in der vorliegenden Arbeit unbestimmt. Durch die Isolierung von hydrogenotrophen und fakultativ autotrophen Sulfatreduzierern aus krustennahen Schichten wird jedoch die Annahme unterstützt, dass molekularer Wasserstoff als anorganischer Elektronendonator nicht nur an Rückenachsen mit hohen magmatischen Gaskonzentrationen eine wichtige Rolle spielt, sondern auch in den oligotrophen Sedimenten über älteren Flanken mittelozeanischer Rücken (Kapitel 3).

Tatsächlich wurden kürzlich über ein „Circulation Obviation Retrofit Kit“, CORK 1301A, das während der IODP Expedition 301 am Juan-de-Fuca-Rücken installiert wurde, geringe Wasserstoffkonzentrationen in den hydrothermalen Fluiden nachgewiesen (0,5 µM; Lin et al., 2012). Neben verschiedenen biogenen Prozessen in den Fluidkanälen (Lin et al., 2012) sind am Untersuchungsstandort Serpentinisierungsprozesse des eisenhaltigen Basaltbestandteils Olivin (Lever et al., 2013) die wahrscheinlich wichtigsten Quellen für Wasserstoff, der über diffusive Austauschprozesse in die darüberliegenden Sedimentschichten gelangt. Dort werden einer Studie von Lever et al. (2010) zufolge hydrogenotrophe Acetogeneseprozesse stimuliert, was die „leichten“ Acetatisotope im Porenwasser der Sedimente erklären würde. Inwiefern hydrogenotrophe Sulfatreduktion eine Rolle spielt oder ob die dort vorkommenden Sulfatreduzierer zur Homoacetogenese fähig sind, könnte in einer künftigen Studie geklärt werden (siehe auch Kapitel 6).

5.2 Anpassung von Mikroorganismen an hohe Temperaturen und hohen Druck

Die im Rahmen der Doktorarbeit durchgeführten Druckexperimente mit *Desulfovibrio*-Stamm P23 lieferten wertvolle Informationen über die Ökophysiologie eines piezophilen Vertreters der Tiefen Biosphäre (Kapitel 4). Durch die komplementäre Analyse der Gesamtfettsäuren und Membranlipide konnten zelluläre homöostatische Anpassungsmechanismen entschlüsselt werden. Insgesamt zeigte sich, dass die Temperatur deutlichere Effekte hervorruft als Druck und dass Stamm P23 seine Membranviskosität und -funktion über den Ornithingehalt und durch strukturelle Änderungen der Hauptphospholipide reguliert. Unter Druck wurden erhöhte Anteile an (AEG-basierten) Phospholipiden, längere Seitenketten und ein höherer Sättigungsgrad induziert.

5.2.1 Wachstum eines Vertreters der Tiefen Biosphäre unter Druck

An Site U1301 sind die Bakterien, die in Krustennähe leben, hohen Temperaturen und einem hohen hydrostatischen Druck ausgesetzt. Piezophilie ist eine wichtige Eigenschaft von marinen Mikroorganismen, die bei Stammbeschreibungen nur selten berücksichtigt wird. Es zeigte sich, dass *Desulfovibrio*-Stamm P23 moderat piezothermophil ist, da er unter Druck bis zu einer Temperatur von 50 °C wuchs und hoher Druck (10-30 MPa) das Zellwachstum förderte. Mikrobielle Aktivitäten dieser Bakterienart unter *in situ*-Bedingungen konnten in der vorliegenden Studie nicht demonstriert werden. Obwohl Stamm P23 ursprünglich aus ca. 60 °C heißen Sedimenten stammt, wurden unter Druck bei dieser Temperatur im Untersuchungszeitraum weder heterotrophes Wachstum noch Sulfatatmung beobachtet. Da unter Druck jedoch Verschiebungen im Temperaturbereich für Wachstum möglich sind und sich Druck auch auf abiotische Faktoren wie beispielsweise die Löslichkeit von Gasen auswirkt, wäre es noch interessant gewesen, autotrophes Wachstum von Stamm P23 unter Druck zu analysieren. Hierfür wäre jedoch eine technische Ausstattung nötig gewesen, die eine Kultivierung mit gasförmigen Substraten ermöglicht. In einer künftigen Studie wäre die Nutzung von gasdichten Glasspritzen (Takai et al., 2008) denkbar oder ein spezielles Hochdruck-Inkubationssystem, wie es jüngst von Sauer et al. (2012) entwickelt wurde. Berücksichtigt werden sollte auch, dass Stamm P23 unter Druck bei dieser Temperatur möglicherweise nur äußerst langsam wächst.

Der Schlüssel für die erfolgreiche Kultivierung von Mikroorganismen liegt in der möglichst nahen Nachahmung der Umweltbedingungen im Habitat. Die meisten Anreicherungskulturen werden unter *in situ*-Temperaturen inkubiert. Hydrostatischer Druck wird bei der Kultivierung von Tiefseebakterien dagegen nur selten berücksichtigt. Durch unsere Druckexperimente wurde deutlich, dass sich fakultativ piezothermophile Mikroorganismen tatsächlich erfolgreich unter normalen Laborbedingungen, also unter Atmosphärendruck, kultivieren lassen, wenn gleichzeitig die *in situ*-Temperatur verringert wird. Neben der geeigneten Wahl des Mediums könnte dieses Vorgehen zukünftig als Isolierungsstrategie zur Gewinnung piezomesophiler oder -thermophiler Reinkulturen eingesetzt werden. Zunutze macht man sich in diesem Fall die essenzielle Fähigkeit von Mikroorganismen, sich durch die Regulierung ihrer Membranfluidität an sich verändernde Umweltbedingungen anzupassen („homeoviskose Anpassung“).

5.2.2 Molekulare Analyse von Membranlipiden

Informationen über die homeoviskose Anpassung von Membranlipiden sind hauptsächlich aus Studien mit psychropiezophilen Tiefseebakterien oder grampositiven Thermophilen bekannt. Die molekulare Analyse der zellulären Lipide unseres Stammes P23 hat aufschlussreiche Ergebnisse zur Anpassung eines moderat piezothermophilen, gramnegativen Bakteriums an hohe Temperaturen und Druck geliefert (Kapitel 4). Da die biophysikalischen Eigenschaften von Zellmembranen wesentlich durch die in den Membranlipiden gebundenen Fettsäuren beeinflusst werden (Zhang & Rock, 2008), lag der Hauptmechanismus der homeoviskosen Anpassung wie erwartet in der Veränderung der Struktur der Fettsäureketten. Durch die Analyse der Gesamtfettsäuren hat sich gezeigt, dass Stamm P23 hohe Anteile verzweigtkettiger FAs (58-79 %) besitzt, wie es für die Gattung *Desulfovibrio* (Vainshtein et al., 1992) und viele thermophile Bakterien (Russell & Fukunaga, 1990) charakteristisch ist. Bei hohen Temperaturen und unter Druck wirkten hohe Anteile hochschmelzender gesättigter Fettsäuren einer zu hohen Membranfluidität entgegen. Interessanterweise erfolgte bei Stamm P23 die Regulierung in zwei Stufen. Es wurde zunächst der Grad der Unsättigung abgebaut und als eine Art „Feinregulierung“ bei noch höheren Temperaturen der Grad der Verzweigung reduziert. Interessant wäre noch gewesen herauszufinden, welche piezothermostabilen Enzyme dabei eine Rolle spielen.

Variationen in der Lipidklasse werden ebenfalls als Mechanismus zur Regulierung des Schmelzpunktes interpretiert (z.B. Russell & Fukunaga, 1990). Von größerer Bedeutung könnte jedoch sein, dass durch die veränderten IPL-Gehalte eine Membranstruktur erreicht wird, um essenzielle Zellfunktionen, zum Beispiel die Permeabilität oder den Elektronentransport in der Atmungskette, aufrecht zu erhalten (z.B. Zhang & Rock, 2008). Die quantitativ wichtigsten Membranlipide in Stamm P23 sind die Phospholipide Phosphatidylethanolamin (PE) und Phosphatidylglycerol (PG) sowie phosphatfreie Ornithinlipide (OL) (Kapitel 4). Durch die massenspektrometrische Lipidanalyse wurden Phospholipide bis in ihre Grundstruktur identifiziert. Dies ermöglichte den Vergleich von Temperatur- und Druckeffekten auf Kopfgruppen, Seitenketten und das Glycerolgrundgerüst. Das für die Gattung *Desulfovibrio* charakteristische Diphosphatidylglycerol (Makula & Finnerty, 1975) wurde nicht nachgewiesen. Dafür wurden vier weitere Phospholipide detektiert, deren Kopfgruppe nicht bestimmbar war. Möglicherweise stellen sie deshalb neue IPL dar, was für die Biomarkerfoschung interessant sein könnte (für nähere Erläuterungen siehe Logemann, 2013).

Die Phospholipide lagen vorwiegend als Diacylglycerole (DAG) vor, während PG zusätzlich als Acyletherglycerol (AEG) detektiert wurde. Im Fall von Stamm P23 zeigte sich, dass Phospholipide bis zur Art ihrer Basisstruktur dem Einfluss von Temperatur und Druck unterlagen. Höhere Temperaturen und Druck förderten den Einbau von AEG-Phospholipiden. Über AEG-Kernlipide ist generell wenig bekannt. Bisher wurden sie nur in mesophilen sulfatreduzierenden Bakterien nachgewiesen (Rütters et al., 2001). Inwiefern derartige strukturelle Änderungen in Zusammenhang mit der Schmelzpunktregulierung der Membranlipide stehen oder zur Membranstabilisierung und -funktion beitragen, bleibt zu klären.

Ornithinlipide sind in Bakterien weit verbreitet (Aygun-Sunar et al., 2006; Geiger et al., 2010). Vertreter der Gattung *Desulfovibrio* sind bisher jedoch die einzigen Deltaproteobakterien, in denen OL bisher nachgewiesen wurden (Makula & Finnerty, 1975; Seidel et al., 2013). Ornithinlipide werden in Abhängigkeit von den Wachstumsbedingungen produziert. In unserer Studie wurde bestätigt, dass die OL-Gehalte in der Cytoplasmamembran mit ansteigenden Temperaturen zunehmen. Diese Temperaturabhängigkeit wurde auch bei Seidel et al. (2013) gefunden, der verschiedene *Desulfovibrio acrylicus*-Stämme untersucht hat.

Darüber hinaus liefert unsere Studie erstmals Informationen über den Einbau von OL unter Druck: Unter hohem Druck wurden weniger OL zugunsten der Phospholipide eingebaut. Interessanterweise wurden keine wesentlichen strukturellen Veränderungen in den Seitenketten der OL unter Druck gefunden. Dies könnte ein Hinweis darauf sein, dass die Membranviskosität vorwiegend über die Seitenketten der Phospholipide und weniger über die Seitenketten der OL reguliert wird.

Über die (öko)physiologische Rolle von Ornithinlipiden wird in der Fachwelt generell spekuliert (siehe z.B. Aygun-Sunar et al., 2006; Seidel, 2009; Geiger et al., 2010). Neben der Regulierung membrangebundener Proteine wird die Biosynthese von phosphatfreien Ornithinlipiden als Anpassung an geringe Phosphatkonzentrationen im Habitat angenommen. Weissenmayer et al. (2002) zeigte, dass der OL-Gehalt unter Phosphatlimitierung zunahm. Tatsächlich wurde Stamm P23 aus phosphatarmen Schichten isoliert (Kapitel 2, 3), die Druckexperimente jedoch wurden mit phosphathaltigem Medium durchgeführt. Weiterführende Experimente mit Stamm P23 unter phosphatlimitierten Bedingungen könnten klären, welche mögliche Rolle Ornithinlipide unter hohen Temperaturen und Druck tatsächlich spielen.

6 Ausblick

6.1 CO_2-Fixierung von *Desulfovibrio*-Stamm P23

Physiologische Untersuchungen haben gezeigt, dass *Desulfovibrio*-Stamm P23 chemolithoautotroph mit CO_2 als einziger Kohlenstoffquelle wachsen kann (Kapitel 3). Fakultativ autotrophe Sulfatreduzierer nutzen zur Fixierung von CO_2 entweder den reduktiven Citratzyklus oder den reduktiven Acetyl-CoA-Weg (Fuchs, 2008). Bei heterotrophem Wachstum nutzen sie den entsprechend umgekehrten Weg, um Acetat oder andere organische Substrate vollständig über Acetyl-CoA als Intermediat zu Kohlendioxid zu oxidieren (Schauder et al., 1986; Muyzer & Stams, 2008). Autotrophes Wachstum eines *Desulfovibrios* ist insofern überraschend, da die Vertreter der Gattung für die Verwertung von Wasserstoff als Elektronendonator gewöhnlich Acetat als Kohlenstoffquelle benötigen und unvollständige Oxidationsprofile aufweisen (Schauder et al., 1986; Fuchs, 2008). Es stellt sich daher die Frage, über welchen Stoffwechselweg Isolat P23 Kohlendioxid assimiliert.

Erste enzymatische Untersuchungen in Zusammenarbeit mit Dr. Alexander Galushko (MPI Bremen) nach Methoden von Galushko & Schink (2000) und Kuever et al. (2001) deuten auf den nichtzyklischen, reduktiven Acetyl-CoA-Weg hin. In zellfreien Extrakten von Stamm P23 wurde das entsprechende Schlüsselenzym Kohlenmonoxid(CO)-Dehydrogenase/Acetyl-CoA-Synthase mit einer schwachen spezifischen Aktivität von 50 nmol min^{-1} (mg protein)$^{-1}$ nachgewiesen. Eine aktive, für den reduktiven Citratzyklus charakteristische 2-Oxoglutarat:Elektronenakzeptor Oxidoreduktase war nicht nachweisbar. Bei Wachstum von Stamm P23 mit Lactat war Acetat als Endprodukt im Medium nachweisbar. Es ist deshalb davon auszugehen, dass Stamm P23 unfähig ist, Acetyl-CoA zu oxidieren und damit gattungstypisch zu den unvollständigen Oxidierern zu zählen. Während Wachstum mit Acetat als Substrat nicht beobachtet wurde, wuchs Stamm P23 jedoch mit Formiat (Fichtel et al., 2012, Kapitel 3), welches ein Intermediat im reduktiven Acetyl-CoA-Weg ist.

Die bisherigen Ergebnisse sollten ein Anlass sein, weitere Enzymanalysen sowie physiologische und molekularbiologische Untersuchungen zur Abschätzung des metabolischen Potenzials von Isolat P23 durchzuführen. Der Nachweis weiterer aktiver Schlüsselenzyme, zum Beispiel der Formiat-Dehydrogenase (Fuchs, 1986; Galushko & Schink, 2000), könnte den reduktiven Acetyl-CoA-Weg untermauern, der auch in autotrophen Metha-

nogenen und acetogenen Bakterien gefunden wird (Schauder et al., 1986, und darin enthaltene Referenzen). Es sollte außerdem untersucht werden, ob *Desulfovibrio*-Stamm P23 dazu fähig ist, neben Formiat auch andere C_1-Verbindungen wie Kohlenmonoxid oder methylierte Verbindungen als Energie- bzw. Kohlenstoffquelle zu nutzen (Fuchs, 1986), insbesondere auch unter sulfatfreien Bedingungen (Homoacetogenese).

6.2 Genomanalyse von *Desulfovibrio*-Stamm P23

Unser moderat piezothermophiler, autotropher *Desulfovibrio*-Stamm P23 repräsentiert eines der wenigen Isolate der marinen Tiefen Biosphäre. Die vollständige Genomanalyse könnte einen umfassenden Einblick in das Anpassungsvermögen des Sulfatreduzierers an die extremen Bedingungen geben, unter denen er tief im Sediment nahe der Ozeankruste lebt. Besonderes Augenmerk sollte dabei auf Gene gelegt werden, die 1) im Zusammenhang mit Anpassungsstrategien an hydrostatischen Druck und hohe Temperaturen stehen, beispielsweise Gene für Biosynthesewege von Fettsäuren, Ornithinen und Phospholipiden, und 2) Hinweise auf den CO_2-Fixierungsweg geben. Die Ergebnisse sollten mit den jüngst veröffentlichten Genomdaten anderer *Desulfovibrio*-Arten verglichen werden, zum Beispiel den piezophilen Arten *D. hydrothermalis* (Ji et al., 2013) und *D. piezophilus* (Pradel et al., 2013).

6.3 Erweiterte Stammbeschreibung von *Desulfovibrio indonesiensis*

Unseren phylogenetischen Analysen entsprechend gehört Stamm P23 der Gattung *Desulfovibrio* an. Auf Basis der vollständigen 16S DNA zeigte er eine 99 %ige Sequenzähnlichkeit zum Typenstamm *Desulfovibrio indonesiensis* Ind1^{T} und stellt deshalb keine neue Art dar. Unsere ermittelten Daten bezüglich seiner teils außergewöhnlichen physiologischen Merkmale (Autotrophie, Piezophilie, Temperaturbereich für Wachstum) und seiner Membranlipide rechtfertigen jedoch eine erweiterte Stammbeschreibung.

6.4 Verwendung von Reinkulturen für weitere Studien

Aus der vorliegenden Arbeit gingen 40 Isolate aus verschiedenen phylogenetischen Gruppen hervor (Kapitel 3). Nicht alle konnten vollständig charakterisiert werden, obwohl es sich wahrscheinlich bei den meisten um neuarti-

ge Bakterien mit möglicherweise bisher nicht beschriebenen physiologischen Fähigkeiten handelt. Kultivierte Vertreter der Tiefen Biosphäre wie Stamm P23 sollten nicht nur in zukünftige mikrobielle Studien, zum Beispiel zu Phagen-Wirt-Beziehungen oder in „*Starvation*"-Experimente, mit einbezogen werden, sondern auch als genetische Modellorganismen dienen. Diese könnten helfen, die Diskrepanz zwischen den Ergebnissen molekularbiologischer und kultivierungsabhängiger Diversitätsstudien zu minimieren. Mit Hilfe verbesserter Primerpaare und Sonden könnte über die qPCR von *dsr*-Genen oder über CARD-FISH das Vorkommen von Sulfatreduzierern und der Anteil einzelner Populationen an der Gemeinschaft sulfatreduzierender Prokaryoten genauer ermittelt werden, insbesondere in Krustennähe sowie an anderen ähnlichen Standorten (siehe Edwards et al., 2012).

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Danksagung

An dieser Stelle sage ich all denjenigen „Danke“, die mich während meiner Studienzeit und Promotion unerlässlich unterstützt haben: Heribert Cypionka und Bert Engelen: Seit 2001 bin ich Eurer AG Paläomikrobiologie angehaftet, als Studentin, Hiwi, Diplomandin und zuletzt Doktorandin. In dieser Zeit habt Ihr mir vieles beigebracht, was für den Beruf und im Leben wichtig ist und was man als Wichtig erachten sollte. Danke, dass Ihr mir Euer Vertrauen geschenkt und mich immer unterstützt habt, vor allem in der Zeit nach der Geburt meiner Zwillinge Franka und Luis und trotz einer Entfernung von 600 km. Euer Engagement und Eure Geduld sind bemerkenswert! Bert, Dir sei besonders gedankt für die Bereitstellung des Promotionsthemas innerhalb Deines Projekts, Dein Vertrauen, das Bestmöglichste aus dem wertvollen Probematerial „herauszuholen“ und Deine stete freundschaftliche Kollegialität. Und, warum einfach, wenn's auch kompliziert geht;-)

Jacqueline „Jezi“ Süß, Wilm Martens-Habbena, Reinhard Wilms, Martin Könneke, Andrea Schlingloff und Michael Pilzen: Ihr habt mir nicht nur mikro- und molekularbiologisches Grundhandwerk sondern darüber hinaus viel fachliches Wissen, Praktisches und Zwischenmenschliches beigebracht. Diese Zeit und die gemeinsamen Tagungsteilnahmen werden mir immer in Erinnerung bleiben.

Jörn Logemann: Wer hätte gedacht, dass aus einer Mittagessensbegleitung, einem Volleyball-Zuspieler und Doktorandenschicksalsteiler einmal ein Arbeitspartner werden würde. Der Zufall wollte es wohl. Dein Fachwissen und Lipid-analytisches Know-How haben mich beeindruckt und meine Sicht der Dinge ungemein bereichert. Und schön, dass Du immer einmal wieder auf einen Kaffee bei uns in Burghaslach vorbeischaust.

Antje Gittel, Stephanie „Steph“ Reischke, Heike Simon, Jöran März, Björn Klein und vor allem Annka Scherf-Klein sowie Michael Seidel: Ihr habt meine Zeit am ICBM und darüber hinaus freundschaftlich begleitet. Danke Euch für die vielen Unternehmungen, Kino- und Tatortabende, Oscarnächte, Kaffeepausen und Volleyball-Matches, einfach für die lustige Zeit.

Gebührlicher Dank gilt auch meinen Lieben hier in Hosla, für ihren Ansporn und die ewig berechtigte Frage, wie lange ich noch „herumdoktern“ möchte: Alex, Chrissi, Ruth, Daniel und Nicole.

Mein Mann Jörg: Dir gebührt innigster Dank und tiefe Liebe dafür, dass Du mich neben unserem familiären Glück (Kinder und Hausbau) und trotz

mancher Diskussion immer unterstützt und ermutigt hast, meine Doktorarbeit abzuschließen: Und, irgendwann werde ich sie doch aufgeben, folgende Annonce: „Zum Zwecke der Gründung einer Interessensgemeinschaft: Meeresmikrobiologen in Mittelfranken gesucht“.

Ruth und Peter: Euch sei ebenfalls sehr gedankt dafür, dass Ihr uns bis zum Einzug ins eigene Häuschen ertragt, uns in mannigfaltiger Weise unterstützt und uns Zeit für Freizeit schenkt. Ihr habt wohl auch nicht geahnt, was da auf Euch zukommt, oder?

Matthias und Hannes: Danke dafür, dass Ihr Franka und Luis lustige Paten seid und Euer „Amt“ ernst nehmt.

Nicht zuletzt danke ich von ganzem Herzen meinen Eltern Marianne und Helmut sowie meiner Schwester Andrea für Ihre stete Liebe und geduldige Unterstützung. *Seine Familie kann man sich nicht aussuchen - aber so mancher Mensch scheint Glück gehabt zu haben - wie ich.*

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Tagungsbeiträge

Fichtel, K., Engelen, B., Cypionka, H., 2014. Hydrothermal fluids from the ocean crust support microbial life in deeply buried sediments (IODP Exp. 301). *IODP/ICDP Kolloquium*, Erlangen, Vortrag.

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Erklärung

Hiermit versichere ich, dass ich die vorliegende Arbeit selbstständig angefertigt habe und keine anderen als die angegebenen Quellen und Hilfsmittel verwendet habe. Zusätzlich erkläre ich, dass diese Dissertation weder in ihrer Gesamtheit noch in Teilen einer anderen wissenschaftlichen Hochschule zur Begutachtung in einem Promotionsverfahren vorliegt oder vorgelegen hat.

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