Microbial Methane Formation in Abandoned Coal Mines in the Ruhr Basin of Germany

Dissertation

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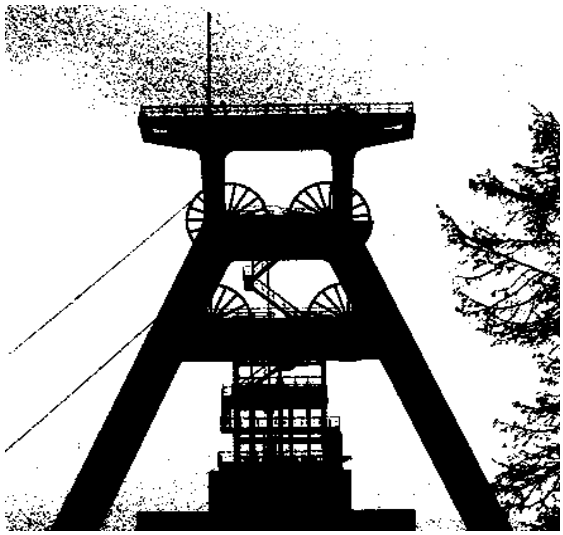

„Wer aber soll hausen in jenen Welten, wenn sie bewohnt sein sollten?

Sind wir oder sie die Herren?

Und ist dies alles dem Menschen gemacht?“

Johannes Kepler

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Zusammenfassung

Das Grubengas aus Kohleflözen wurde innerhalb der letzten Jahre zunehmend für die Produktion von Strom und Wärme genutzt (Thielemann *et al*. 2004). Global verursacht der Kohlebergbau etwa 7% der jährlichen Methanemissionen in die Atmosphäre (Denman *et al*. 2007). Die Kohlenstoff- und Wasserstoffisotopien des Methans in den Minenatmosphären zeigten, dass das Methan thermogenen und biogenen Ursprungs ist (Thielemann *et al.* 2004).

Das Ziel dieser Studie war der Nachweis der rezenten mikrobiellen Methanproduktion. Die verantwortlichen Mikroorganismen sollten identifiziert und physiologisch charakterisiert werden. Die aktiven methanogenen Archaeen sollten identifiziert und quantitativ erfasst werden, aber auch die aktiven Bakterien, die die methanogenen Prozesse begleiten. Für die folgenden geochemischen und mikrobiologischen Untersuchungen wurde Steinkohle und Grubenholz aus stillgelegten Kohleschächten zweier Zechen im Ruhrgebiet in Deutschland entnommen und unter *in situ* Bedingungen inkubiert.

Unsere Untersuchungen zeigten, dass die mikrobielle Methanproduktion eine rezente Methan-Ressource darstellt. Das Methan in den Minenatmosphären wies eine Gaszusammensetzung auf, die biogenen und thermogenen Ursprungs ist, wobei der biogene Anteil mit bis zu 80% überwiegt. Identische Isotopien wurden auch in den *in situ* Methanemissionen aus der Kohle und dem Grubenholz sowie in den Langzeitinkubationen nachgewiesen. Die Langzeitinkubationen, in denen Kohle und Holz als alleinige Kohlenstoffquelle vorhanden waren, zeigten eine konstante Methanproduktion über eine Inkubationszeit von 9 Monaten mit einer höheren Methanproduktion in den Holzinkubationen. In den mit methanogenen Substraten, Acetat und H_2+CO_2 angereicherten Kulturen wurde vorwiegend die acetoclastische Methanogenese stimuliert. Die Methanemissionsraten aus dem Holz waren 3-4mal höher im Vergleich zur Kohle.

An der Methanbildung in diesem Minensystem ist eine komplexe Lebensgemeinschaft aktiv involviert. Phylogenetische Analysen zeigten die Präsenz einer Gemeinschaft aus Holz abbauenden Pilzen (*Ascomyceten* und *Basidiomyceten*) und fakultativ anaerober Bakterien (α - ε) *Proteobacteria*, *Bacteroidetes*, *Tenericutes, Actinobacteria*, *Chlorobi* and *Chloroflexi*). Die Archeen wurden von Vertretern der *Methanosarcinales* und der *Crenarchaeen* repräsentiert. Die methanogene Gemeinschaft in den Anreicherungen und in den Originalproben wurde von den *Methanosarcinales,* nahe verwandt mit *Methanosarcina barkeri*, dominiert. Die acetoclastische Methanbildung erfolgte nicht nur in den Acetat Anreicherungen sondern auch in den H_2+CO_2 Stimulationen von Kohle und Holz. H_2+CO_2

wurde hauptsächlich von den Acetogenen, nahe verwandt mit *Pelobacter acetylenicus* und *Clostridium* sp., genutzt. Diese bildeten Acetat welches dann den Methanogenen zur Verfügung steht. Obwohl *Methanosarcina* sp. dafür bekannt ist sowohl H_2+CO_2 als auch Acetat für die Methanogenese zu nutzen, verwerteten die von uns identifizierten das leichter zugängliche Acetat als den thermodynamisch günstigeren Wasserstoff. Die mikrobielle Gemeinschaft scheint sehr gut an die geringen Wasserstoff Konzentrationen in den Kohleminen angepasst zu sein und nuzten Acetat als Hauptvorstufe für das biogene Methan.

Summary

Mine gas has come into the focus of the power industry and is being used increasingly for heat and power production worldwide (Thielemann *et al*. 2004). About 7% of the annual methane emissions originate from coal mining (Denman *et al*. 2007). In many coal deposits, stable carbon and hydrogen isotopic signatures of methane indicate a mixed thermogenic and biogenic origin (Thielemann *et al*. 2004).

The present study focused on the identification of recently produced coal-mine methane as well as on the diversity, abundance, and activity of the microorganisms linked directly to methanogenesis. We aimed to unravel the active methanogens being responsible for the methane release but also the active bacteria involved in the metabolic network. Therefore, weathered hard coal and mine timber were collected in two abandoned coal mines in the Ruhr Basin of Germany.

The combined geochemical and microbiological investigations identified microbial methanogenesis as a recent source of methane. Mine timber and hard coal showed an *in situ* production of methane with isotopic signatures similar to those of the methane in the mine atmosphere. Long-term incubations of coal and timber as sole carbon sources formed methane over a period of 9 months. Predominantly, acetoclastic methanogenesis was stimulated in enrichments containing acetate or H_2+CO_2. Highest methane formation rates were detected in the timber incubations (3-4 times higher than in the coal incubations). The methane-formation processes are complex. Wood-degrading fungi (*Ascomycetes* and *Basidiomycetes*) and a broad spectrum of facultative anaerobic bacteria (α-ε *Proteobacteria*, *Bacteroidetes*, *Tenericutes, Actinobacteria*, *Chlorobi* and *Chloroflexi*) were detected. Archaea were represented by members of *Methanosarcinales* and *Crenarcheaota*. Thus, the methanogenic community in the enrichments and unamended samples was dominated by *Methanosarcinales* closely related to *Methanosarcina barkeri*. The formation of methane was due to acetoclastic methanogenesis in the acetate but also in the H_2+CO_2 cultures of coal and timber. The H_2+CO_2 was mainly used by acetogens similar *to Pelobacter acetylenicus* and *Clostridium* species forming acetate as intermediate and providing it to the methanogens. Although, *Methanosarcinales* are known to use both, hydrogen as well as acetate, those identified rather utilized the easier accessible acetate than the thermodynamically more preferential hydrogen. The microbial communities appeared highly adapted to the low H_2 concentrations in the coal mine with acetate as the main precursor of the biogenic methane.

List of publications

The results presented in this dissertation have been published in international journals summarized below.

1. Krüger M, **Beckmann S**, Engelen B, Thielemann T, Cramer B, Schippers A, and Cypionka H. (2008) *Microbial methane formation from hard coal and timber in an abandoned coal-mine*. Geomicrobiology Journal 25:315-321.

   Concept: M.K., S.B., B.E., H.C., B.C., T.T.; Analysis of results: S.B., M.K.; Sampling: M.K., S.B., B.E.; Geochemical analysis: M.K.; Molecular biological analysis: S.B., B.E.; First draft of manuscript: M.K., S.B., B.E.; Revision by: H.C.

2. **Beckmann S**, Krüger M, Engelen B, Gorbushina A, and Cypionka H. (2011) *Role of Bacteria, Archaea and Fungi involved in methane release in abandoned coal mines.* Geomicrobiology Journal 28(4).

   Concept: S.B., B.E., H.C.; Analysis of results: S.B.; Sampling: S.B., B.E., M.K.; Geochemical analysis: S.B., M.K.; Molecular biological analysis: S.B.; Molecular analysis of *Fungi*: S.B., A.A.G.; First draft of manuscript: S.B.; Revision by: B.E., A.A.G., H.C.

3. **Beckmann S**, Lüders T, Krüger M, von Netzer F, Engelen B, and Cypionka H. (2010) *Activity of acetoclastic Methanosarcinales and acetogens in abandoned coal mines proven by stable isotope probing.* Submitted to Applied and Environmental Microbiology.

   Concept: S.B., B.E., H.C.; Analysis of results: S.B., T.L.; Sampling: S.B., B.E., M.K.; Geochemical analysis: S.B., M.K.; Molecular biological analysis: S.B.; SIP analysis: S.B., T.L.; First draft of manuscript: S.B.; Revision by: T.L., M.K., B.E., H.C.

Further publications not included in this thesis

Krüger M, **Beckmann S**, Engelen B, Cypionka H, and Thielemann T. (2007) *Microbial methane-formation from coal and wood – possible sources for biogenic methane in abandoned coal-mines.* Geophysical Research Abstracts, Vol.9, 01264.

Labrenz M, **Beckmann S**, Martens-Habbena W, and Jürgens K. (2005) *Impact of different in vitro electron donor/acceptor conditions on potential chemolithoautotrophic communities from marine pelagic redoxclines.* Applied and Environmental Microbiology 71: 6664-6672.

Presentations in national and international meetings

2010 **Beckmann S**, Krüger M, Engelen B, Gorbushina A, Lüders T, and Krüger M. *Role of Bacteria, Archaea and Fungi involved in Methane Release in Abandoned Coal Mines.* Annual Meeting of the German Society of General and Applied Microbiology (VAAM). **Oral presentation**

2009 **Beckmann S**, Engelen B, Cypionka H., and Krüger M. *Microbial Methane Formation from Coal and Mine Timber in Abandoned Coal Mines*. Goldschmidt Conference, Davos, Schwitzerland. **Oral presentation**

2009 **Beckmann S**, Krüger M, Cypionka H, and Engelen B. *Microbial Communities releasing Methane in Abandoned Coal Mines.* Annual Meeting of the German Society of General and Applied Microbiology (VAAM). **Poster**

2008 **Beckmann S**, Krüger M, Cypionka H, and Engelen B. *Microbial Communities releasing Methane in Abandoned Coal Mines.* International Symposium for Subsurface Microbiology (ISSM), Shizuoka, Japan. **Poster**

2008 **Beckmann S**, Krüger M, Engelen B, and Cypionka H. *What is the Origin of Biogenic Methane in Abandoned Coal Mines.* Annual Meeting of the German Society of General and Applied Microbiology (VAAM). **Poster**

2007 **Beckmann S**, Krüger M, Engelen B, Cypionka H, and Thielemann T. *Microbial Methane-Formation from Coal and Wood – possible Sources for Biogenic Methane in Abandoned Coal-Mines.* Annual Meeting of the German Society of General and Applied Microbiology (VAAM). **Poster**

2006 **Beckmann S**, Jost G, Martens-Habbena, Jürgens K, and Labrenz M. *The Impact of Mn(IV) and Fe(III) on Chemolithoautotrophic CO_2 Dark Fixation in Pelagic Redoxclines of the Central Baltic Sea.* Annual Meeting of the German Society of General and Applied Microbiology (VAAM). **Poster**

List of abbreviations

BES	2-Bromoethanesulfonate
BLAST	Basic Local Alignment Search Tool
bp	Base pair
BSA	Bovine Serum Albumin
C	Carbon
°C	Degree Celsius
CH_4	Methane
CO_2	Carbondioxide
CsCl	Cesium Chloride
d	Day
D	Deuterium
DAD	Diode Array Detector
DAPI	4`,6-Diamidino-2-phenylindol
DGGE	Denaturing Gradient Gel Electrophoresis
DNA	Deoxyribonucleic Acid
dNTP	Deoxyribonucleoside triphosphate
DSMZ	Deutsche Sammlung von Mikroorganismen und Zellkulturen (German Resource Centre for Biological Material)
dsr	Dissimilatory sulfite reductase
ed.	Editor
eds.	Editors
EDTA	Ethylene Diamine Tetraacetic Acid Biology Laboratory
e.g.	Exempli gratia (for instance)
EMBL	European Molecular Biology Laboratory
et. al.	Et alii
f	Forward
Fig.	Figure
FISH	Fluorescence *In Situ* Hybridization
g	Gram
g	Gravitational acceleration
h	Hour
H_2	Hydrogen
H_2SO_4	Sulfuric acid
HPLC	High Performance Liquid Chromatography
ITS	Internal Transcribed Spacer
KCl	Potassium Chloride
l	Liter
µ	Micro-
m	Milli-

M	Molar
mcr	Methylcoenzyme M reductase
min	Minute
mm	Millimeter
Mt	Million tons
n	Nano-
N_2	Nitrogen
NaCl	Sodium Chloride
NCBI	National Center for Biotechnology Information
NVT	Near Vertical Tube
p	Pico-
PAH	Polycyclic Aromatic Hydrocarbon
PBS	Phosphate-buffered saline
PCR	Polymerase Chain Reaction
pH	Power of hydrogen
qPCR	Quantitative Polymerase Chain Reaction
r	Reverse
rcf	Relative centrifugal force
rpm	Revolutions per minute
rRNA	Ribosomal RiboNucleic Acid
s	Seconds
SDS	Sodium dodecyl sulfate
SIP	Stable Isotope Probing
sp.	species (singular)
spp.	Species (plural)
SRB	Sulfate-Reducing Bacteria
Tab.	Table
TAE	Tris-Acetate-EDTA
TE	Tris-EDTA
Tris	Trishydroxymethylaminoethane
U	Units
UV	Ultra violet
V	Voltage
vol	Volume
vol/vol	Volume per volume
w/v	Weight per volume
wt	Weight
wt/vol	Weight per volume

1 Introduction

1.1 Coal-mine methane: A feasible energy source and hazard

Worldwide, mine gas emissions of active and abandoned coal mines release substantial amounts of methane contributing up to seven percent of the global methane formation (Denman *et al*. 2007). Mine gas emissions contain between 25 and 45 Mt of methane (Beck *et al*. 1993, Clayton *et al*. 1993, Khalil *et al*. 1993). The uncontrolled release of methane from abandoned mines receives current attention owing to its impact on the overall atmospheric methane budget, and thus for climate change. The mine gas is not only a green house gas, but also a hazard. Its presence has long been recognized because of explosions that have occurred (Boyer *et al.* 1990) and still occur during underground mining. Only recently has the mine gas in coal beds been recognized as a large untapped energy resource (Rice 1993). Within the last ten years, more and more countries utilized the methane of the mine gas for energy production. In Germany, the mine gas from active and abandoned mining areas is increasingly used for heat and power production (Thielemann *et al.* 2004), especially after the introduction of the "renewable energy law" in 2000, which discriminates between fossil and renewable energy sources with fiscal benefits for the later one. Other reasons for this trend are an increasing deployment of abandoned mining areas, and a more effective and sustainable utilization in active mining.

1.2 Methane formation in coal mines

1.2.1 Biogenic or thermogenic methane?

Methane can be generated by different reaction pathways. Each pathway leads to a certain isotopic signature (carbon and hydrogen isotopes) of the components. An elegant method to track the process of methanogenesis is based on the analysis of stable isotopes of carbon (C) and hydrogen (H). Schoell (1980, 1988) and Whiticar (1990, 1996, 1999) identified the different methane pathways and types using the partitioning of the light and heavy isotopes of carbon and hydrogen and the resultant isotope signatures. Whiticar (1999) used the characteristic isotopic differences to develop a classification scheme (CD-diagram), which is

commonly used for interpretation (Fig. 1). The combination of $\delta^{13}C_{CH4}$ and δD_{CH4} values defines the various natural sources of methane. Thermogenic methane is generally enriched in ^{13}C and therefore isotopically heavier ($\delta^{13}C_{CH4}$ range is roughly -50‰ to -20‰, Fig. 1) compared with biogenic methane that is depleted in ^{13}C ($\delta^{13}C_{CH4}$ range is roughly -50‰ to - 110‰, Fig. 1). The biogenic reduction of CO_2 or reduced carbon substrates (acetate, methanol or methylated amines) to methane is characterized with a kinetic isotope effect for carbon which discriminates against ^{13}C. Additionally, hydrogen isotope ratios of methane are helpful to define the type of biogenic methane. Hydrogen isotope effects during methanogenesis of methylated substrates lead to larger deuterium depletions (δD_{CH4} range is -250‰ to -531‰) compared with the CO_2-reduction pathway (δD_{CH4} range is -170‰ to -250‰, Fig. 1, Whiticar 1999). In many coal deposits worldwide, the analysis of the stable carbon isotopic composition of methane has shown that the produced gas is a mixture that originates from both, thermogenic and biogenic sources (Smith and Pallasser 1996, Kotarba 2001, Hosgörmez *et al*. 2002, Thielemann *et al*. 2004, Tao *et al*. 2007, Fig. 1)

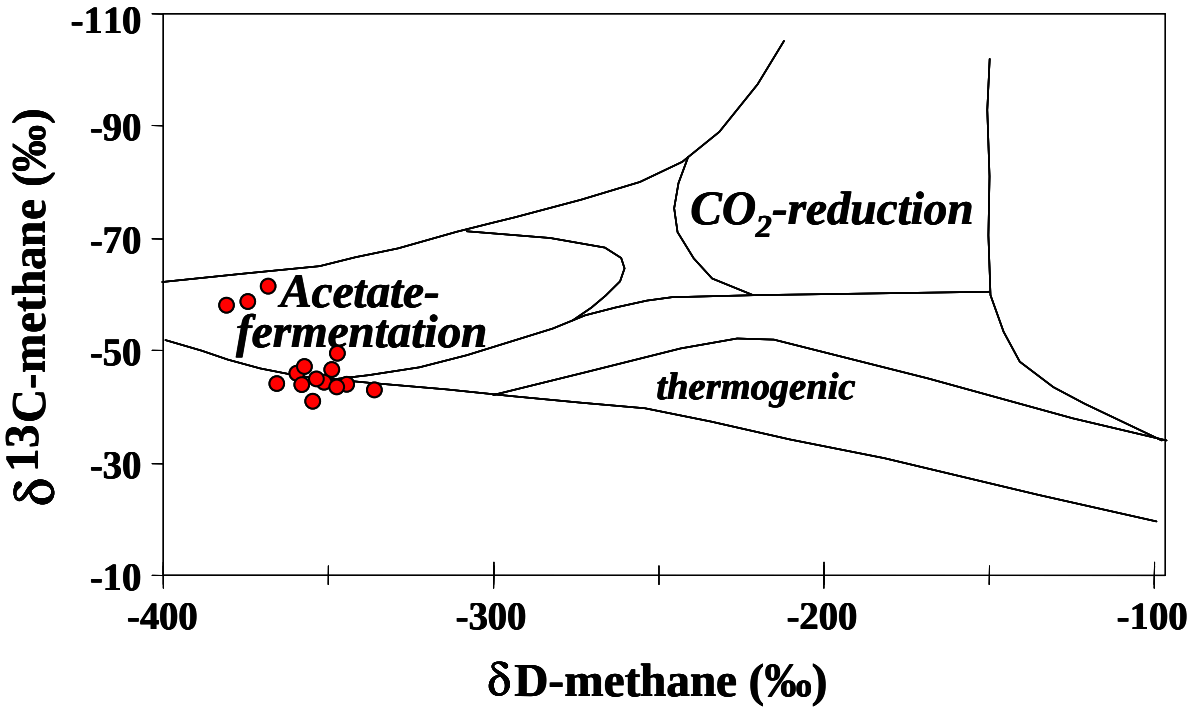

Figure 1: Isotopic composition of methane formed by biogenic and thermogenic reactions (Krüger 2008 modified according to Whiticar 1999). The red circles show the isotopic composition of methane from different abandoned coal mines in Germany indicating a mixture of biogenic and thermogenic origin.

Thermogenic methane is the result of thermal cracking of sedimentary organic matter occurring during coalification. The main process took place in Carboniferous time. In Early Permian time, folding, uplift, and erosion resulted in degassing of the coal beds (Rice 1993). The pressure from overlying rock and surrounding water cause the mine gas to bond to the surface of the coal and be absorbed into the solid matrix of the coal as free gas within micropores and cleats, as dissolved gas in water, as adsorbed gas by micropores, cleats and within the molecular structure of the coal. Therefore thermogenic methane is a remainder of geological processes but biogenic methane formation is still going on.

Biogenic methane production is the result of microbial metabolism and the age of its formation is not known, yet. The microbial communities and activities involved remained uncharacterized. Interestingly, time series of measurements in coal mines in the Ruhr Basin, Germany, showed an increasing proportion of the biogenically produced methane during the last years, indicating a recent origin (Thielemann *et al.* 2004). As well for the industrial utilization as for risk assessment it is important to know, whether the methane is currently produced or has been formed in the geological past. Hints on an ongoing biological formation of methane were obtained from isotopic analyses over the last years (Thielemann *et al*. 2004).

1.2.2 Biogenic methane formation

The breakdown of organic matter leading to methanogenesis is performed in a complex series of processes by diverse microbes, each of which contributes to the partial oxidation of organic matter. Methanogens belong to the Archaea domain (Woese *et al*. 1990) and are obligate anaerobes that metabolize only the final steps of anaerobic degradation of organic matter utilizing relatively few and simple compounds to obtain energy and cell carbon. Thus, methanogens rely on bacteria and microbes that successively break down larger molecules providing acetate and hydrogen as their main substrates. While hydrogen is energetically favourable, acetate is the quantitatively more available substrate. Some methanogens utilize acetate which typically accounts for 70% of methane formed in diverse habitats (Zinder 1993). Others are specialists for H_2+CO_2 as methane precursors, while some methanogens, e.g. those belonging to the *Methanosarcina* spp., are able to use both acetate as well as H_2 as their substrates.

1.2.3 Coal conversion to methane

First reports about microbial coal conversion have been published by Potter (1908) and Fischer (1932). Research has been intensified in the 1980`s when Fakoussa (1981) showed first effects on hard coal caused by *Pseudomonas* strains. Further studies focused on different solubilization mechanisms of coal (Cohen and Gabriele 1982, Scott *et al*. 1986, Cohen *et al*. 1990). A summary of the investigations in this field ranging from 1981 to 1997 has been reviewed by Fakoussa and Hofrichter (1999). However, most of the research was concentrated on brown coal and lignite rather on hard coal, and it was focused on aerobic conditions. Fakoussa (1981, 1988, 1990) showed that some aerobic bacteria and fungi can use hard coal as the sole carbon source. Deobald and Crawford (1987) showed that the rate limiting step is the initial solubilization of utilizable substrates that is achieved by microbial and especially extracellular fungal enzymes. Later studies observed that the presence of oxygen and water promotes the biodegradation (Fakoussa 1990, Scott and Fleet 1994, Fakoussa and Hofrichter 1999). While the microbial degradation of coal under aerobic conditions is well known (Hofrichter and Fakoussa, 2001), only Budwill *et al*. (2004) observed higher methane production rates in sewage sludge amended with coal than without, indicating an anaerobic microbial degradation of fossil organic matter. The presence of methanogens and methanogenesis is well known for water-flooded oil reservoirs (Edwards and Grbic-Galic 1994, Nazina *et al.* 1995), but no comparable microbiological studies are available concerning coal mines.

1.3 Abandoned coal mines: Sampling underground

Two sampling campaigns were performed in 2006 and 2007 in sealed compartments of coal mines closed in the 1960`s in the Ruhr Basin of Germany (Fig. 2). After the end of mining, the mines were closed with several metre thick concrete walls. Only embedded tunnels (Fig 2A) served as connectors between the sealed compartments (Fig. 2B-G) and the open coal-mine. The sampling proceeded 800 metres underground. Since there was only marginal ventilation via small empty pipes or cracks, the atmospheric conditions in these parts were characterized by 100% air humidity and low oxygen content with less than 4% oxygen. *In situ* temperatures were 35-37 °C. Wet floor and walls are covered by biofilms and thick layers of fungi grow on mine timber (Fig. 2C-E). The mines harbour weathered hard coal (also called "rock coal") that belongs to the fat coals according to the German classification (Thielemann *et al*. 2004 and references therein, Fig. 2B and H). Besides the coal, large amounts of timber could be a second possible source for biogenic methane formation within coal mines. The mine timber (coniferous wood) were used for the construction of mines and left behind after the end of mining (Fig. 2B). In the sealed mining compartments, weathering of coal and timber proceed in a suboxic atmosphere. Microbial activity causes oxygen depletion and creates anoxic zones in the ground, where methane is being formed.

Large pieces of coal and mine timber were collected aseptically and immediately stored under N_2 for further processing (Fig. 2H). *In situ* methane emission rates were measured using flux chambers exposed in different parts of the mine over coal and timber (Fig. 2F).

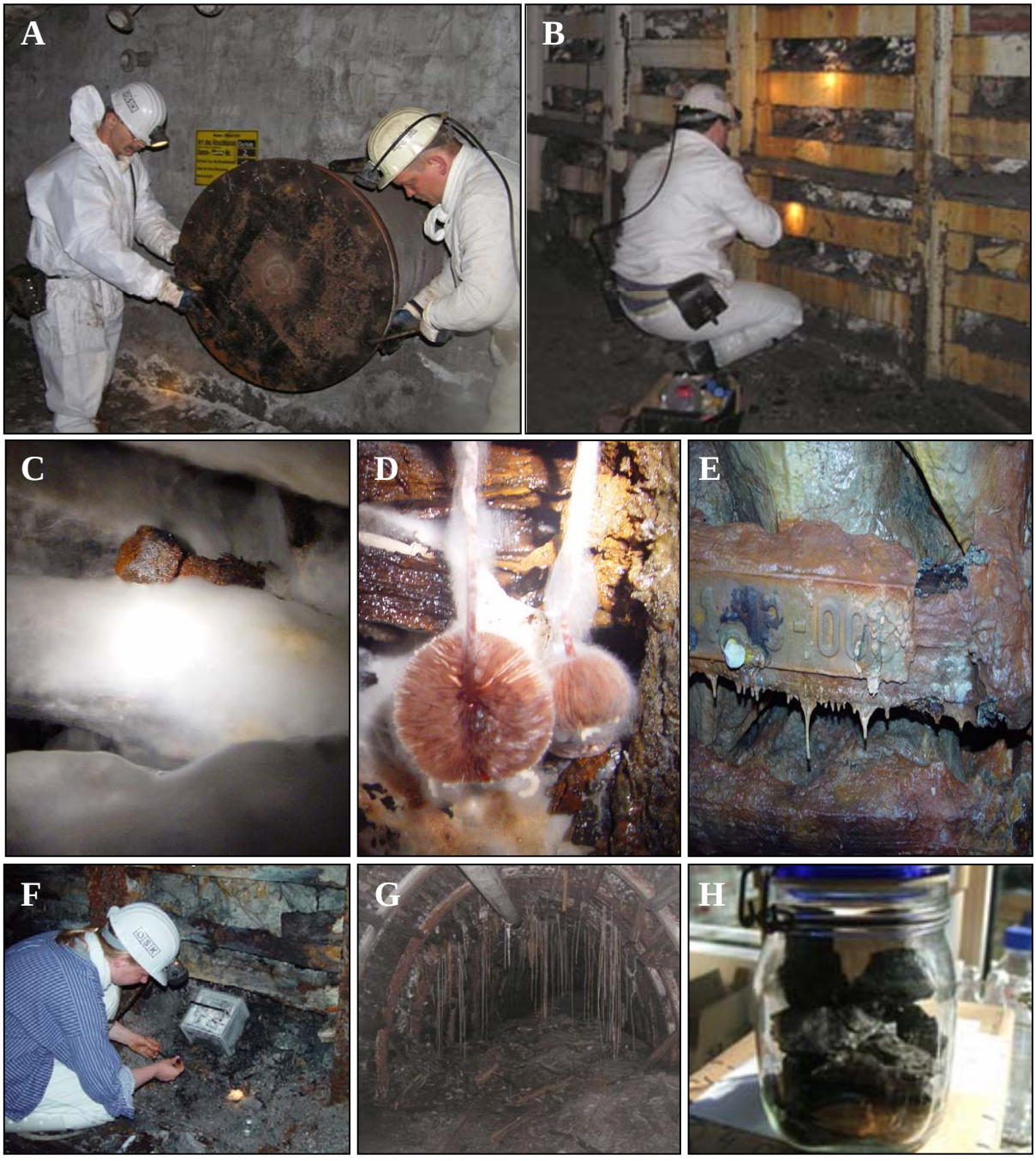

Figure 2: Sampling site. Snap-shots of two sampling campaigns performed in different abandoned coal mines in the Ruhr Basin, Germany.

1.4 Thesis outline

The aim of this thesis was to identify ongoing methanogenesis and to unravel the microbial processes as well as the active microorganisms involved in methane formation.

In order to identify ongoing methanogenesis, we combined geochemical and microbiological investigations. We have measured *in situ* methane fluxes and isotopic signatures of methane and carbon dioxide, and collected samples for microbiological and phylogenetical investigations. A selective enrichment approach of coal and timber amended with methanogenic substrates (acetate, H_2+CO_2, methylamine) was chosen to promote the growth of methanogens and therefore methanogenesis. In order to ascertain whether the methanogens were also present in the original coal and timber samples, *in situ* fluorescence hybridization (FISH) were applied.

The main aim of the present work was to understand the microbial processes leading to methane release and to identify the active microorganisms involved in the metabolic network. Therefore, we have assessed not only the archaeal community composition, but also the bacterial and fungal communities. Coal and mine timber samples and anaerobic enrichments were subjected to denaturing gradient gel electrophoresis (DGGE) and quantitative PCR. Besides the 16S-and 18SrRNA genes, functional key genes that encode for the dissimilatory sulfate reductase (*dsr*) and the methyl-coenzyme M reductase (*mcr*) were analysed. Hints on the abundance of different groups were derived from quantitative PCR. As a further step, stable-isotope labeled ^{13}C-acetate and ^{13}CO+H_2 were fed into microbial communities in stimulation experiments with coal and timber, respectively. Stable isotopic signatures of the CH_4 and CO_2 revealed the flux of ^{13}C-label. Direct identification of the ^{13}C-substrate assimilating microorganisms was obtained via DNA-Stable isotope probing (SIP) coupled to subsequent quantitative PCR and DGGE analyses.

1.5 References

Beck LL, Piccot SD, Kirchgessner DA. 1993. Industrial resources. In: Khalil MAK (Eds.). Atmospheric Methane: Sources, Sinks, and Role in Global Change. Berlin: Springer. P 399-431.

Boyer CM II, Kelefant JR, Kuuskraa VA, Manger KC. 1990. Methane emissions from coal mining: issues and opportunities for reduction: U.S. Environmental Protection Agency, Air and Radiation (ANR-445), EPA/400/9-90/008,86 p. plus appendices.

Budwill K, Beaton A, Bustin M, Muehlenbachs K, Gunter WD. 2004. Methanogenic activity on coal and sequestered CO_2 for enhanced methane Technologies, Kyoto, Japan.

Clayton JL, Oremland RS, Leventhal JS, Dudley DR, Pashin JC, Mosher B, Czepiel P. 1993. Atmospheric methane flux from coals – preliminary investigation of coal mines and geologic structures in the Black Warrior Basin, Alabama. In: Howell DG (Ed.). The future of energy gases, 1570: U.S. Geological Survey. P 471-492.

Cohen MS, Gabriele PD. 1982. Degradation of coal by the fungi *Polyporus versicolor* and some properties of laccase A. Acta Chem Scandin 21:2367-2378.

Cohen MS, Feldmann KA, Brown CS, Grey ET. 1990. Isolation and identification of the coal-solubilizing agent produced by *Trametes versicolor*. Appl Environ Microbiol 56:3285-3290.

Denman K, Brasseur G, Chidthaisong A, Ciais P, Co P, Dickinson R, Hauglustaine D. 2007. Couplings between changes in the climate system and biogeochemistry. In: Solomon S, Qin D, Manning M, Chen Z, Marquis M, Averyt KB, Tignor M, Miller HL. (Eds.), Climate Change 2007: The Physical Science Basis. Contribution of Working Group I to the Fourth Assessment Report of the Intergovernmental Panel on Climate Change. Cambridge and New York. Cambridge University Press.

Deobald LA, Crawford DL. 1987. Activities of cellulose and other extracellular enzymes during lignin solubilization by Streptomyces-Viridosporus. Appl Microbiol Biotechnol 26:158-163.

Edwards EA, Grbic-Galic D. 1994. Anaerobic degradation of toluene and oxylene by a methanogenic consortium. Appl Environ Microbiol 60:313-322.

Fakoussa RM. 1981. Kohle als Substrat für Mikroorganismen: Untersuchungen zur mikrobiellen Umsetzung nativer Steinkohlen. (Ph.D. thesis). Bonn: University of Bonn.

Fakoussa RM. 1988. Production of water-soluble coal substances by partial microbial liquefaction of untreated coal. Resour Conserv Rec 1:251-260.

Fakoussa RM. 1990. Microbiological treatment of German hard coal. In: Wise DL (Ed.). Bioprocessing and Biotreatment of Coal. New York: Marcel Dekker. P 95-107.

Fakoussa RM, Hofrichter M. 1999. Biotechnology and microbiology of coal degradation. Appl Microbiol Biotechnol 52:25-40.

Fischer F. 1932. Biologie und Kohle. Angewandte Chemie 45:185-194.

Hofrichter M, Fakoussa RM. 2001. Lignin, humic substances and coal. In: Steinbüchel A (Eds.). Biopolymers, I. Weinheim: Wiley-VCH. P 393-429.

Hosgörmez H, Yalcin NM, Cramer B, Gerling P, Faber E, Schaefer RG, Mann U. 2002. Isotopic and molecular composition of coal-bed gas in the Amasra region (Zonguldak basin – western Black Sea). Organ Geochem 33:1429-1439.

Khalil MAK, Rasmussen RA, Shearer MJ, Ge S, Rau JA. 1993. Methane from coal burning. Chemosphere 26:473-477.

Kotarba MJ. 2001. Composition and origin of coalbed gases in the Upper Silesian and Lublin basins, Poland. Organ Geochem 32:163-180.

Nazina TN, Ivanova AE, Borzenkov IA, Belyaev SS, Ivanov MV. 1995. Occurrence and geochemical activity of microorganisms in high-temperature, water-flooded oil fields of Kazakhstan and Western Siberia. Geomicrobiol J 13:181-192.

Potter MC. 1908. Bakterien als Agentien bei der Oxidation amorpher Kohle. Zentralblatt Bakteriol Parasitenkunde II 21:262-280.

Rice, DD. 1993. Composition and origins of coalbed gas. In: Law BE, Rice DD (Eds.). Hydrocarbons from coal. Amer. Assoc. Petrol. Geo.l Studies in Geol 38:159-184.

Schoell M. 1980. The hydrogen and carbon isotopic composition of methane from natural gases of various origins. Geochim Cosmochim Acta 44:649-661.

Schoell M. 1988. Multiple origins of methane in the earth. Chem Geol 71:1-10.

Scott CD, Strandberg GW, Lewis SN. 1986. Microbial solubilization of coal. Biotechnol Prog 2:131-139.

Scott AC, Fleet AJ. 1994. Coal and coal-bearing strata as oil-prone source rocks: current problems and future directions. Geological Society of London, Special Publications 77:201-205.

Smith JW, Pallasser RJ. 1996. Microbial origin of Australian coalbed methane. AAPG Bull 80:891-897.

Tao MX, Shi BG, Li JY, Wang WC, Li XB, Gao B. 2007. Secondary biological coalbed gas in the Xinji area, Anhui province, China: Evidence from the geochemical features and secondary changes. Inter J Coal Geol 71:358-370.

Thielemann T, Cramer B, Schippers A. 2004. Coalbed methane in the Ruhr Basin, Germany: a renewable energy resource? Organ Geochem 35:1537-1549.

Van Krevelen DW. 1993 . Coal: typology – chemistry – physics – constitution. Coal science and technology 3, 3rd edn. Elsevier, Amsterdam.

Whiticar MJ. 1990. A geochemical perspective of natural gas and atmospheric methane. Organ Geochem 16:531-547.

Whiticar MJ. 1996. Isotope tracking of microbial methane formation and oxidation. Mitteilungen der Internationalen Vereinigung für Limnologie 25:39-54.

Whiticar MJ, Snowdon LR. 1999. Geochemical characterization of selected Western Canada oils by C-5 – C-8 Compound Specific Isotope Correlation (CSIC). Org Geochem 30:1127 - 1161.(USA) 87:4576-4579.

Woese CR, Kandler O, Wheelis ML. 1990. Towards a natural system of organisms: proposal for the domains Archaea, Bacteria, and Eucarya: Proceedings of the National Academy of Science

Zinder SH. 1993. Physiological ecology of methanogens. Ferry JC (Eds.). Methanogenesis: ecology, physiology, biochemistry and genetics. New York. Chapman & Hall.

2 Publications

2.1 Publication 1

Microbial methane formation from hard coal and timber in an abandoned coal mine

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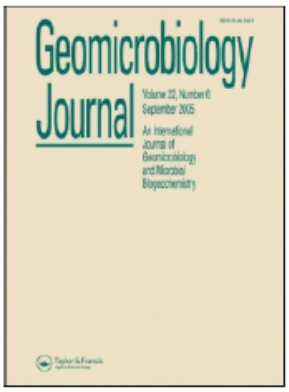

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Microbial Methane Formation from Hard Coal and Timber in an Abandoned Coal Mine

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About 7% of the global annual methane emissions originate from coal mining. Also, mine gas has come into focus of the power industry and is being used increasingly for heat and power production. In many coal deposits worldwide, stable carbon and hydrogen isotopic signatures of methane indicate a mixed thermogenic and biogenic origin. In this study, we have measured in an abandoned coal mine methane fluxes and isotopic signatures of methane and carbon dioxide, and collected samples for microbiological and phylogenetic investigations. Mine timber and hard coal showed an *in-situ* production of methane with isotopic signatures similar to those of the methane in the mine atmosphere. Enrichment cultures amended with mine timber or hard coal as sole carbon sources formed methane over a period of nine months. Predominantly, acetoclastic methanogenesis was stimulated in enrichments containing acetate or hydrogen/carbon dioxide. Molecular techniques revealed that the archaeal community in enrichment cultures and unamended samples was dominated by members of the *Methanosarcinales*. The combined geochemical and microbiological investigations identify microbial methanogenesis as a recent source of methane in abandoned coal mines.

Keywords Coal mine gas, stable isotope fractionation, methanogenic Archaea, hydrocarbon degradation, diversity, DGGE

INTRODUCTION

Total methane emissions from hard coal mines on a global scale range between 25 and 45 Mt (Boyer et al. 1990; Beck et al. 1993; Clayton et al. 1993; Khalil et al. 1993), contributing up to seven percent to global methane emissions (Denman et al. 2007). Within the last 10 years, more and more countries utilized a part of the coal gases for energy production. In Germany, the gas from active and abandoned mining areas is increasingly used for heat and power production, especially after the introduction of the "renewable energy law" in 2000, which discriminates between fossil and renewable energy sources with fiscal benefits for the latter.

Other reasons for this trend are an increasing deployment of abandoned mining areas, and a more effective and sustainable utilization in active mining. Coal gas can be generated by different reaction pathways. Each pathway leads to a certain isotopic signature (hydrogen and carbon isotopes) of the components. Schoell (1980, 1988) and Whiticar (1990, 1996) used characteristic isotopic differences to develop a classification scheme, which is commonly used for interpretation.

In many coal deposits worldwide, the analysis of the stable carbon isotopic composition of methane showed that the produced gas is a mixture that originates from thermogenic and biogenic sources (Smith and Pallasser 1996; Kotarba 2001; Hosgörmez et al. 2002; Thielemann et al. 2004; Tao et al. 2007). However, the time point for the formation of the latter fraction is not known, and the microbial communities and mechanisms involved remained uncharacterised. Interestingly, time series of measurements in coal mines in the Ruhr Basin, Germany, showed an increasing proportion of the biogenic, microbially produced methane during the last years, indicating a recent origin (Thielemann et al. 2004). As well for the industrial utilization as for risk assessment it is important to know, whether the methane is currently produced or has been formed in the geological past. Hints on a recent biological formation of methane were obtained from isotopic analyses over the last years (Thielemann et al. 2004).

First reports about microbial coal conversion have been published by Potter (1908) and Fischer (1932). Research has been intensified in the 1980s (Fakoussa 1981; Cohen and Gabriele 1982), and the work in this field has been reviewed previously (Hofrichter and Fakoussa 2001). Most of the research was concentrated on brown coal and lignite rather than on hard coal, and it was focussed on aerobic conditions. Fakoussa (1981, 1988,

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1990) showed that some aerobic bacteria and fungi can use hard coal as the sole carbon source.

While the microbial degradation of coal under aerobic conditions is well known (Hofrichter and Fakoussa 2001), only recently Budwill et al. (2004) observed higher methane production rates in sewage sludge amended with coal than without, indicating an anaerobic microbial degradation of fossil organic matter. A study by Zengler et al. (1999) showed the conversion of hexadecane to methane by a microbial enrichment culture from a freshwater ditch. The presence of methanogens and methanogenesis is well known for water-flooded oil reservoirs (Edwards and Grbic-Galic 1994; Nazina et al. 1995), but no respective microbiological studies are available concerning coal mines.

Therefore, in this study we measured *in situ* isotopic signatures and methane fluxes in a coal mine in the Ruhr Basin, which had been abandoned and sealed in the 1960s. Furthermore, we collected samples for microbiological and phylogenetic investigations on methane production and the involved microbial populations to find indications for the presence and intensity of recent methanogenesis.

MATERIAL AND METHODS

Sampling

Samples were collected in February 2006 and May 2007 in a sealed part of a coal mine closed in the 1960's. Large pieces of mine timber and coal as well as H_2O were collected and immediately stored under N_2. *In situ* temperatures were 35–36°C with 100 % humidity. Flux chambers made of Plexiglas, 20 × 20 × 20 cm, were exposed in different parts of the mine over coal, timber or control areas to measure methane emission rates. Gas samples were taken through a septum regularly in 15 minutes intervals over 1–2 h and stored on saturated NaCl until analysis for CH_4, CO_2, and stable isotopes as previously described by Krüger et al. (2001).

Enrichment Cultures

Processing of coal, timber and water samples was done in an anaerobic chamber under N_2 atmosphere. Samples were homogenized and distributed in hungate tubes containing 5 ml of mineral medium (Widdel and Bak 1992) with a salinity of 15 PSU, according to *in situ* values. In controls, 10 mM BES (2-Bromoethanesulfonate) were added to detect abiotic degassing via inhibiting methanogenesis. In stimulation experiments either 10 mM acetate or H_2/CO_2 (80/20) was added. All incubations were carried out at least in triplicates. The increase of methane in the headspace as well as carbon stable isotopes of methane and carbon dioxide produced were continuously measured over a period of 9 months and analysed as previously described by Krüger et al. (2001).

DNA Extraction and PCR Amplification

Genomic DNA was extracted from 0.5 g of coal and mine-timber samples using the FastDNA® Spin Kit (Q-BIOgene, Carlsbad, CA), according to the manufacturer's instructions. A nested PCR was used in order to investigate the archaeal community composition as described by Vetriani et al. (1999). The domain-specific primer pair S-D-Arch-0025-a-S-17 (5′-CTG GTT GAT CCT GCC AG-3′) and S-*-Univ-1517-a-A-21 (5′-ACG GCT ACC TTG TTA CGA CTT-3′) was used for the amplification of the almost-complete archaeal 16S rRNA gene. For denaturing gradient gel electrophoresis (DGGE), a 550-bp fragment of the archaeal 16S rRNA gene was amplified by using the primers S-D-Arch-0344-a-S-20 (5′-ACG GGG CGC AGC AGG CGC GA-3′) and 907r (5′-CCG TCA ATT CCT TTG AGT TT-3′). All PCRs were checked by electrophoresis on a 1.5% (wt/vol) as described previously (Wilms et al. 2006).

Denaturing Gradient Gel Electrophoresis (DGGE) Analysis

PCR amplicons and loading buffer (40% [wt/vol] glycerol, 60% [wt/vol] 1 × Tris-acetate-EDTA [TAE], and bromphenol blue) were mixed in a ratio of 1:2. DGGE was performed using an INGENYphorU-2 system (Ingeny, Goes, The Netherlands). PCR products of the 16S rRNA gene were loaded onto polyacrylamide gels (6% [wt/vol]) in 1 × TAE (40 $mmol^{-1}$ Tris, 20 $mmol^{-1}$ acetate, 1 $mmol^{-1}$ EDTA), with a denaturing gradient adjusted from 30% to 80% (with 100% denaturant corresponding to 7 M urea and 40% formamide). Electrophoresis was accomplished at a constant voltage of 100 V and a temperature of 60°C for 20 h. After electrophoresis, the gels were stained for 2 h in 1 × SybrGold solution (Molecular Probes, Eugene, Oreg.) in 1 × TAE, washed for 20 min with distilled water, and documented using a digital imaging system (BioDocAnalyze; Biometra, Göttingen, Germany).

Sequence Analysis

DGGE bands were excised for sequencing and treated as described by Del Panno et al. (2005). PCR products were purified by using the QIAquick PCR purification Kit (Quiagen GmbH, Hilden, Germany) and eluted in 30 μl of PCR water (Ampuwa, Fresenius, Bad Homburg, Germany). DNA yields were quantified fluorometrically in a microtiterplate reader (FLUOstar Optima, BMG Labtechnologies, Offenburg, Germany) using a 1:200 diluted PicoGreen reagent (Molecular Probes, Eugene, OR) as described in detail by Wilms et al. (2007). The 16S rRNA gene sequences were obtained by cycle sequencing using the DNA Sequencing System 4000 (Li-COR Inc., Lincoln, Neb.) with the IRDye™ 800-labeled primer 907r and the DYEnamic direct cycle sequencing kit (Amersham Biosciences, Little Chalfont, UK) in accordance with the manufacturer's instructions. Sequences were compared to those in GenBank using the BLAST function of the National Center for Biotechnology

Information server and have been deposited in the GenBank nucleotide sequence database under the accession No. AM850093-AM850104.

Fluorescence *in situ* Hybridization (FISH)

A specific detection of Archaea was carried out by FISH as described in detail by Amann et al. (1990) and Pernthaler et al. (2001) using the general probe Arch915 (5′-GTG CTC CCC CGC CAA TTC CT-3′). The probe was synthesized and labeled with the sulfoindocyanine dye Cy 3 (Biomers, Ulm, Germany). Briefly, cells of the supernatant of coal or mine timber incubations were fixed in 4% paraformaldehyde (2% [vol/vol] final concentration) for 3h at 4°C. Fixed cells were stored at −20°C until further processing. Glas slides with ten separate hybridization wells per slide were used for the hybridization. The slides were treated with a gelatine solution (0.1% gelatine, 0.01% chromium potassium sulfate).

Samples of 4 μl were spread on each well and dried on air. A moisture chamber equilibrated with 5 M sodium chloride was used for the hybridization. A 7 μl sample of hybridization mixture containing 35% formamide, 5 M sodium chloride, 1 M Tris/HCL and 1% SDS was applied to each well. After 30 min of incubation at the hybridization temperature of 46°C, 50 ng of the fluorescent probe was added. Hybridization was carried out for 2 h at 46°C. The hybridization buffer was removed by flushing the slide with wash solution (5 M sodium chloride, 1 M Tris/HCL, 1% SDS, 0.05 M EDTA). The slides were incubated in wash solution for 20 min at 48°C, followed by thorough rinsing with distilled water and stored in the dark.

RESULTS AND DISCUSSION

To test the hypothesis that the strong biogenic component of mine gas in abandoned coal mines has a recent origin, two sampling campaigns were performed in the Ruhr Basin, Germany, in a coal mine that had been closed in the 1960s. Mine water collected from small ditches and pools sometimes also containing mine timber or coal pieces showed an almost neutral pH and slightly elevated carbonate and salt concentrations (Table 1). The δ^{13}C-signature of methane in the mine atmosphere was −45.6 ‰ (Table 2), indicating a mixture of biogenic and thermogenic gas (Whiticar and Snowdon 1999).

Gas emissions were measured*in situ* in galleries and tunnels with flux chambers that were placed over residual pieces of mine timber or the weathered coal, the only possible sources for recent methane formation (Figure 1). The δ^{13}C-values of the emitted methane varied from 39.8 to 49.5‰ in the gas phase collected over different areas with coal or mine timber, respectively. Methane emission rates accumulated to 12 and 42 g m^{-2} a^{-1}, being up to 1000-fold lower than e.g., in wetlands or rice

TABLE 1
Mine water chemistry.

General parameters			
Conductivity (μS/cm) (25°C)	3090	Temperature (°C)	24
pH-value	7.60	Carbon density (mmol/l)	7.16
Complete density (mmol/l)	3.79	Σdissolved ions (mg/l)	2657
Cations (mg/l)		Anions (mg/l)	
K^+	17.8	Cl^-	133
Na^+	647	SO_4^-	851
Mg^{++}	50	HCO_3^-	875
Ca^{++}	69.6	BO_2^-	10
Li^+	0.39	Non-ionic substances (mg/l)	
Sr^{++}	2.5	SiO_2	15.3
Inorganic traces (μg/l)			
PO_4^{3-}	160	Cu	<5
Al	10	Pb	<30
NH_4	30	Cd	<2
Ba	20	Fe(II)	37
Ti	<1	Zn	<3
Br^-	300	Cr	<5
Co	<5	Mn	41
Ni	<5	As	<20
Be	<0,5	Sc	<1
V	<5		

TABLE 2
Concentration, stable carbon and hydrogen isotopic composition of methane and carbon dioxide collected in the atmosphere of an abandoned coal mine, taken from flux chamber measurements at the same location*, or produced in laboratory incubations of coal and mine timber samples at the end of incubations (mean ± SE, n = 3–5).

	CH_4 (%)	$\delta^{13}CH_4$ (‰)	CO_2 (%)	$\delta^{13}CO_2$ (‰)
Mine atmosphere	2–5	−45.6 ± 4.32	0.5–1.2	−16.3 ± 1.07
Flux chamber		−45.0 ± 2.15		−19.1 ± 1.07
Coal incubation		−47.4 ± 1.31		−16.1 ± 0.73
Mine timber incubation		−55.8 ± 3.25		−19.7 ± 0.43

*Measured before ventilation and entering of the mine.

fields (Krüger et al. 2001; Heyer et al. 2002). Control sites in the mine without coal or timber showed no methane emissions. In none of the gas samples collected underground the potential substrate hydrogen was detected.

Samples of timber, coal and mine water were collected aseptically and incubated anaerobically under *in situ* conditions without substrates. While no methane formation was observed in water samples, all mine timber incubations showed a constant and long-term (>9 months) formation of methane (Figure 2). Surprisingly, methanogenesis was also found in several incubations with hard coal samples as sole source of carbon and energy. No methane (or hydrogen) release was observed of control incubations with the methanogenic inhibitor BES (2Bromoethanesulphonate), thus excluding an abiotic degassing of adsorbed methane from the incubated samples. Methane production rates were with 0.22 ± 0.13 and 1.24 ± 0.51 $\mu g\ g^{-1}_{wet\ weight}\ d^{-1}$ for coal and mine timber in a similar range as observed for example in freshwater lake sediments (Chan et al. 2005; Eller et al. 2005).

The stable carbon isotopic signatures of the produced methane were with −47.4 for coal and −55.8‰ for timber incubations in a similar range as those in the gas samples collected *in situ* in the closed mine (Table 2). The more positive $\delta^{13}C$-signatures in the mine atmosphere show a still ongoing contribution of desorbing thermogenic fossil methane, probably originating from other mine regions, to the overall signal.

In the absence of oxygen and other alternative electron acceptors, like iron(III), nitrate or sulfate, methanogenesis is the terminal electron-accepting process. We investigated which of the two dominant pathways, the hydrogenotrophic or the acteoclastic, is important for coal and timber degradation. The addition of acetate to the incubations lead to a strong and relatively

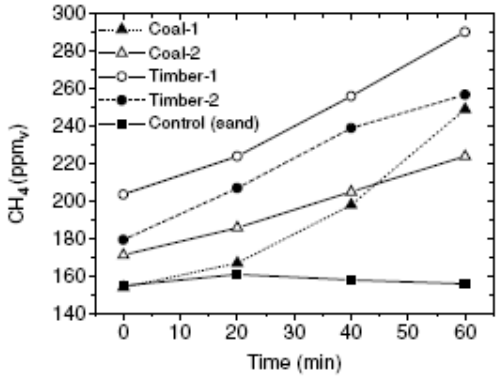

FIG. 1. In situ methane emissions over time. Methane concentrations were measured on-site in flux chambers that were mounted above coal residues, different timber samples and a sandy control site.

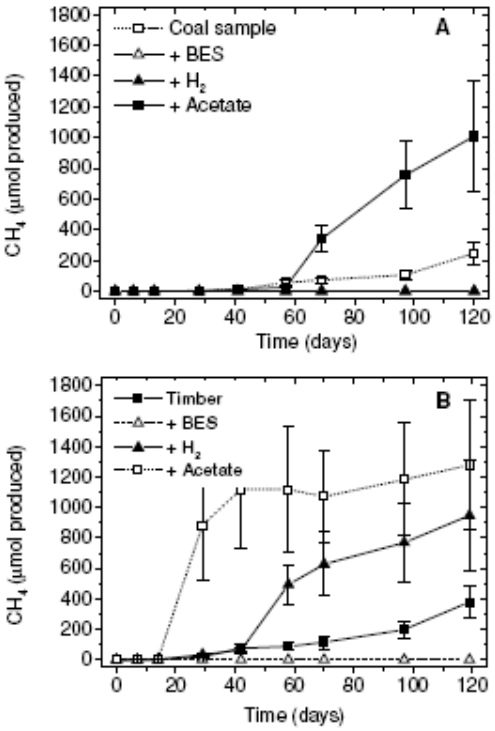

FIG. 2. Microbial methane formation in long-term incubations from an abandoned coal mine. a, coal and b, mine timber samples. (mean ± standard error, n = 3).

rapid stimulation of methane production, while the effect of hydrogen was less pronounced (Figure 2). Together with the comparably positive stable carbon isotopic signature of the produced methane, this indicates that acetate might be a central intermediate in the degradation processes of timber and coal.

In the control incubations with BES but also in unamended cultures, small amounts of both, acetate and hydrogen, were detected (data not shown). Low concentrations of acetate and hydrogen agree with their proposed role as key intermediates during methanogenesis from natural organic compounds or hydrocarbons (Schink 1997; Zengler et al. 1999). Decomposition of such compounds by anaerobic bacteria produces acetate and hydrogen, which are consumed by methanogenic Archaea and hence maintained at very low concentrations.

Microscopic screening showed a large variety of microbial cells in the different enrichment cultures. Especially the mine timber incubations were characterised by complex aggregates of timber particles with differently shaped cells (Figure 3a), while the hard coal incubations were dominated by coccoid cells. The molecular analysis of the original samples and of enrichment cultures showed the presence of a diverse archaeal community. Fluorescence-in-situ-hybridisation (FISH) microscopy with Archaea-specific probes reflected the different morphological types (Figure 3b). Furthermore, methanogenic Archaea that were associated with mine timber could directly be visualised using their typical autofluorescence at 420 nm (Figure 3c).

As expected from the more rapid methane formation in the incubations with acetate (Figure 2), a dominance of acetoclastic methanogens was found by DGGE (denaturing gradient gel electrophoresis) and subsequent 16S rRNA gene sequencing in unamended enrichments and in the acetate-stimulated cultures. Different acetoclastic members of the *Methanosarcinales* were detected (Table 3), closely related to *Methanosarcina* species (*M. barkeri*, *M. lacustris* and *M. siciliae*) and to *Methanosaeta harundinaceae*. Typical cell forms of these species were also present in the enrichment cultures (Figure 3c-e). The results of the community analysis confirmed that acetate is a central intermediate in recent mine gas formation.

Hydrogenotrophic and methylotrophic methanogens closely related to *Methanolobus taylorii* were selectively enriched in specifically designed culture media. However, they were neither detected by DGGE in the original coal or and timber samples nor in the unamended enrichments and probably play a minor role *in situ*. Surprisingly, a presently uncultured member of the Crenarcheota belonging to the *Thermoprotei* subgroup was found in the unamended and the hydrogen-stimulated enrichments. This subgroup was previously detected in petroleum reservoirs (Li et al. 2007) and a subsurface gold mine (Nunoura et al. 2005), and is known to exhibit a chemoorganotrophic lifestyle, to reduce sulfur compounds, or to utilize hydrogen. Isolation of the methanogenic key players in mine gas formation is currently in progress. The first colonies

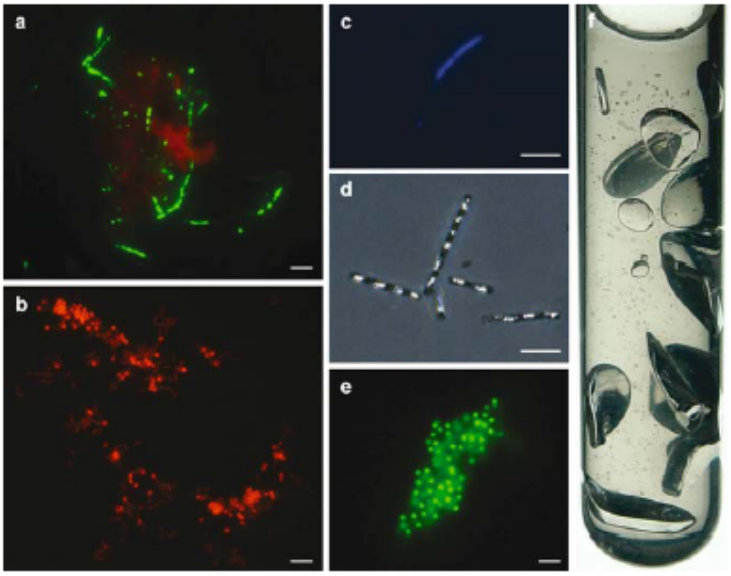

FIG. 3. Microscopic analysis of methanogenic Archaea in hard coal and mine timber samples (scale bars = 5 μm). a, Mine timber particle (red) colonised by differently shaped microorganisms (Sybr Green I staining). b, Coccoid and rod-shaped Archaea from an enrichment culture amended with mine timber and acetate (fluorescence in situ hybridisation, FISH). c, Methanogenic rod-shaped cell (autofluorescence). d, *Methanosaeta*-like cells from an acetate-enrichment (phase contrast). e, *Methanosarcina*-like cells from an acetate-enrichment (Sybr Green I staining). f. Methane bubbles formed around methanogenic colonies (tiny dots) in deep agar dilution series.

TABLE 3
Phylogenetic affiliation of DGGE bands after amplification of the archaeal 16S rRNA gene

	Coal incubations	Mine timber incubations		
Substrate	Closest relative	Sequence similarity (%)	Closest relative	Sequence similarity
Unamended	*Methanosarcina barkeri*	96	*Methanosarcina barkeri*	96
			Methanosarcina siciliae	99
			Methanosaeta harundinaceae	98
			Uncultured Crenarchaeota	96
H_2/CO_2	*Methanosarcina barkeri*	98	*Methanolobus taylorii*	97
			Methanosaeta harundinaceae	93
			Uncultured Crenarchaeota	97
Acetate	*Methanosarcina lacustris*	97	*Methanosarcina barkeri*	99
			Methanosaeta harundinaceae	98

showing gas production were grown in deep agar dilution series (Figure 3f).

In this study, for the first time the presence and activity of methanogenic Archaea in abandoned coal mines and the conversion of hard coal and the mine timber to methane could be demonstrated. Since hard coal is effectively sterilised due to the high temperatures during its formation, the best explanation for the presence of these diverse microbial communities is a recolonisation introduced by the anthropogenic mining activities. Possibly, also the transport of microbes via water in faults might provide a further source of microbial life, as postulated e.g. for gold mines (Lollar et al. 2006).

However, methanogenesis is only the terminal step in the degradation of complex organic substrates. Especially for mine timber it is expected, that a close interaction of Fungi and Bacteria are mediating the first decompositon of polymeric substances to provide the carbon sources for the methanogenic Archaea. Surprisingly, besides the mine timber also weathered hard coal was degraded by these microbial consortia, providing another possibly long-term source of methane in the mine gas. However, even after the end of the mining activities oxygen is remaining for a long time in the system. This residual oxygen probably initiates weathering of the coal and timber, thus facilitating a subsequent microbial degradation under anoxic conditions.

In conclusion, the stable isotope analysis indicated a strong biogenic component of coal-mine methane originating from acetoclastic methanogenesis. This was confirmed by the fast stimulation of methane formation in acetate-amended enrichment cultures. The respective acetoclastic Archaea were identified by the detailed microscopical and molecular biological analysis. The predominance of these microorganisms and the ongoing methane formation in the unamended hard coal and mine timber incubations furthermore indicate that acetoclastic methanogenesis is an important process *in situ*, which is in contrast to previous observations.

In gas samples collected aboveground at different mining plants also in the Ruhr Basin, Thielemann et al. (2004) observed similar $\delta^{13}C$- but δD-values between −190 and −260‰ for the mine gas methane, which they explained by the contribution of hydrogenotrophic methanogens. Similar observations were made in gas-isotopic studies at other mining areas (Smith and Pallasser 1996; Kotarba 2001). However, the underlying microbiology and environmental conditions might be completely different from those at the present study site.

Overall, our new results support the assumption that abandoned coal reservoirs have a potential to supply methane gas for energy production over extended time scales. The worldwide increased mining activity will go along with an increased coal weathering and the formation of biogenic methane. On the other hand, the uncontrolled release of methane from abandoned mines might become even more important for the overall atmospheric budget of methane, and thus for climate change.

REFERENCES

Amann RI, Krumholz L, Stahl DA. 1990. Fluorescent-Oligonucleotide probing of whole cells for determinative, phylogenetic, and environmental studies in microbiology. J Bacteriol 172:762–770.

Beck LL, Piccot SD, Kirchgessner DA. 1993. Industrial sources. In: Khalil MAK (Eds.). Atmospheric Methane: Sources, Sinks, and Role in Global Change. Berlin: Springer. P 399–431.

Boyer CM, Kelafant JR, Kuuskraa VA, Manger KC. 1990. Methane emissions from coal mining: Issues and opportunities for reduction. U.S. Environmental Protection Agency, Washington, DC. 90 p.

Budwill K, Beaton A, Bustin M, Muehlenbachs K, Gunter WD. 2004. Methanogenic activity on coal and sequestered CO_2 for enhanced methane recovery. Proceedings of the 4th International Conference on GHG Control Technologies, Kyoto, Japan.

Chan OC, Claus P, Casper P, Ulrich A, Lueders T, Conrad R. 2005. Vertical distribution of structure and function of the methanogenic archaeal community in Lake Dagow sediment. Environ Microbiol 7:1139–1149.

Clayton JL, Oremland RS, Leventhal JS, Dudley DR, Pashin JC, Mosher B, Czepiel P. 1993. Atmospheric methane flux from coals - preliminary investigation of coal mines and geologic structures in the Black Warrior Basin, Alabama. In: Howell DG (Ed.). The future of energy gases, 1570: U.S. Geological Survey. P 471–492.

Cohen MS, Gabriele PD. 1982. Degradation of coal by the fungi *Polyporus versicolor* and some properties of laccase A. Acta Chem Scandin 21:2367–2378.

Del Panno M, Morelli IS, Engelen B, Berthe-Corti L. 2005. Effect of petrochemical sludge concentrations on microbial communities during a soil bioremediation process. FEMS Microbiol Ecol 53:305–316.

Denman KL, Brasseur G, Chidthaisong A, Ciais P, Cox PM, Dickinson RE, Hauglustaine D. 2007. Couplings between changes in the climate system and biogeochemistry. In: Solomon S, Qin, D, Manning M, Chen Z, Marquis M, Averyt KB, Tignor M, Miller, HL (Eds.), Climate Change 2007: The Physical Science Basis. Contribution of Working Group I to the Fourth Assessment Report of the Intergovernmental Panel on Climate Change. Cambridge and New York: Cambridge University Press.

Edwards EA, Grbic-Galic D. 1994. Anaerobic degradation of toluene and o-xylene by a methanogenic consortium. Appl Environ Microbiol 60:313–322.

Eller G, Deines P, Grey J, Richnow H-H, Krüger M. 2005. Methane cycling in lake sediments and its influence on chironomid larvae. FEMS Microbiol Ecol 54:339–350.

Fakoussa RM. 1981. Kohle als Substrat für Mikroorganismen: Untersuchungen zur mikrobiellen Umsetzung nativer Steinkohlen [Ph.D. thesis]. Bonn: University of Bonn.

Fakoussa RM. 1988. Production of water-soluble coal substances by partial microbial liquefaction of untreated coal. Resour Conserv Rec 1:251–260.

Fakoussa RM. 1990. Microbiological treatment of German hard coal. In: Wise DL (Ed). Bioprocessing and Biotreatment of Coal. New York: Marcel Dekker. P 95–107.

Fischer F. 1932. Biologie und Kohle. Angewandte Chemie, 45. P 185–194.

Heyer J, Berger U, Kuzin IL, Yakovlev ON. 2002. Methane emissions from different ecosystem structures of the subarctic tundra in Western Siberia during midsummer and during the thawing period. Tellus Ser B Chem Phys Meteorol 54:231–249.

Hofrichter M, Fakoussa RM. 2001. Lignin, humic substances and coal. In: Steinbüchel A (Eds.). Biopolymers, 1. Weinheim: Wiley-VCH. P 393–429.

Hosgörmez H, Yalcin NM, Cramer B, Gerling P, Faber E, Schaefer RG, Mann U. 2002. Isotopic and molecular composition of coal-bed gas in the Amasra region (Zonguldak basin - western Black Sea). Organ Geochem 33:1429–1439.

Khalil MAK, Rasmussen RA, Shearer MJ, Ge S, Rau JA. 1993. Methane from coal burning. Chemosphere 26:473–477.

Kotarba MJ. 2001. Composition and origin of coalbed gases in the Upper Silesian and Lublin basins, Poland. Organ Geochem 32:163–180.

Krüger M, Frenzel P, Conrad R. 2001. Microbial processes influencing methane emission from rice fields. Glob Change Biol 7:49–61.

Li H, Yang SZ, Mu BZ, Rong ZF, Zhang J. 2007. Molecular phylogenetic diversity of the microbial community associated with a high-temperature petroleum reservoir at an offshore oilfield. FEMS Microbiol Ecol 60:74–84.

Lollar BS, Lacrampe-Couloume G, Slater GF, Ward J, Moser DP, Gihring TM, Lin LH, Onstott TC. 2006. Unravelling abiogenic and biogenic sources of methane in the Earth's deep subsurface. Chem Geol 226:328–339.

Nazina TN, Ivanova AE, Borzenkov IA, Belyaev SS, Ivanov MV. 1995. Occurence and geochemical activity of microorganisms in high-temperature, water-flooded oil fields of Kazakhstan and Western Siberia. Geomicrobiol J 13:181–192.

Nunoura T, Hirayama H, Takami H, Oida H, Nishi S, Shimamura S, Suzuki Y, Inagaki F, Takai K, Nealson KH, Horikoshi K. 2005. Genetic and functional properties of uncultivated thermophilic crenarchaeotes from a subsurface gold mine as revealed by analysis of genome fragments. Environ Microbiol 7:1967–1984.

Pernthaler J, Glöckner FO, Schönhuber W, Amman R. 2001. Fluorescence *in situ* hybridization (FISH) with rRNA-targeted oligonucleotide probes. Meth Microbiol 30:207–226.

Potter MC. 1908. Bakterien als Agentien bei der Oxidation amorpher Kohle. Zentralblatt Bakteriol Parasitenkunde II 21:647–665.

Schink B. 1997. Energetics of syntrophic cooperation in methanogenic degradation. Microbiol Mol Biol Rev 61:262–280.

Schoell M. 1980. The hydrogen and carbon isotopic composition of methane from natural gases of various origins. Geochim Cosmochim Acta 44:649–661.

Schoell M. 1988. Multiple origins of methane in the earth. Chem Geol 71:1–10.

Smith JW, Pallasser RJ. (1996) Microbial origin of Australian coalbed methane. AAPG Bull 80:891–897.

Tao MX, Shi BG, Li JY, Wang WC, Li XB, Gao B. 2007. Secondary biological coalbed gas in the Xinji area, Anhui province, China: Evidence from the geochemical features and secondary changes. Inter J Coal Geol 71:358–370.

Thielemann T, Cramer B, Schippers A. 2004. Coalbed methane in the Ruhr Basin, Germany: a renewable energy resource? Organ Geochem 35:1537–1549.

Vetriani C, Jannasch HW, MacGregor BJ, Stahl DA, Reysenbach AL. 1999. Population structure and phylogenetic characterization of marine benthic *Archaea* in deep-sea sediments. Appl Environ Microbiol 65:4375–4384.

Whiticar MJ. 1990. A geochemical perspective of natural gas and atmospheric methane. Organ Geochem 16:531–547.

Whiticar MJ. 1996. Isotope tracking of microbial methane formation and oxidation. Mitteilungen der Internationalen Vereinigung für Limnologie 25:39–54.

Whiticar MJ, Snowdon LR. 1999. Geochemical characterization of selected Western Canada oils by C-5-C-8 Compound Specific Isotope Correlation (CSIC). Org Geochem 30:1127–1161.

Widdel F, Bak F. 1992. Gram-negative mesophilic sulfate-reducing bacteria. In: A. Balows HGT, M. Dworkin, W. Harder and K.-H. Schleifer (Eds.). The Prokaryotes, IV. New York: Springer-Verlag. P 3352–3378.

Wilms R, Sass H, Köpke B, Cypionka H, Engelen B. 2007. Methane and sulfate profiles within the subsurface of a tidal flat are reflected by the distribution of sulfate-reducing bacteria and methanogenic archaea. FEMS Microbiol Ecol 59:611–621.

Wilms R, Sass H, Köpke B, Köster J, Cypionka H, Engelen B. 2006. Specific bacterial, archaeal, and eukaryotic communities in tidal-flat sediments along a vertical profile of several meters. Appl Environ Microbiol 72:2756–2764.

Zengler K, Richnow HH, Rossello-Mora R, Michaelis W, Widdel F. 1999. Methane formation from long-chain alkanes by anaerobic microorganisms. Nature 401:266–269.

2.2 Publication 2

Role of *Bacteria, Archaea* and *Fungi* involved in methane release in abandoned coal mines

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ABSTRACT

Worldwide, abandoned coal mines release substantial amounts of methane which is largely of biogenic origin. The aim of this study was to understand the microbial processes involved in mine-gas formation. Therefore, coal and timber samples and anaerobic enrichments from two abandoned coal mines in Germany were subjected to DGGE analyses and quantitative PCR. The primers used were specific for *Bacteria*, *Archaea*, *Fungi*, and the key functional genes for sulfate reduction (*dsrA*) and methanogenesis (*mcrA*). A broad spectrum of facultative anaerobic bacteria and acetogens belonging to all five groups (α-ε) of the *Proteobacteria*, as well as the *Bacteroidetes*, *Tenericutes*, *Actinobacteria*, *Chlorobi* and *Chloroflexi* were detected. *Archaea* were represented by acetoclastic *Methanosarcinales* and *Crenarchaeota* with an unknown metabolism. *Fungi* formed thick biofilms particularly on timber, and were identified as typical wood degraders belonging to the *Ascomycetes* and *Basidiomycetes*. The community analysis as well as the environmental conditions and the metabolites detected in a previous study are consistent with the following scenario of methane release: Weathering of coal and timber is initiated by wood-degrading *Fungi* and *Bacteria* under a suboxic atmosphere. In the lower, oxygen-depleted layers *Fungi* and *Bacteria* perform incomplete oxidation and release reduced substrates which can be channeled into methanogenesis. Acetate appeared to be the main precursor of the biogenic methane in the investigated coal mines.

INTRODUCTION

Gas emissions of active and abandoned coal mines account for seven percent of the globally released methane (Denman et al. 2007). Mine gas is not only a possible hazard but also an energy source, which is increasingly coming into focus for industrial utilization worldwide. Stable carbon and hydrogen isotopic signatures indicate that methane in mine-gas in many regions has a mixed thermogenic and biogenic origin (Hosgörmez et al. 2002; Thielemann et al. 2004; Tao et al. 2007). Thermogenic methane is a remainder of geological processes. Biogenic methane formation is still going on.

In a recent study we have measured methane formation rates and detected methanogenic archaea (Krüger et al. 2008). However, the communities and metabolic pathways involved remained unknown. Generally, coal is not a good microbial substrate and only partially degradable. The rate-limiting step is the initial solubilisation of utilisable substrates that is achieved by microbial and especially extracellular fungal enzymes (Deobald and Crawford

1987). The presence of oxygen and water promotes the biodegradation (Fakoussa 1990; Scott andFleet 1994; Fakoussa and Hofrichter 1999; Strapoc et al. 2008a). Another possible source for biogenic methane formation within coal mines are large amounts of timber that were used for the construction of mines and left behind after the end of mining. In the sealed mining compartments, weathering of coal and timber proceed in a suboxic atmosphere at temperatures around 37°C and at 100 % humidity. Wet floor and walls are covered by biofilms. Thick layers of fungi grow on mine timber (Figure 1a-b). Microbial activity causes oxygen depletion and creates anoxic zones in the ground, where methane is being formed. Methanogens rely on syntrophic organisms catalysing the initial decomposition steps and providing acetate and hydrogen as their main substrates. Some methanogens utilise acetate which typically accounts for 70% of methane formed in diverse habitats (Zinder 1993). Others are specialists for H_2 (+ CO_2) as methane precursors, while some methanogens belonging to the genus *Methanosarcina* are able to use both acetate as well as H_2 as their substrates.

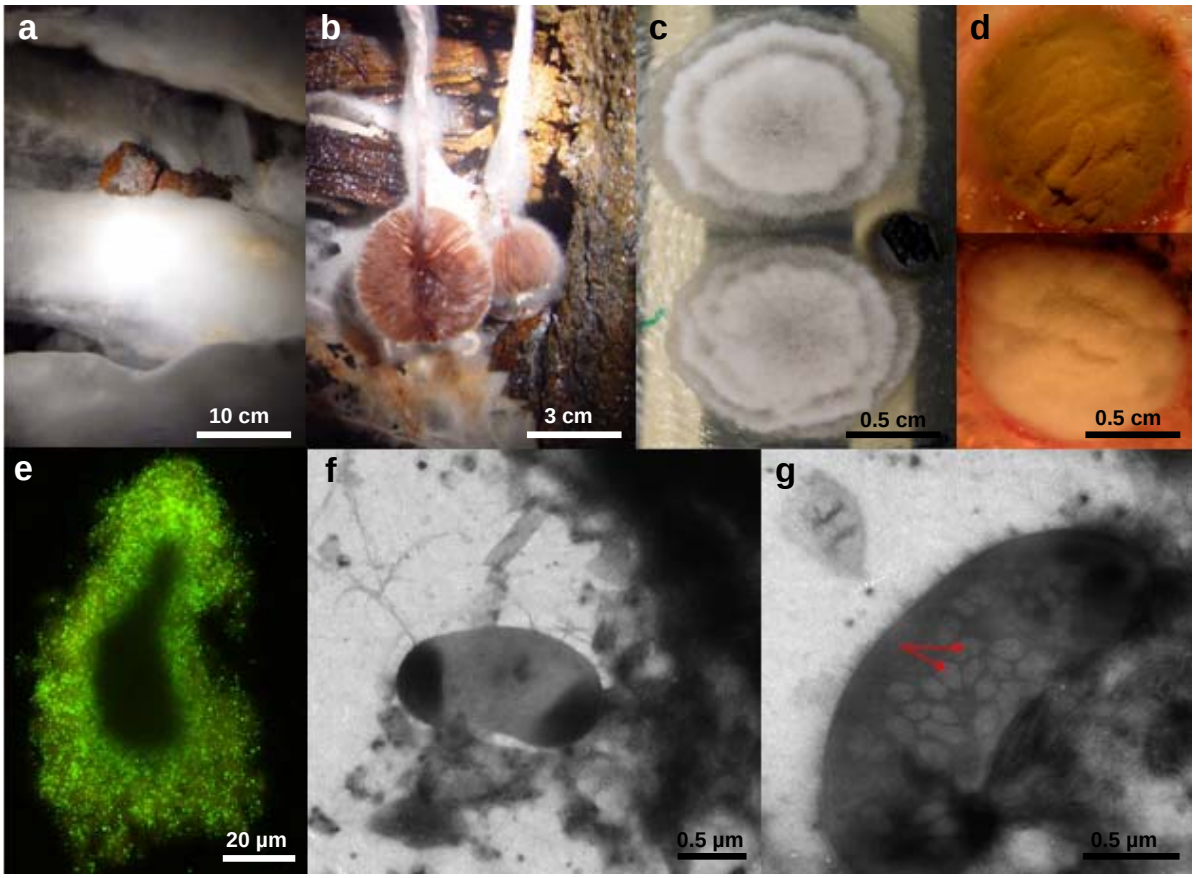

FIG. 1: *Archaea*, *Bacteria* and *fungi* in the coal mines. (a, b) Mine timber overgrown by *fungi*. (c, d) Fungal isolates (*Ascomycetes* and *Basidiomycetes*). (e) Biofilm from mine timber (Sybr Green I staining). (f, g) *Archaea* with vesicles (arrows) from biofilm (Transmission Electron Microscopy).

In a recent study on the microbial communities in a coal mine, we have shown the presence and activity of methanogenic archaea converting acetate to methane (Krüger et al. 2008). Acetate was found to accumulate in assays where methanogens were inhibited by 2-bromoethanesulfonate (BES). This suggested acetoclastic methanogenesis to be the dominant pathway. In contrast, earlier investigations reported that methanogenesis is mainly driven by H_2-utilizing archaea (Flores 2008; Strapoc et al. 2008b). However, these studies refer to water samples collected aboveground, and not to coal or timber samples, directly.

In the present investigation we have assessed not only the archaeal community composition, but also the bacterial and fungal communities. Besides the 16S- and 18SrRNA genes, functional key genes that encode for the dissimilatory sulfate reductase (*dsr*) and the methyl-coenzyme M reductase (*mcr)* were analysed. Hints on the importance of different groups were derived from quantitative PCR. The main objective was not the phylogenetic assessment but to understand the process of methane formation from coal and timber by linking the physiological features of the detected community members.

MATERIAL AND METHODS

Sample collection and enrichment cultures

Samples were collected in February 2006 and May 2007 in sealed compartments of coal mines closed in the 1960`s. The mines harbour weathered hard coal (also called "rock coal") belonging to the fat coals according to the German classification (Thielemann *et al*. 2004 and references therein). Large pieces of coal (2-15 cm) and mine timber (2-10 cm) were collected aseptically in glass bottles that were immediately flushed with N_2 and stored at 4 °C until further processing. *In situ* temperatures were 35-37 °C with 100 % air humidity. Processing of coal and mine timber samples was done in an anaerobic chamber under nitrogen atmosphere to prevent oxidation. Coal and timber samples (1 g) were homogenized and distributed in hungate tubes containing 10 mL of sulfate-free mineral medium (Widdel and Bak 1992) with a salinity of 15 PSU, according to *in situ* values of mine water. Controls were supplemented with 10 mM 2-bromoethanesulfonate (BES) to inhibit methanogenesis. Enrichment cultures were amended with either 10 mM acetate, H_2/CO_2 (80/20), 5 mM methanol, or 10 mM trimethylamine. All

incubations were carried out at least in triplicate. The increase of methane in the headspace as well as the stable isotopes of methane and carbon dioxide produced were continuously monitored over 9 months and analyzed by gas chromatography/mass spectrometry (GC/MS) as described previously by Krüger et al. (2001 and 2008).

DNA extraction and amplification

Genomic DNA from 0.5 g and 10 g (wet weight) of coal and mine-timber samples, respectively, was obtained either by freeze-thawing of cell pellets or by using the FastDNA® Spin Kit (Q-BIOgene, Carlsbad, CA, USA) and the UltraClean Soil DNA Kit Mega Prep (MO BIO Laboratories, Inc., Carlsbad, CA, USA), according to the manufacturer's instructions. The DNA was concentrated by ethanol precipitation, and dissolved in either 30 µL or 100 µL of sterile distilled water. The extracted DNA was used as the target for polymerase chain reaction (PCR). For denaturing gradient gel electrophoresis (DGGE), a 550-bp fragment of the archaeal 16S rRNA gene was amplified by using the primers S-D-Arch-GC-0344-a-S-20 (5`-ACG GGG CGC AGC AGG CGC GA-3`) and 907r (5`-CCG TCA ATT CCT TTG AGT TT-3`). For the analysis of bacterial composition, the primers GC357f (5`-CCT ACG GGA GGC AGC AG-3`) and 907r were used to amplify partial 16S rRNA genes (Muyzer et al. 1995). For the amplification of the fungal 18S rRNA gene and the fungal ITS gene, the primer pairs EF4 (5`-GGA AGG GRT GTA TTT ATT AG-3`)/EF3 (5`-TCC TCT AAA TGA CCA AGT TTG-3`), EF4/NS3 (5`-GGC TGC TGG CAC CAG ACT TGC-3`) and ITS1F (5`-CTT GGT CAT TTA GAG GAA GTA-3`)/ITS4 (5`- CGC CGT TAC TGG GGC AAT CCC TG-3`) were used (Larena et al. 1999; Smit et al. 1999; Brodie et al. 2003). At the 5`-end of each forward primer, an additional 40-nucleotide GC-rich sequence (GC-clamp) was added to obtain a stable melting point of the DNA fragments in the DGGE according to Muyzer et al. (1993). PCR amplification was performed using an Eppendorf Thermal Cycler system (Mastercycler, Eppendorf, Hamburg, Germany) as follows: 2 µL (1-100 ng) of template DNA, 1 U of *Taq* DNA polymerase, the manufacturers`recommended buffer as supplied with the polymerase enzyme, 0.2 mM dNTP`s, 100 pM of each of the appropriate primers, and 10 mM of BSA were adjusted to a total volume of 50 µL with PCR water (Ampuware, Fresenius, Bad Homburg, Germany). The PCR-program included an initial denaturation step for 4 min at 96 °C. For the PCR of archaeal DNA, a first cycle step was carried out for 30 s at 96 °C; 1 min at 48 °C; and 1 min at 72 °C. For amplification of bacterial DNA, 30 s at 94 °C; 45 s at 55 °C; and 1 min at

72 °C was used. For PCR of the fungal 18S rRNA and the ITS region the following thermocycling pattern was used: 1 min at 94°C, 1 min at 50°C (18S rRNA) and 55°C (ITS), and 2 min at 72°C. The total number of cycles was 35. Primer extension was carried out for 10 min at 72 °C. Aliquots (5 µL) of the PCR products were analyzed by agarose gel electrophoresis in 1.5% (wt/vol) agarose gels and ethidium bromide staining (0.8 ng mL^{-1}) for 20 min on a UV transilluminator as described previously (Wilms et al. 2007).

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

DNA standards for quantitative (real-time) PCR were prepared as described by Wilms et al. (2007) and Engelen et al. (2008). Briefly, the bacterial 16S rRNA gene of *Desulfovibrio vulgaris*^{T}(DSM 644) and the archaeal 16S rRNA gene of *Methanosarcina barkeri*^{T}(DSM 800) were amplified using the bacterial primer pair 8f and 1492r (Lane 1991) or the domain specific primer set S-D-Arch-0025-a-S-17 and S-*-Univ-1517-a-A-21 (Vetriani et al. 1999). The PCR amplicons were purified using the Quia quick purification kit (Quiagen, Hilden, Germany) in accordance with the manufacturer`s instructions and quantified using PicoGreen staining as described by Wilms et al. (2007). The purified PCR products were diluted from $1:10^3$ to $1:10^{10}$ and served as a target for the qPCR standard curves.

Quantitative PCR was used to determine bacterial and archaeal abundances in the original samples. Bacterial and archaeal targets were measured in at least three different dilutions of DNA extracts (1:10 to 1:1000) and in triplicate. Primer sets specific for different phylogenetic domains and functional genes were used according to Wilms et al. (2007). For the quantitative PCR assay of *Crenarchaea* and *Methanosarcinales,* the specific primer sets Cren28F (5`-AAT CCG GTT GAT CCT GCC GGA CC-3`)/ Cren457R (5`-TTG CCC CCC GCT TAT TCS CCC G-3`) (Schleper et al. 1997) and MSL812F (5`-GTA AAC GAT RYT CGC TAG GT-3`) / MSL1159R (5`-GGT CCC CAC AGW GTA CC-3`) (Shin et al. 2008) were used, respectively. The qPCR mixtures contained 12.5 µL of the premix solution of a DyNAmo HS SyberGreen qPCR Kit (New England Biolabs, Inc., Hitchin, UK), 1 µL of each primer and 10 µL standard or DNA extract as a template in a final reaction volume of 25 µL. The PCR was carried out in a Rotor-Gene-3000 cycler (Corbett Research, Sydney, Australia). After initial denaturation at 95°C for 15 min, 50 cycles followed. Each cycle consisted of denaturation for 10 s at 94°C, annealing for 20s 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 also measured at the end of each cycle for 20 s at 82°C for *Bacteria* and 80°C for *Archaea*. After the last cycle, a melting curve was recorded by increasing the temperature from 50°C to 99°C (1°C every 10 s). The numbers of bacterial and archaeal 16S rRNA targets were calculated from the DNA concentrations according to Süss et al. (2006). Cell numbers of *Archaea* and *Bacteria* were estimated using an average 16S rRNA copy number of 4.1 and 1.5, respectively (http://rrndb.cme.msu.edu/).

Denaturing gradient gel electrophoresis (DGGE) analysis

DGGE was performed using an INGENYphorU-2 system (Ingeny, Goes, The Netherlands). PCR products and loading buffer (40% [wt/vol] glycerol, 60% [wt/vol] 1x Tris-acetate-EDTA [TAE], and bromphenol blue) were mixed in a ratio of 1:2. The PCR amplicons were applied directly onto 6% (wt/vol) polyacrylamide gels with a linear gradient of 30-80% denaturant for archaeal and 50-70% for bacterial PCR products (with 100% denaturant corresponding to 7 M urea and 40% [vol/vol] formamide). Electrophoresis was accomplished in 1xTAE buffer (40 mM Tris-acetate [pH 7.4], 20 mM sodium acetate, 1 mM Na_2EDTA), at a constant 100 V and a temperature of 60°C for 20 h. After electrophoresis, the gels were stained for 2 h in 1xSybrGold solution (Molecular Probes, Eugene, USA) in 1 x TAE and washed for 20 min with distilled water. The gel was digitized using the digital imaging-system (BioDocAnalyze; Biometra, Göttingen, Germany) with UV transillumination (302 nm).

Reamplification and sequencing of DGGE bands

Denaturant gradient gel electrophoresis bands were excised for sequencing using a sterile scalpel and treated as described by Del Panno et al. (2005). Briefly, DGGE bands were transferred into 50 µL of PCR water and incubated for 48 h at 4 °C. For reamplification, 2 µL of the supernatant was taken as a template using the same reaction mix as described above with a final volume of 50 µL. The PCR protocol was adjusted for the reamplification: 96 °C for 30 s, 48 °C for 1 min, and 72 °C for 1 min for the reamplification of the archaeal DNA and 94 °C for 30 s, 55 °C for 45 s, 72 °C for 1 min for the reamplification of the bacterial DNA. The total number of cycles was 25 and the final elongation was carried out at 72 °C for 10 min. Some

amplicons were loaded onto a second DGGE gel for purification and were excised again. The bands were treated as described above and served as a target for sequencing the amplified fragments of the 16S rRNA genes. PCR products were purified by using the QIAquick PCR purification Kit (Quiagen GmbH) and eluted in 30 µL of PCR water. DNA yields were estimated fluorometrically in a microtiterplate reader (FLUOstar Optima, BMG Labtechnologies, Offenburg, Germany) using a 1:200 diluted PicoGreen reagent according to a modified manufacturer`s protocol (Molecular Probes, Eugene, USA) as described in detail by Wilms et al. (2007). Only one tenth part of each volume and 1 µL of the extracted DNA and lambda-DNA (in concentrations ranging from 100 ng μL^{-1} to 1 ng μL^{-1}) were used. The 16S rRNA gene sequences were obtained by cycle sequencing using the DNA Sequencing System 4000 (Li-COR Inc., Lincoln, NE, USA) with the IRDye^{TM}800-labeled primer 907r and the DYEnamic direct cycle sequencing kit (Amersham Biosciences, Little Chalfont, UK) in accordance with the manufacturer`s instructions. Sequences were compared to those in GenBank using the BLAST tool of the National Center for Biotechnology Information server (Altschul et al. 1997) and have been deposited in the GenBank nucleotide sequence database under the Accession Nos. FN13616-FN13723.

RESULTS

Potential fermenters dominate the bacterial community

DGGE fingerprints from original samples and cultures showed that both mines harboured similar bacterial communities with differences between coal and timber samples (Figure 2, Table 1). A total of 48 bacterial 16S rRNA gene sequences were obtained (Table 1). Most sequences were derived from Gram-negative bacteria of all five groups (α-ε) of the *Proteobacteria*, the *Bacteroidetes*/*Chlorobi* group and the *Chloroflexi.* The detected Gram-positive bacteria exclusively belonged to the *Firmicutes*.

Besides the phylogenetic analysis we made a physiological assessment of the closest cultured relatives of the detected groups. The majority of the microbial community in both, coal and timber was represented by different bacterial populations that were related to fermenting bacteria (Table 1). Many of the relatives are capable of fermenting sugars,

including mono- and polysaccharides (cellulose, chitin). These potential fermenters belonged to the *Proteobacteria* (*Thalassobaculum*, *Janthinobacterium* sp.) and *Firmicutes* (*Clostridium*, *Gracilibacter*, *Acholeplasma* sp.). Another group is capable of utilising hydrocarbons represented by sequences of *Bacteroidetes/Chlorobi* group (*Pelobacter* sp.) and some *Proteobacteria* (*Hydrogenophaga*, *Pedobacter, Pseudoaminobacter* sp.).

Furthermore, sequences were derived from nitrate-, sulfate- or sulfur reducers. The majority of them was obtained from timber samples and belonged to *Proteobacteria* capable of using acetate as electron donor. In coal samples, only one sequence affiliated with a nitrate reducer with nitrogen-fixing abilities (*Azoarcus* sp.) and one from a sulfur reducer (*Desulfurivibrio alkaliphilus*) were found.

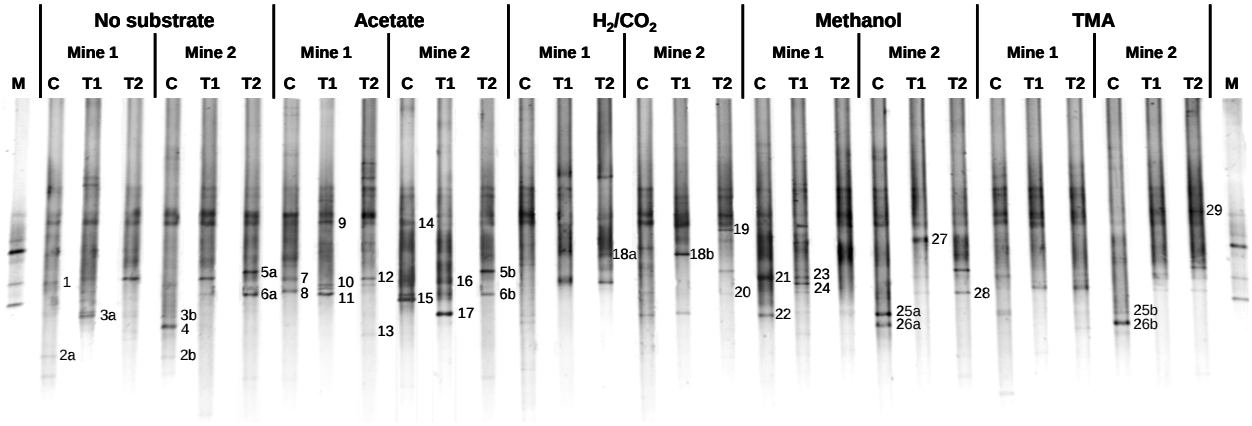

FIG. 2: Denaturing gradient gel electrophoresis (DGGE) of the bacterial community in unamended and amended cultures of weathered coal and mine timber from two different coal mines. The numbered bands were excised and sequenced. Small letters indicate bands of the same migration behaviour and sequence. The sequence similarity to the closest cultivated phylotype is given in Table 1. Bands with equivalent positions in different lanes (indicated by small letters) always corresponded to the same sequences.

TABLE 1. Bacterial diversity detected by denaturing gradient gel electrophoresis (DGGE) from original and amended/unamended incubation samples of weathered coal and mine timber.

	Coal mine 1			
	Closest cultured relative	Similarity (%)	Band no. in Fig. 2	Accession no.
Original sample	*Anaeromyxobacter* sp.*	96		
	Actinobacterium sp.*	99		
	Acholeplasma sp.	95		
	Dechloromonas hortensis	94		
	Azoarcus sp.	98		
	Gracilibacter thermotolerans	89		
	Desulfuromonas michiganensis	94		
	Desulfuromonas acetexigens	89		
	Chloroflexi sp.	87		
Cultures				
Unamended	*Janthinobacterium lividum**	96	1	
	*Pelobacter acetylenicus**	95	2a	
	Desulfurivibrio alkaliphilus	95	3a	
Acetate	*Chlorobi* sp.*	84	7	
	Azoarcus sp.*	99	8	
	Desulfocapsa thiozymogenes	72	9	
	Halochromatium sp.	91	10	
	Desulfobulbus sp.	91	11	
	Thiorodospira sibirica	94	12	
	Desulfosalina propionicus	93	13	
H_2/CO_2	*Acholeplasma brassicae*	90	18a	
Methylamine	*Halochromatium* sp.*	94	21	
	*Halochromatium salexigens**	93	22	
	Nitratireductor aquibiodomus	98	23	
	Acholeplasma sp.	94	24	

*Coal original samples and incubations

Methanosarcinales and *Crenarchaeota* represent the *Archaea*

Methanogenic archaea were enriched in all amended (acetate, hydrogen and methylamine) and unamended cultures and were also present in the original samples of coal and timber in both coal mines. Most of them were related to acetoclastic *Methanosarcinales* (*Methanosarcina* sp., *Methanosaeta* sp., Table 2). Methylotrophic *Methanosarcinales* related to *Methanolobus* sp. were selectively enriched from mine timber and coal in cultures containing methylamine.

Sequences originating from uncultured *Crenarchaeota* were observed in coal and timber cultures and in original samples of both mines (Table 2). The crenarchaeal sequences belonged to unclassified *Thermoprotei* and to members of the marine archaeal group 1. The only crenarchaeal sequence giving hints on a possible metabolism was observed in methylamine cultures, and was related to the ammonia-oxidising *Nitrosopumilus maritimus*. Two euryarchaeal sequences derived from members of *Halobacteriales* and *Thermoplasmatales* were detected in coal. Furthermore, the use of archaea-specific primers yielded one 18S fungal rRNA sequence (see below).

TABLE 2. Phylogenetic affiliation of DGGE bands after amplification of the archaeal 16S rRNA gene in the untreated samples and in the unamended/amended incubations of weathered coal and mine timber samples from two different coal mines.

	Coal mine 1 2006			Coal mine 1 2007			Coal mine 2 2007		
Substrate	Closest relative	Similarity (%)	Acc. no.	Closest relative	Similarity (%)	Acc. no.	Closest relative	Similarity (%)	Acc. no.
Original	Uncultured *Crenarchaeote**	83		Uncultured *Euryarchaeote**	97		*Natronomonas pharaonis**	94	
	Uncultured *Thermoplasmatales**	83		Uncultured *Thermoplasmatales**	82		Uncultured *Methanosarcineaceae*	87	
	Uncultured *Crenarchaeote*	99		Uncultured *Methanosarcinaceae*	95		Uncultured *Crenarchaeote*	87	
	Uncultured *Methanosarcinaceae*	95		Uncultured *Crenarchaeote*	99		*Serpula himantioides*	96	
	Methanomethylovorans hollandica	96		Uncultured marine *Crenarchaeote*	96				
Unamended	*Methanosarcina barkeri**	96		Uncultured *Euryarchaeote**	97		Uncultured *Crenarchaeote**	99	
	Methanosarcina barkeri	93		Uncultured *Crenarchaeote**	98		Uncultured *Methanosarcinales*	98	
	Methanosarcina siciliae	99		*Methanosarcina barkeri*	98				
	Uncultured *Crenarchaeote*	96		*Methanolobus taylorii*	97				
Acetate	*Methanosarcina lacustris**	97		*Methanolobus taylorii**	97		Uncultured *Crenarchaeote**	99	
	Methanosarcina barkeri	98		*Methanosarcina barkeri*	98		Uncultured *Methanosarcinaceae*	99	
	Methanosaeta harundinaceae	98		*Methanosaeta harundinaceae*	98				
H_2/CO_2	*Methanosarcina barkeri**	98		Uncultured *Crenarchaeote**	99		Uncultured *Crenarchaeote**	99	
	Methanosarcina barkeri	98		*Methanosarcina barkeri*	98		Uncultured *Methanosarcinaceae*	99	
	Methanolobus taylorii	97		*Methanolobus taylorii*	96				
	Methanosaeta harundinaceae	93							
	Methanomethylovorans hollandica	93							
	Uncultured *Crenarchaeote*	97							
Methylamine	*Methanolobus taylorii*	97		*Methanolobus taylorii**	97		Uncultured *Methanosarcinaceae**	98	
	Methanolobus oregonensis	97		*Methanosarcina barkeri*	99		*Methanolobus taylorii*	94	
	Methanolobus tindarius	95		*Methanolobus taylorii*	97		*Nitrosopumilus maritimus*	89	
				Uncultured *Crenarchaeote*	96				

*Coal original samples and incubations

Biofilms harbour specific bacterial communities

The host-rock walls and the timber truss of the mines were largely covered by biofilms (Figure 1e), which were analysed separately. The microbial community composition differed from those of coal and timber. A total of 14 sequences were affiliated to members of *Nitrospirae*, *Firmicutes*, *Bacteroidetes/Chlorobi* group and the *Proteobacteria* (Table 3). The archaeal domain was represented by methanogenic archaea (Figure 1f-g) belonging to the obligate hydrogenotrophic *Methanococcales* and methylotrophic *Methanosarcinales* (Table 3). Furthermore, sequences originating from uncultured *Crenarchaeota* simlar to those detected in coal and timber were found. However, a few sequences were affiliated with the ammonia-oxidizing Candidatus *Nitrososphaera gargensis* (97% similarity).

The host rock harboured a completely different microbial community represented by sequences derived from the *Bacteroidetes/Chlorobi* group (uncultured *Psychroflexus*, 87% sequence similarity), the *Firmicutes* (*Butyrivibrio fibrisolvens*, 90%) and the *Gammaproteobacteria* (*Legionella feelei*, 93%). With archaeal primers, only sequences related to uncultured *Crenarchaeota* were found.

TABLE 3. Procaryotic diversity detected in the biofilm covering large parts of the host-rock walls and timber truss in mine 1.

	Biofilm on host-rock wall		
	Closest cultured relative	Similarity (%)	Accession no.
Bacteria	*Balneola* sp.	91	
	Legionella feeleii	87	
	Uncultured *Psychroflexus*	87	
	Nitrospira moscoviensis	84	
	Rubrivivax sp.	91	
	Hydrogenophaga sp.	87	
Archaea	*Methanomethylovorans hollandica*	99	
	Methanotorris formicicum	81	
	Candidatus *Nitrososphaera gargensis*	97	
	Uncultured *Crenarchaeote*	100	
	Biofilm on timber truss		
Bacteria	*Fusibacter paucivorans*	97	
	Legionella feeleii	91	
	Balneola sp.	87	
	Nitrospira moscoviensis	84	
Archaea	*Methanomethylovorans hollandica*	99	
	Uncultured *Crenarchaeote*	100	

*Coal original samples and incubations

Typical wood-degrading fungi colonise mine timber

A total of 13 species belonging to the *Ascomycetes* and *Basidiomycetes* were detected from timber samples by DGGE fingerprinting and sequencing. Of these, 10 were isolated (Table 4, Figure 1c-d). Members of the *Ascomycetes* predominated the fungal communities in both mines, although the community composition was different. A higher diversity of *Fungi* was detected in coal mine 1, where large fungal mats covered the timber (Figure 1a-b). Mine 2 harboured less species. The majority of the detected sequences could be affiliated with relatives of wood-degrading *Fungi*. As mentioned above, the use of archaea-specific primers yielded 18S fungal rRNA related to *Serpula himantioides* (96% similarity), a timber-degrading fungus belonging to the *Basidiomycetes*.

TABLE 4. *Fungi* detected by DGGE in original samples of mine timber from two different coal mines.

Coal mine 1		Coal mine 2	
Closest relative	Similarity (%)	Closest relative	Similarity (%)
Ascomycetes			
Aspergillus sydowii	77	*Pseudallescheria boydii*	84
Hypocrea lixii	94	*Nectria mauritiicola*	88
Penicillium islandicum	86		
Penicillium cyclopium	86		
Acremonium sp.	94		
Verticillium antillanum	94		
Basidiomycetes			
Oligoporus balsameus	97	*Bjerkandera adusta*	98
Hyphodontia hastata	97		
Trechispora alnicola	98		
Bridgeoporus nobilissimus	96		

Quantitative PCR confirms the abundance of dominant groups

In order to assess the abundance of selected groups, quantitative PCR was performed on the original samples. Broad-range 16S rRNA primer sets were used to evaluate the bacterial *vs*. archaeal abundances. *Bacteria* ranging between 10^6 and 10^8 cells g^{-1} were the most abundant prokaryotes in coal as well as in timber (Figure 3). The numbers were higher in timber than in

coal. For the quantitative detection of sulfate reducers, the dissimilatory sulfite reductase gene (*dsrA*) was targeted. Consistently low abundances of sulfate reducers (0.01-1% of total bacteria) were found with moderately higher abundances in timber than in coal.

Archaeal numbers (10^3 to 10^4 cells g^{-1} coal and 10^4 to 10^5 cells g^{-1} timber) were 3-4 orders of magnitude lower than those of the bacteria (Figure 3). Specific 16S rRNA primer sets were applied for the differentiation of the *Methanosarcinales* and the *Crenarchaeota*. These two groups accounted for almost 90 % of the *Archaea (Methanosarcinales* 35%, and *Crenarchaeota* 55%). Furthermore, the quantification of the methylcoenzyme M reductase gene (*mcrA)* as a proxy for methanogenic archaea showed that *Methanosarcinales* accounted for most of the the methanogens.

The abundance of fungal copy numbers was determined by fungi-specific 18S rRNA primers. Fungal targets reached numbers of 10^5 and 10^8 copies per g coal and timber, respectively.

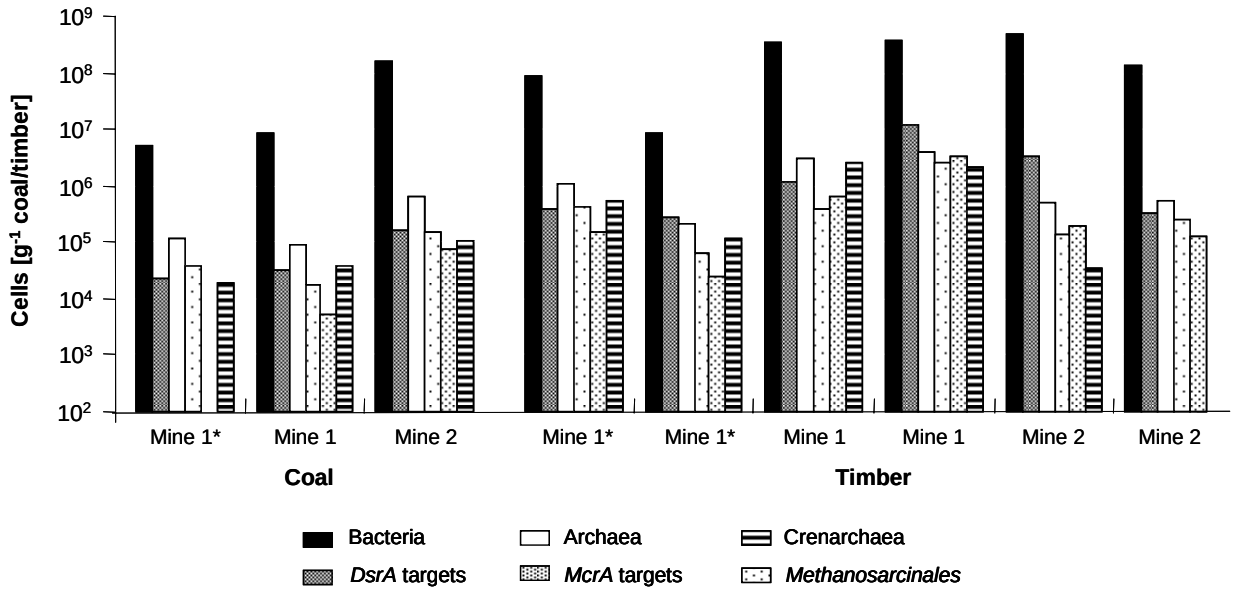

FIG. 3. Profile of the microbial abundances in coal and mine timber of two different coal mines from the sampling campaigns in 2006* and 2007. The results of the timber samples were gained from two different sampling sites inside the same coal mine. Relative abundances of *Archaea*, *Bacteria* and *fungi* and functional genes (*dsrA* and *mcrA*-genes) were determined via quantitative PCR. Additionally, the abundances of *Methanosarcinales* and *Crenarchaea* as important subgroups within the domain *Archaea* were determined. The calculated standard deviations for replicate quantifications of one sample were constantly between 10 and 50%.

DISCUSSION

In this study we have identified not only the methane-producing archaea in two abandoned coal mines, but also the populations feeding them and the possible substrates utilised for this process (Figure 4). For the first time, a quantitative assessment of the prokaryotic communities in coal and timber was performed. The following scenario regards the environmental situation and the fact that acetate is the precursor of methane. We assumed that the identified populations have similar metabolic capacities as their cultured relatives. This assumption is hypothetical and not yet confirmed by activity measurements.

Oxic conditions promote weathering of coal and timber

After the end of mining in the 1960's, the mines of this study were closed with several metre thick concrete walls. Since there was only marginal ventilation via small empty pipes or cracks, the atmospheric conditions in these parts were characterised by high humidity and a low oxygen content with less than 4% oxygen. However, oxygen was still available. Weathering of coal normally starts by a microbial attack under oxic conditions (Fakoussa 1990). However, the observation of thick fungal mats on timber lets us assume that timber provides the main precursors of methane. Wood-degrading *Fungi* are aerobes that usually carry out complete oxidation of their substrates. Underneath the mats, oxygen depletion might occur and create anoxic conditions, particularly at lowered atmospheric O_2 concentrations. Under these conditions, *Fungi* might perform incomplete oxidation and release reduced substrates which can be channeled into methanogenesis.

A way to overcome potential nitrogen limitations

Timber and coal have low nitrogen content, and communities feeding on them might possibly run into nitrogen limitation. This was found for fungal timber degradation especially in the later decomposition stages (Spano et al. 1982). In case of nitrogen limitation, two potential diazotrophs (*Clostridium* sp. and *Azoarcus* sp.) found in our samples might potentially help to overcome these deficiencies (Jurgensen 1973; Larsen et al. 1978; Jurgensen et al. 1984). A beneficial effect of N_2-fixing wood-inhabiting bacteria on fungal growth was found in coal seams before (Hurek and Reinholdhurek 1995; Shin et al. 2008). Interestingly, relatives of

these bacteria were described as tunneling bacteria paving the way for fungal hyphae through decaying wood (Rogers and Baecker 1991; Clausen 1995).

Facultative anaerobic bacteria and acetogens are assumed to fuel methanogenesis

The major bacterial groups were also observed in deep coal layers in recent investigations by Fry *et al.* (2009). The relaivtives of most of them are capable of both, oxygen respiration and fermentation. Only few sequences are derived from strict anaerobes (Figure 4). Some relatives of the detected bacteria are related to acetogens using hydrogen as electron donor (Küsel et al. 2000). Acetogenic *Clostridia* were found in earlier surveys in coal-mine sediments (Shimizu et al. 2007; Shin et al. 2008). The presence of the acetogens might explain the low abundance of hydrogenotrophic methanogens, and thus favouring acetoclastic methanogenesis as the dominant process.

Methane is released by acetoclastic *Methanosarcinales*

Our recent detection of acetate-consuming methanogens in abandoned coal mines (Krüger et al. 2008) was confirmed on a quantitative basis. All methanogens found in original samples and unamended enrichments belonged to the *Methanosarcinales.* Their nearest cultured relatives can utilise acetate, some of them additionally hydrogen. Our observations are in accordance with the detection of high methane formation rates in enrichment cultures with acetate, and also with the accumulation of acetate in incubations after inhibition of methanogens by 2-bromoethanesulfonate (Krüger et al. 2008). Obviously, acetate is the main precursor of methane in the investigated coal mines. Acetoclastic methanogenesis was also dominant in two other coal seams investigated recently (Green et al. 2008; Ulrich and Bower 2008). Methanogen sequences derived from *Methanosarcinales* were also observed in deep coal deposits (Fry et al. 2009). However, in mine water and drainages sampled aboveground, hydrogenotrophic methanogens were prevailing (Flores 2008; Warwick et al. 2008; Strapoc et al. 2008a). Furthermore, methylotrophic methanogenesis appears to play a role in coal seam groundwaters investigated by Shimizu et al. (2007). We selectively enriched methylotrophic methanogens (with methanol or trimethylamine), but did not find a quantitative impact on the i*n situ* processes.

The methanogens constituted about half the *Archaea*, the other half belonged to uncultured *Crenarchaeota*. A few sequences were affiliated with ammonia-oxidising *Nitrosopumilus maritimus* and *Nitrososphaera gargensis*. *N. maritimus* has already been detected in coal waters (Shimizu et al. 2007). However, no *Crenarchaeota* were found in one timber sample of mine 2 and until now nothing is known about the physiological features of the majority of the *Crenarchaeota* present in our samples.

In conclusion, in the sealed compartments of the coal mines a complex community of microbes is involved in methane formation, inhabiting very distinct ecological niches. The presence of biogenic methane not only in mined and pristine coal seams but also in oil reservoirs (Head et al. 2004) or black shales (Martini et al. 2008) underlines the large potential of this unconventional biological energy resources for future exploitation. Our findings provide the basis for a deeper understanding of the underlying processes and timescales, and thus should help to get more reliable estimates of global methane inventories.

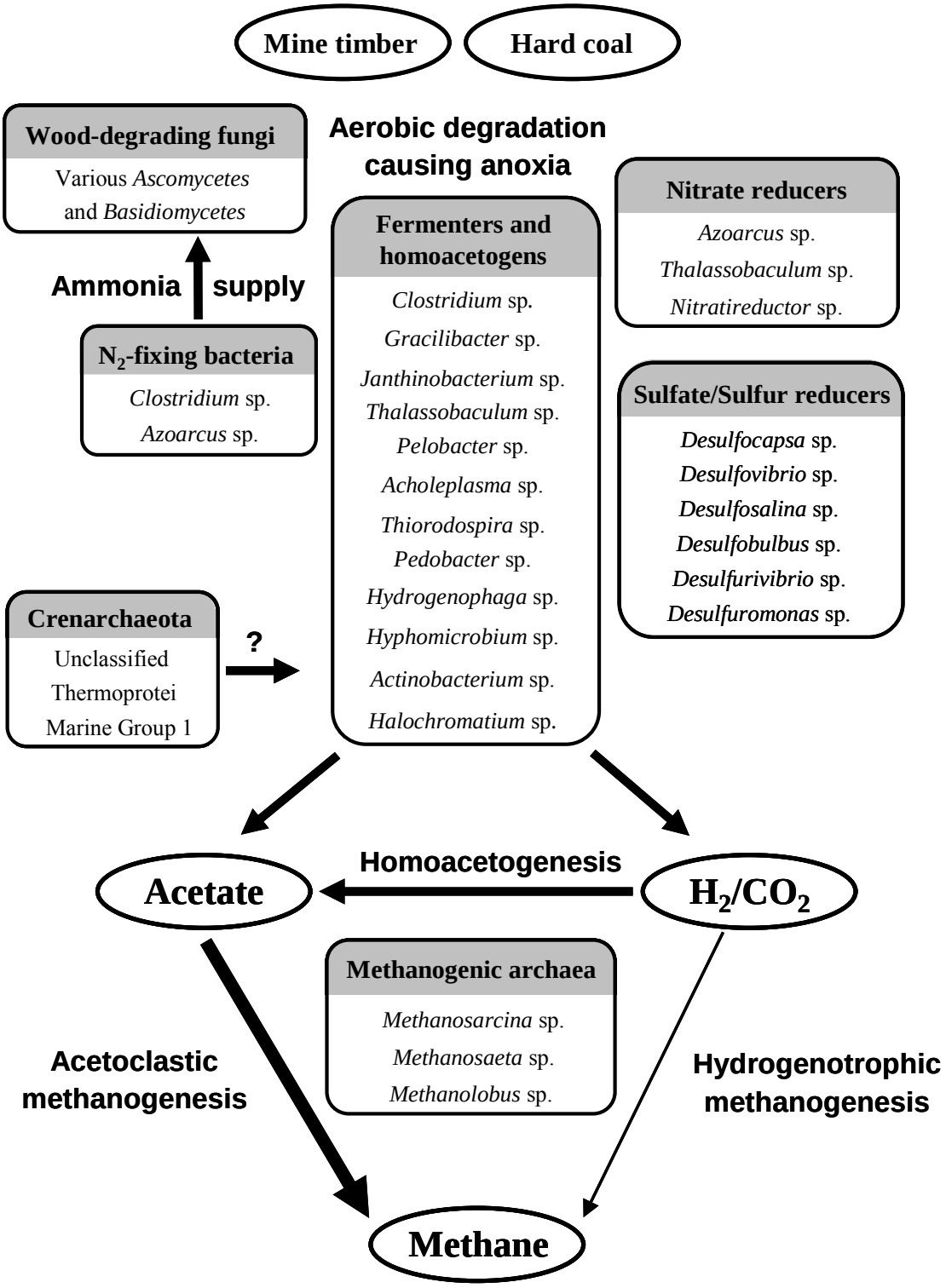

FIG. 4. Overview of the main organism groups and their feasible involvement in the degradation of hard coal and mine timber in two investigated coal mines.

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REFERENCES

Altschul SF, Madden TL, Schaffer AA, Zhang JH, Zhang Z, Miller W, Lipman DJ. 1997. Gapped BLAST and PSI-BLAST: a new generation of protein database search programs. Nucleic Acids Research 25:3389-3402.

Brodie E, Edwards S, Clipson N. 2003. Soil fungal community structure in a temperate upland grassland soil. Fems Microbiology Ecology 45:105-114.

Clausen CA. 1995. Dissociation of the multienzyme complex of the brown-rot fungus postia-placenta. FEMS Microbiology Letters 127:73-78.

Del Panno M, Morelli I, Engelen B, Berthe-Corti L. 2005. Effect of petrochemical sludge concentration on microbial communities during a soil bioremediation process. FEMS Microbiology Ecology 53:305-316.

Denman K, Brasseur G, Chidthaisong A, Ciais P, Cox P, Dickinson R, Hauglustaine D. 2007. Couplings between changes in the climate system and biogeochemistry. In: Solomon S, Qin D, Manning M, Chen Z, Marquis M, Averyt KB, Tignor M, Miller HL. (Eds.), Climate Change 2007: The Physical Science Basis. Conribution of Working Group I to the Fourth Assessment Report of the Intergovernmental Panel on Climate Change. Cambridge and New York. Cambridge University Press.

Deobald LA, Crawford DL. 1987. Activities of cellulase and other extracellular enzymes during lignin solubilization by Streptomyces-Viridosporus. Applied Microbiology and Biotechnology 26:158-163.

Engelen B, Ziegelmüller K, Wolf L, Köpke B, Gittel A, Cypionka H, Treude T, Nakagawa S, Inagaki F, Lever MA, Steinsbu BO. 2008. Fluids from the oceanic crust support microbial activities within the deep biosphere. Geomicrobiology Journal 25:56-66.

Fakoussa RM. 1990. Microbiological treatment of German hard coal. In: Wise DL (Ed). Bioprocessing and biotreatment of coal. New York: Marcel Dekker. P95-107.

Fakoussa RM, Hofrichter M. 1999. Biotechnology and microbiology of coal degradation. Applied Microbiology and Biotechnology 52:25-40.

Flores RM. 2008. Microbes, methanogenesis, and microbial gas in coal. International Journal of Coal Geology 76:1-2.

Fry CJ, Horsfield B, Sykes R, Cragg BA, Heywood C, Kim GT, Mangelsdorf K, Mildenhall DC, Rinna J, Vieth A, Zink K-G, Sass H, Weightman AJ, Parkes RJ. 2009. Prokaryotic populations and activities in an interbedded coal deposit, including a previously deeply buried section (1.6-2.3 km) above ~150 Ma basement rock. Geomicrobiology Journal 26:163-178

Green MS, Flanegan KC, Gilcrease PC. 2008. Characterization of a methanogenic consortium enriched from a coalbed methane well in the Powder River Basin, USA. International Journal of Coal Geology 76:34-45.

Head IM, Jones DM, Larter SR. 2003. Biological activity in the deep subsurface and the origin of heavy oil. Nature 426:344-52.

Hosgörmez H, Yalcin NM, Cramer B, Gerling P, Faber E, Schäfer RG, Mann U. 2002. Isotopic and molecular composition of coal-bed gas in the Amasra region (Zonguldak basin - western Black Sea). Organic Geochemistry 33:1429-1439.

Hurek T, Reinholdhurek B. 1995. Identification of grass-associated and toluene-degrading diazotrophs, *Azoarcus* spp, by analyses of partial 16S ribosomal DNA-sequences. Applied and Environmental Microbiology 61:2257-2261.

Jurgensen MF. 1973. Relationship between nonsymbiotic and nitrogen fixation and soil nutrient status - a review. Journal of Soil Science 24:512-522.

Jurgensen MF, Larsen MJ, Spano SD, Harvey AE, Gale MR. 1984. Nitrogen fixation associated with increased wood decay in Douglas-Fir Residue. Forest Science 30:1038-1044.

Krüger M, Frenzel P, Conrad R. 2001. Microbial processes influencing methane emission from rice fields. Global Change Biology 7:49-63.

Krüger M, Beckmann S, Engelen B, Thielemann T, Cramer B, Schippers A, Cypionka H. 2008. Microbial methane formation from hard coal and timber in an abandoned coal mine. Geomicrobiology Journal 25:315-321.

Küsel K, Dorsch T, Acker G, Stackebrandt E, Drake HL. 2000. *Clostridium scatologenes* strain SL1 isolated as an acetogenic bacterium from acidic sediments. International Journal of Systematic and Evolutionary Microbiology 50:537-546.

Lane D. 1991. *16S/23S rRNA sequencing.* In: Stackebrandt E, Goodfellow, M. (Eds.). Nucleic acid techniques in bacterial systematica. Chichester, UK: Wiley. P 205-248

Larena I, Salazar O, Gonzalez V, Julian MC, Rubio V. 1999. Design of a primer for ribosomal DNA internal transcribed spacer with enhanced specificity for ascomycetes. Journal of Biotechnology 75:187-194.

Larsen MJ, Jurgensen MF, Harvey AE. 1978. N_2 fixation associated with wood decayed by some common fungi in Western Montana. Canadian Journal of Forest Research-Revue Canadienne De Recherche Forestiere 8:341-345.

Martini AM, Lynn MW, McIntosh JC. 2008. Identification of microbial and thermogenic gas components from Upper Devonian black shale cores, Illinois and Michigan basins. American Association of Petroleum Geologists Bulletin 3:327-339.

Muyzer G, Dewaal EC, Uitterlinden AG. 1993. Profiling of complex microbial-populations by denaturing gradient gel-electrophoresis analysis of polymerase chain reaction-amplified genes-coding for 16S ribosomal-RNA. Applied and Environmental Microbiology 59:695-700.

Muyzer G, Teske A, Wirsen CO, Jannasch HW. 1995. Phylogenetic-relationships of *Thiomicrospira* species and their identification in deep-sea hydrothermal vent samples by denaturing gradient gel-electrophoresis of 16S rDNA fragments. Archives of Microbiology 164:165-172.

Rogers GM, Baecker AAW. 1991. *Clostridium-Xylanolyticum* sp-nov, an anaerobic xylanolytic bacterium from decayed Pinus-Patula wood chips. International Journal of Systematic Bacteriology 41:140-143.

Schleper C, Holben W, Klenk HP. 1997. Recovery of crenarchaeotal ribosomal DNA sequences from freshwater-lake sediments. Applied and Environmental Microbiology 63:321-323.

Scott AC, Fleet AJ. 1994. Coal and coal-bearing strata as oil-prone source rocks: current problems and future directions. Geological Society of London, Special Publications 77:201-205.

Shimizu S, Akiyama M, Naganuma T, Fujioka M, Nako M, Ishijima Y. 2007. Molecular characterization of microbial communities in deep coal seam groundwater of northern Japan. Geobiology 5*:*423-433.

Shin SG, Lee CS, Hwang K, Ahn JH, Hwang S. 2008. Use of order-specific primers to investigate the methanogenic diversity in acetate enrichment system. Journal of Industrial Microbiology & Biotechnology 35:1345-1352.

Smit E, Leeflang P, Glandorf B, van Elsas JD, Wernars K. 1999. Analysis of fungal diversity in the wheat rhizosphere by sequencing of cloned PCR-amplified genes encoding 18S rRNA and temperature gradient gel electrophoresis. Applied and Environmental Microbiology 65:2614-2621.

Spano SD, Jurgensen MF, Larsen MJ, Harvey AE. 1982. Nitrogen-fixing bacteria in Douglas-Fir Residue decayed by *Fomitopsis-Pinicola*. Plant and Soil 68:117-123.

Strapoc D, Picardal FW, Turich C, Schaperdoth I, Macalady JL, Lipp JS, Lin YS, Ertefai TF, Schubotz F, Hinrichs KU, Mastalerz M, Schimmelmann A. 2008a. Methane-producing microbial community in a coal bed of the Illinois basin. Applied and Environmental Microbiology 74:3918-3918.

Strapoc D, Mastalerz M, Schimmelmann A, Drobniak A, Hedges S. 2008b. Variability of geochemical properties in a microbially dominated coalbed gas system from the eastern margin of the Illinois Basin, USA. International Journal of Coal Geology 76:98-110.

Süss J, Schubert K, Sass H, Cypionka H, Overmann J, Engelen B. 2006. Widespread distribution and high abundance of *Rhizobium radiobacter* within Mediterranean subsurface sediments. Environmental Microbiology 8:1753-1763.

Tao MX, Shi BG, Li JY, Wang WC, Li XB, Gao B. 2007. Secondary biological coalbed gas in the Xinji area, Anhui province, China: Evidence from the geochemical features and secondary changes. International Journal of Coal Geology 71:358-370.

Thielemann T, Cramer B, Schippers A. 2004. Coalbed methane in the Ruhr Basin, Germany: a renewable energy resource? Organic Geochemistry 35:1537-1549.

Ulrich G, Bower S. 2008. Active methanogenesis and acetate utilization in Powder River Basin coals, United States. International Journal of Coal Geology 76:25-33.

Vetriani C, Jannasch HW, MacGregor BJ, Stahl DA, Reysenbach AL. 1999. Population structure and phylogenetic characterization of marine benthic archaea in deep-sea sediments. Applied and Environmental Microbiology 65:4375-4384.

Warwick PD, Breland FC, Hackley PC. 2008. Biogenic origin of coalbed gas in the northern Gulf of Mexico Coastal Plain, USA. International Journal of Coal Geology 76:119-137.

Widdel F, Bak F. 1992. Gram-negative mesophilic sulfate-reducing bacteria. In: Balows A, Dworkin, M., Harder, W., Schleifer, K.H. (Eds.). The Prokaryotes. New York: Springer-Verlag. P 3352-3378

Wilms R, Sass H, Köpke B, Cypionka H, Engelen B. 2007. Methane and sulfate profiles within the subsurface of a tidal flat are reflected by the distribution of sulfate-reducing bacteria and methanogenic archaea. FEMS Microbiology Ecology 59:611-621.

Zinder SH. 1993. Physiological ecology of methanogens. Ferry JC (Ed). Methanogenesis: ecology, physiology, biochemistry and genetics. New York. Chapman & Hall.

2.3 Publication 3

Activity of acetoclastic *Methanosarcinales* in abandoned coal mines proven by stable isotope probing

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Activity of Acetoclastic *Methanosarcinales* in Abandoned Coal Mines proven by Stable Isotope Probing

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ABSTRACT

In abandoned coal mines, methanogenic archaea are responsible for the production of substantial amounts of methane. The present study aimed to directly unravel the active methanogens mediating the methane release as well as active bacteria potentially involved in the trophic network. Therefore, the stable-isotope labeled precursors of methane, ^{13}C-acetate and H_2+$^{13}CO_2$ were fed to liquid cultures from hard coal and mine timber from a coal mine in Germany. Directed by the methane production, samples for DNA stable-isotope probing (SIP) coupled to subsequent quantitative PCR and DGGE analyses were taken over 6 months. Surprisingly, the formation of ^{13}C-methane was linked to acetoclastic methanogenesis in both, the ^{13}C-acetate and H_2+$^{13}CO_2$-amended cultures of coal and timber. H_2+$^{13}CO_2$ was mainly used by acetogens related to *Pelobacter acetylenicus* and *Clostridium* species. Active methanogens, closely affiliated to *Methanosarcina barkeri*, utilized the readily available acetate rather than the thermodynamical more favourable hydrogen. Thus, we functionally dissect a methanogenic microbial community highly adapted to the low H_2 conditions usually to be found in coal mines.

INTRODUCTION

Worldwide, mine gas emissions of active and abandoned coal mines release substantial amounts of methane contributing up to seven percent of the global methane formation (4). Mine gas is a hazard but also is a potential source of methane for the industry. Stable carbon and hydrogen isotopic signatures indicated that methane in mine-gas has a mixed thermogenic and biogenic origin (33, 31). In abandoned coal mines, the thermogenic methane is a remainder of geological processes, but its biogenic formation is still going on (14). Besides hard coal, possible sources for methane are large amounts of mine timber that was used for the construction of mines and left behind after the end of mining.

Generally, methane is produced either from acetate, hydrogen or methylotrophic substrates as precursors. Recently, we showed that methylotrophic methanogenesis did not have a quantitative impact on the *in situ* processes (2). However, while hydrogen is energetically favourable, acetate is the quantitatively more available substrate (38). In former studies we have revealed that acetate is an important intermediate of the degradation processes and the main precursor of the biogenic methane in abandoned coal mines (14). We have also shown the presence of *Methanosarcina* spp. as the dominating archaea (2). *Methanosarcina* spp. are

able to use acetate as well as H_2+CO_2. While other investigations reported that methanogenesis in coal mines is mainly driven by H_2-utilizing archaea (7, 28), our studies indicated that acetoclastic methanogenesis seems to be the main methanogenic process at least in the abandoned coal mines we have investigated (2).

The activity of methanogens in different habitats can be studied by using stable isotope probing (SIP, 17, 16). DNA-SIP allows the identification of specifically active members of microbial populations based on the incorporation of ^{13}C into the DNA of cells consuming labeled substrates (18). In this technique, labeled DNA is resolved after incubation under label addition by subsequent isopycnic gradient ultracentrifugation (22). Hence, guilds of methanogens that utilize ^{13}C-labeled methanogenic substrates such as ^{13}C-acetate or H_2+$^{13}CO_2$ can be recovered in ^{13}C-enriched DNA. However, both methanogenic precursors can also be utilized by syntrophic acetate oxidatizing and/or homoacetogenic bacteria, respectively (27, 32). Until now, only few SIP studies were performed in coal habitats. Han *et al*. (10) investigated the active methanotrophic community in a Chinese coal deposit. However, no studies are present revealing the activity of microorganisms directly involved in methane production in abandoned coal mines. In our present study, we wanted to identifiy active methanogens and test the hypothesis that acetate is a main precursor of methane even if hydrogen is available as an energetically more favourable electron donor.

MATERIALS AND METHODS

Sample collection and enrichment cultures

Samples were collected in May 2007 in sealed compartments of coal mines closed in the 1960`s. Briefly, large pieces of coal and mine timber were collected aseptically in glass bottles that were immediately flushed with N_2 and stored at 4°C until further processing. *In situ* temperatures were 35-36 °C with 100 % air humidity. Processing of coal and mine timber samples was done in an anaerobic chamber under nitrogen atmosphere to prevent oxidation. Samples were homogenized and distributed in hungate tubes containing 500 mL of sulfate-free mineral medium (36) with a salinity of 15 PSU, according to *in situ* values. Controls were supplemented with 10 mM 2-bromoethanesulfonate (BES) to exclude abiotic degassing by inhibiting methanogenesis. Enrichment cultures were amended with either 10 mM fully ^{13}C-labeled acetate (Campro Scientific) or ^{13}C-bicarbonate (Campro Scientific)

+ H_2. All incubations were frozen at -80°C after an incubation time of 6 month until further processing for DNA-SIP. Subsamples of all incubations were taken at month 0 and 3 of incubation. The increase of methane and hydrogen in the headspace as well as the stable isotopes of methane were continuously monitored over 6 months and analyzed by gas chromatography/mass spectrometry (GC/MS) as described previously by Krüger *et al*. (13). Concentrations of acetate were analyzed by high-performance liquid chromatography (Agilent Technologies) using a Zorbax Eclipse Plus C8 USP L7 column (Agilent technologies) at 60°C. The eluent was a 5 mM H_2SO_4/methanol gradient at 1 ml/min. Acetate was detected by a diode array detector (DAD, Agilent Technologies).

DNA extraction, isopycnic centrifugation and gradient fractionation

DNA was extracted from 0.5 g of incubation slurry after 0, 3 and 6 months using phenol-chloroform extraction as described by Lueders *et al*. (18). Three parallel extractions were carried out and pooled for each incubation treatment. DNA was checked by standard agarose gel electrophoresis and quantified using PicoGreen staining according to Wilms *et al*. (37).

Gradient preparation, isopycnic centrifugation and gradient fractionation were performed as described by Lueders *et al*. (17) with minor modifications. Each gradient consisted of 6.3 mL of CsCl (approximately 1.72 g mL^{-1}, Calbiochem), and ca.1 mL of gradient buffer (100 mM Tris-HCL, pH 8.0, L^{-1}, 100 mM KCL L^{-1}, 1 mM EDTA L^{-1}) including 2000 ng of DNA. Prior to centrifugation, the average density of the centrifugation medium was controlled refractometrically and adjusted to an average density of 1.84 g cm^{-3}. The samples were centrifuged in 6.3 mL polyallomer quick-seal tubes (Beckman) in a NVT 65 near vertical rotor (Beckman) using a Beckman LE-70 Ultracentrifuge (Beckman Instruments). Centrifugation was performed at 20°C for 36 h at 44500 rpm (184000*g*). Gradients were fractionated as described before by Neufeld *et al*. (21). Briefly, the gradients were fractionated from bottom to top into 12 equal fractions (400 µL). A precisely controlled flow rate was achieved by displacing the gradient medium with water at the top of the tube using a Graseby syringe pump 3100 at a flow rate of 1 mL min^{-1}. The density of each collected fraction (a small aliquot of 100 µL) was measured by determining the refractory index using a digital refractometer (AR20; Reichert Analytical Instruments, Depew, NY, USA). Subsequently, the DNA was precipitated using polyethylene glycol 6000 (Aldrich Chemistry). The DNA pellet was washed once with 70% ethanol and dissolved in 25 µL of elution buffer.

Quantification of archaeal and bacterial 16S rRNA genes in density gradient fractions

DNA was precipitated from gradient fractions and quantified fluorometrically and by quantitative PCR using the primer system Ar109f/Ar912rt and Ba27F/907R modified to Lueders *et al*. (17). The qPCR mixtures contained 12.5 µL of the premix solution of a DyNAmo HS SyberGreen qPCR Kit (New England Biolabs, Inc., Hitchin, UK), 1 µL of each primer and 10 µL standard or DNA extract as a template in a final reaction volume of 25 µL. The PCR was carried out in a Rotor-Gene-3000 cycler (Corbett Research, Sydney, Australia). After initial denaturation at 95°C for 15 min, 50 cycles followed. Each cycle consisted of denaturation for 30 s at 94°C, annealing for 20s at 52°C for bacteria and for archaea elongation for 30 s at 70°C, and fluorescence measurement at 70 °C. To check amplification specificity, fluorescence was also measured at the end of each cycle for 20 s at 80°C for. After the last cycle, a melting curve was recorded by increasing the temperature from 50°C to 99°C (1°C every 10 s). The numbers of bacterial and archaeal 16S rRNA gene targets were calculated from the DNA concentrations according to Süss *et al*. (30). DNA standards for quantitative (real-time) PCR were prepared as described by Wilms *et al*. (37) and Engelen *et al*. (6). Bacterial and archaeal targets were measured in at least three different dilutions of DNA extracts (1:10 to 1:1000) and in triplicate.

PCR and denaturing gradient gel electrophoresis (DGGE) analysis

For denaturing gradient gel electrophoresis (DGGE), a 803-bp fragment of the archaeal 16S rRNA gene was amplified by using the primers Ar109f (5`-AC KGC TCA GTA ACA CGT-3`) and Ar912rt (5`-GTG CTC CCC CGC CAA TTC CTT TA-3`). For the analysis of bacterial composition, the primers BA27F (5`-AGA GTT TGA TCM TGG CTC AG -3`) and 907R (5`-CCG TCA ATT CCT TTG AGT TT-3`) were used to amplify a 880-bp fragment of the bacterial 16S rRNA genes. At the 5`-end of each forward primer, an additional 40-nucleotide GC-rich sequence (GC-clamp) was added to obtain a stable melting point of the DNA fragments in the DGGE according to Muyzer *et al.* (20). PCR amplification was performed using an Eppendorf Thermal Cycler system (Mastercycler, Eppendorf, Hamburg, Germany) as follows: 2 µL (1-100 ng) of template DNA, 1 U of *Taq* DNA polymerase, the manufacturers`recommended buffer as supplied with the polymerase enzyme, 10 mM dNTP`s, 50 µM of each of the appropriate primers, and 10 mM of BSA were adjusted to a

total volume of 50 µL with PCR water (Ampuware, Fresenius, Bad Homburg, Germany). The PCR-program included an initial denaturation step for 5 min at 94 °C. For the PCR of archaeal and bacterial DNA, a first cycle step was carried out for 30 s at 94 °C; 30 s at 52°C; and 1 min at 72 °C. The total number of cycles was 30. Primer extension was carried out for 5 min at 72°C. Aliquots (5 µL) of the PCR products were analyzed by agarose gel electrophoresis in 1.5% (wt/vol) agarose gels and ethidium bromide staining (0.8 ng mL^{-1}) for 20 min on a UV transilluminator as described previously (37).

DGGE was performed using an INGENYphorU-2 system (Ingeny, Goes, The Netherlands). PCR products and loading buffer (40% [wt/vol] glycerol, 60% [wt/vol] 1x Tris-acetate-EDTA [TAE], and bromphenol blue) were mixed in a ratio of 1:2. The PCR amplicons were applied directly onto 6% (wt/vol) polyacrylamide gels with a linear gradient of 30-80% denaturant for archaeal and 50-70% for bacterial PCR products (with 100% denaturant corresponding to 7 M urea and 40% [vol/vol] formamide). Electrophoresis was accomplished in 1xTAE buffer (40 mM Tris-acetate [pH 7.4], 20 mM sodium acetate, 1 mM Na_2EDTA), at a constant 100 V and a temperature of 60°C for 20 h. After electrophoresis, the gels were stained for 2 h in 1xSybrGold solution (Molecular Probes, Eugene, USA) in 1 x TAE and washed for 20 min with distilled water. The gel was digitized using the digital imaging-system (BioDocAnalyze; Biometra, Göttingen, Germany) with UV transillumination (302 nm).

Reamplification and sequencing of DGGE bands

DGGE bands were excised for sequencing using a sterile scalpel and treated as described by Del Panno *et al*. (3). Briefly, the bands were transferred into 50 µL of PCR water and incubated for 48 h at 4 °C. For reamplification, 2 µL of the supernatant was taken as a template using the same reaction mix as described above with a final volume of 50 µL. The PCR protocol was adjusted for the reamplification was the same as above with minor modifications. The total number of cycles was 25 and the final elongation was carried out at 72 °C for 10 min. PCR products were purified by using the QIAquick PCR purification Kit (Quiagen GmbH) and eluted in 30 µL of PCR water. DNA yields were estimated fluorometrically in a microtiterplate reader (FLUOstar Optima, BMG Labtechnologies, Offenburg, Germany) using a 1:200 diluted PicoGreen reagent according to a modified manufacturer`s protocol (Molecular Probes, Eugene, USA) as described in detail by Wilms *et al.* (37). Sequence analyses were accomplished by GATC Biotech AG (Konstanz,

Germany). Sequences were compared to those in GenBank using the BLAST tool of the National Center for Biotechnology Information server (1) and have been deposited in the GenBank nucleotide sequence database under the Accession Nos. AA-AX.

RESULTS

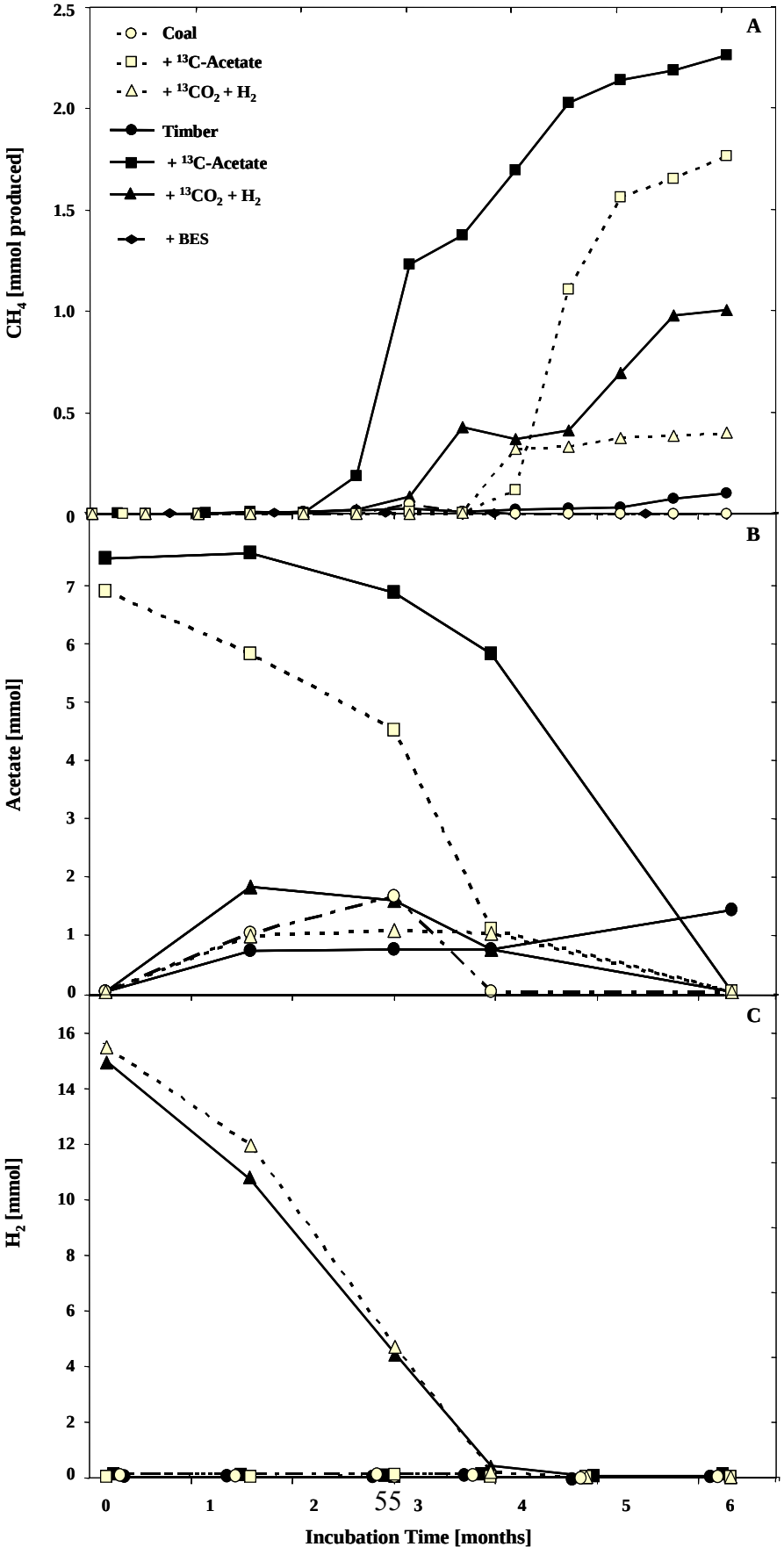
For SIP, microcosms with coal and timber samples were amended with ^{13}C-acetate and H_2+$^{13}CO_2$ and incubated under *in situ* conditions. The accumulation of CH_4 was followed over a period of 6 months. To identify active community members, samples for DNA extraction were taken at the beginning, and after 3 and 6 months (Fig. 1A). The day 0 incubation samples served as SIP-controls.

Acetate is the precursor of methane

CH_4 production was observed in all incubations (Fig. 1A) with higher activities in timber than in coal enrichments. Highest CH_4 formation rates (0.13 µmol per g wet weight and day) were detected in the ^{13}C-acetate enrichments between month 3 and 6 of incubation. The addition of H_2+$^{13}CO_2$ resulted in less stimulation of methanogenesis (maximum rate 0.05 µmol per g wet weight and day). The isotopic signature of CH_4 indicated that methane was formed from the labeled substrates added (data not shown). No methane formation was observed in control incubations with the methanogenesis inhibitor BES (2-bromoethanesulfonate). Therefore, abiotic degassing of adsorbed methane from the incubated samples can be excluded.

While H_2 was largely used up after 3 months, acetate was completely depleted after 6 months (Fig. 1B and C). Interestingly, acetate formation was observed in the H_2+$^{13}CO_2$ cultures, while there was no H_2 formation in the acetate incubation. The fact that acetate formation was also detected in the unamended incubations indicated the central role of acetate in the process of methanogenesis. The isotopic signature of the formed acetate showed a strong labelling indicating its formation from $^{13}CO_2$ (data not shown).

FIG. 1: Long-term incubations of weathered hard coal and mine timber amended with ^{13}C-labeled acetate and H_2+CO_2 and the microbial methane formation **(A)** as well as the acetate- **(B)** and hydrogen **(C)** depletion/formation.



DNA-SIP reveals that methanogenesis is mediated by *Methanosarcina* spp.

In order to identify active community members, samples from the ^{13}C-enriched incubations were analyzed by stable isotope probing (SIP). Density-resolved archaeal DNA was first detected in gradient fractions using archaeal qPCR (Fig. 2 and 3). The amount of archaeal DNA detected in the heavy fractions increased over the incubation time between month 3 and 6. Only the timber cultures showed ^{13}C-labeled archaeal DNA already after 3 months, with maximum quantities in DNA gradients from acetate enrichments. After 6 months, 'light' and 'heavy' DNA-fractions substantiated a clear labelling of archaeal DNA for timber and the coal cultures, under both amended with ^{13}C-acetate and $^{13}CO_2$, respectively (Fig. 2 and 3).

Next, the archaeal community members detected in 'light' and 'heavy' gradient fractions were analyzed by denaturing gradient gel electrophoresis (DGGE) and subsequent band sequencing (Tables 1 and 2). In general, total archaeal community composition was similar in the ^{13}C-acetate and H_2+$^{13}CO_2$ cultures, and dominated by relatives of *Methanosarcina* spp., *Methanosaeta* spp., and uncultured *Crenarchaeota* (Tables 1 & 2). However, clear ^{13}C-labeling was mainly evident for relatives of *Methanosarcina* spp. Increased respective band-intensities reflected larger amounts of labeled ^{13}C-DNA in the acetate cultures (Fig. 2 and 3). To a minor extent, labeled DNA was also observed of a DGGE band related to *Methanosaeta* spp. in the acetate enrichments. Members of the *Crenarchaeota* were abundant in the original coal and timber samples but showed no incorporation of ^{13}C-labeled substrates.

Identification of active *Bacteria*

Quantities of labeled bacterial 16S rRNA genes increased over incubation time (Fig. 2 and 3), as found for the *Archaea*. While strongly labelled bacterial DNA was detected especially in the coal ^{13}C-acetate amendments already after 3 months, H_2+$^{13}CO_2$ cultures showed substantial quantities of ^{13}C-DNA only after 6 months of incubation.

In the acetate-amended coal samples, bacterial DNA labelled after 3 months was affiliated to a lineage of uncultured *Geobacteraceae* and *Pelobacter* spp., and additionally to *Pseudoalteromonas* and *Clostridium* spp. after 6 months. Most surprising, however, abundant unlabeled and labelled DNA was detected for a relative of *Pseudomonas* sp. after 6 months only. In H_2+$^{13}CO_2$-amended coal samples, clearly labelled DNA was evident from gradient

fraction DGGE analysis after 6 months only, and affiliated to *Clostridium*, *Desulfovibrio* and *Pelobacter* spp., as well as again uncultered *Geobacteraceae*.

In ^{13}C-acetate-amended timber cultures, members of chemoautotrophic bacteria (*Hydrogenophaga* and *Hyphomicrobium* spp.) as well as sulfate and sulfur reducers (*Desulfovibrio* and *Desulfuromonas* spp.*)* were primarily detected in 'intermediate' and 'heavy' gradient fractions. Surprisingly, the DGGE band dominating the highest density DNA gradient fractions was related to *Burkholderia* spp. This could also be a methodical effect due to the high GC-DNA content of *Burkohlderia* spp. In H_2+$^{13}CO_2$-amended timber microcosms, *Hydrogenophaga* spp. clearly dominated 'heavy' DNA after 6 months, while a relative of *Desulfovibrio* spp. was detected in 'intermediate' gradient fractions already after 3 months.

TABLE 1. Bacterial and archael diversity in hard-coal incubations amended with ^{13}C-acetate and H_2+$^{13}CO_2$ detected by denaturing gradient gel electrophoresis (DGGE) of density-resolved DNA gradient fractions. Phylogenetic affiliation of the 16S rRNA genes from microbes that incorporated ^{13}C-labeled substrates are highlighted in grey.

Hard coal samples				
Labeled substrate	**Closest cultured relative**	**Similarity (%)**	**Band letter in Fig. 2**	**Accession no.**
+ ^{13}C-Acetate	***Bacteria***			
	Burkholderia cepacia	100	A	
	Pseudoalteromonas sp.	100	B	
	Clostridium sp.	99	C	
	Pseudomonas stutzeri	99	D	
	Desulfovibrio africanus	100	E	
	Pelobacter acetylenicus	95	F	
	Uncultured *Geobacteraceae*	99	G	
	Archaea			
	Methanosaeta sp.	98	a	
	Uncultured *Crenarchaeota*	98	b	
	Methanosarcina barkerii	99	c	
	Methanosarcina sp.	99	d	
+ H_2+$^{13}CO_2$	***Bacteria***			
	Burkholderia cepacea	100	A	
	Desulfovibrio alkaliphilus	92	B	
	Clostridium sp.	99	C	
	Desulfovibrio africanus	100	D	
	Pelobacter acetylenicus	95	E	
	Uncultured *Geobacteraceae*	98	F	
	Xanthomonas sp.	100	G	
	Archaea			
	Methanosaeta sp.	98	a	
	Uncultured *Crenarchaeota*	99	b	
	Methanosarcina barkerii	93	c	

FIG. 2: Quantitative PCR distribution and DGGE-community profiles of density-resolved bacterial and archaeal DNA in SIP centrifugation gradients after 3 and 6 months of ^{13}C-acetate **(Fig. 2A)** and H_2+ $^{13}CO_2$ **(Fig. 2B)** incubations of hard coal. Letters on the DGGE profiles indicate the same band throughout the gradient. Band letters correspond to those in Table 1. Same band letters indicate the same organism

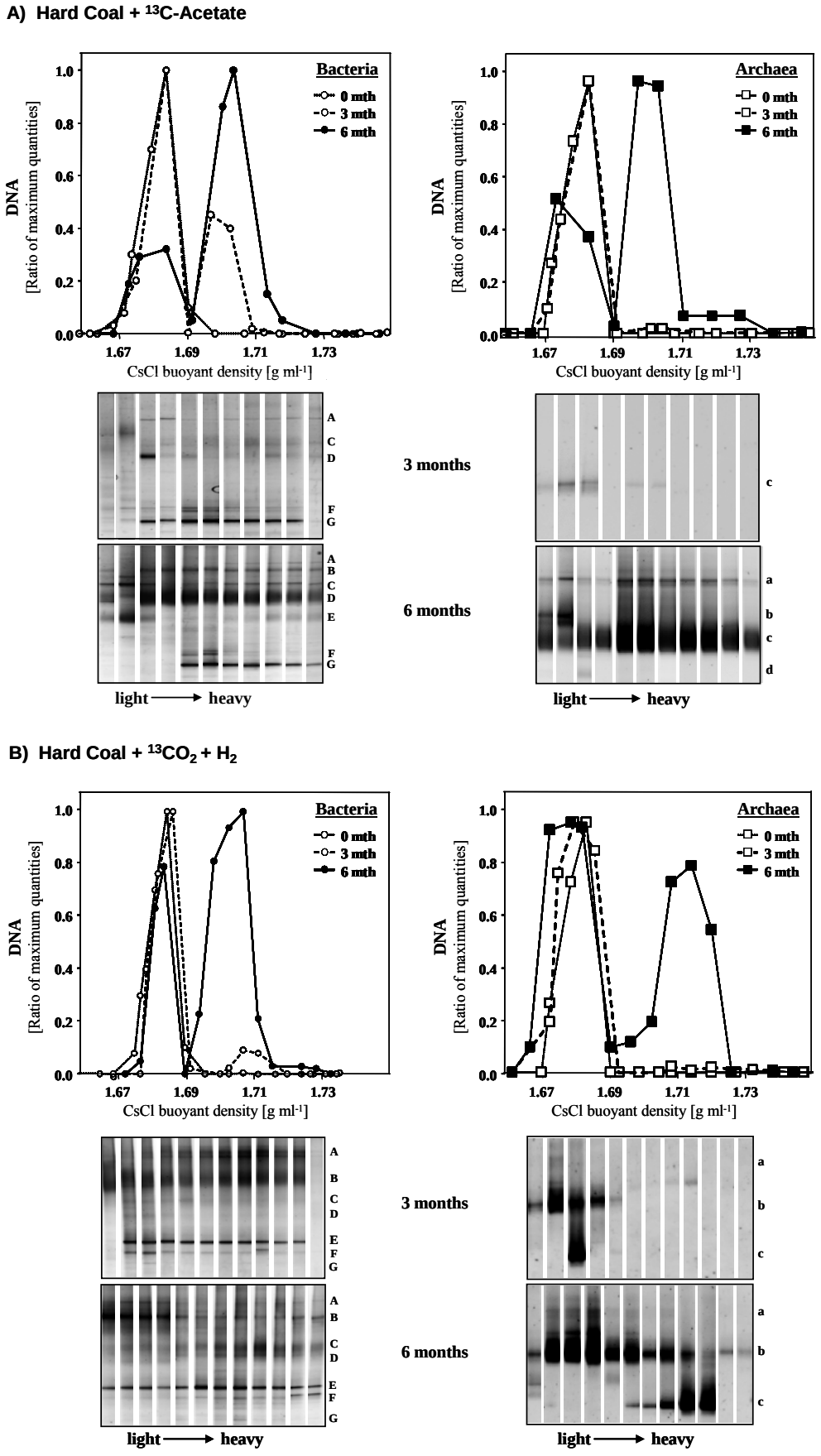
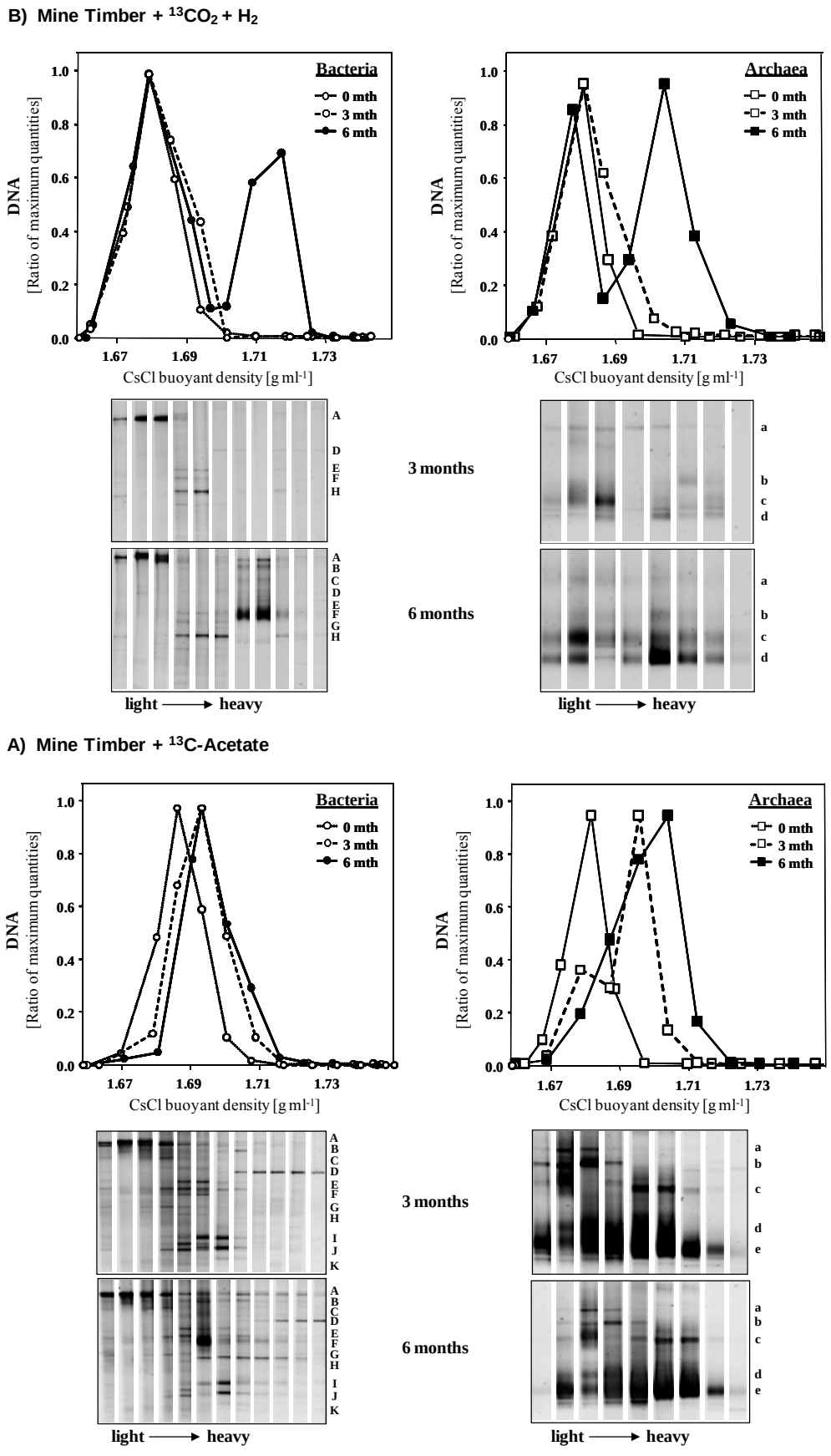


TABLE 2. Bacterial and archael diversity in mine-timber incubations amended with ^{13}C-acetate and H_2+$^{13}CO_2$ detected by denaturing gradient gel electrophoresis (DGGE) of density-resolved DNA gradient fractions. Phylogenetic affiliation of the 16S rRNA genes from microbes that incorporated ^{13}C-labeled substrates are highlighted in grey.

Mine timber samples				
Labeled substrate	**Closest cultured relative**	**Similarity (%)**	**Band letter in Fig. 3**	**Accession no.**
+ ^{13}C-Acetate	***Bacteria***			
	Acholeplasma sp.	95	A	
	Uncultured *Bacteroidetes*	99	B	
	Clostridium sp.	99	C	
	Burkholderia cepacia	100	D	
	Hyphomicrobium sp.	99	E	
	Hydrogenophaga sp.	94	F	
	Desulfovibrio sp.	95	G	
	Desulfovibrio africanus	100	H	
	Halochromatium sp.	92	I	
	Desulfuromonas acetexigens	95	J	
	Uncultured *Geobacteraceae*	96	K	
	Archaea			
	Uncultured *Crenarchaeota*	98	a	
	Uncultured *Crenarchaeota*	98	b	
	Methanosaeta sp.	98	c	
	Methanosarcina barkeri	99	d	
	Methanosarcina barkeri	93	e	
+ H_2+$^{13}CO_2$	***Bacteria***			
	Acholeplasma sp.	95	A	
	Uncultured *Bacteroidetes*	99	B	
	Clostridium sp.	99	C	
	Burkholderia cepacia	99	D	
	Hyphomicrobium sp.	99	E	
	Hydrogenophaga sp.	94	F	
	Desulfovibrio sp.	95	G	
	Desulfovibrio africanus	95	H	
	Archaea			
	Uncultured *Crenarchaeota*	98	a	
	Methanosarcina sp.	100	b	
	Methanosarcina sp.	100	c	
	Methanosarcina barkeri	99	d	

FIG. 3: Quantitative PCR distribution and DGGE-community profiles of density-resolved bacterial and archaeal DNA in SIP centrifugation gradients after 3 and 6 months of ^{13}C-acetate **(Fig. 3A)** and H_2+ $^{13}CO_2$ **(Fig. 3B)** incubations of mine timber. Letters on the DGGE profiles indicate the same band throughout the gradient. Band letters correspond to those in Table 2. Same band letters indicate the same organisms.



DISCUSSION

In the present study we have identified active microbes responsible for methane formation in samples taken from abandoned coal-mines. We demonstrated that acetate is the main precursor of methane and identified the microbes involved in the processes leading to methane formation.

Within the last few years, methane release was also observed and studied in other coal mines, worldwide. Several of the community members we have detected in our samples have been found previsouly in other coal mine deposits (especially *Pelobacter acetylenicus*, *Clostridium* sp., *Pseudomonas* sp., Uncultured *Geobacteraceae*, *Methanosarcinales* sp.) indicating their potential role in the process of methane release (25, 26, 8, 12). A predominance of acetoclastic *Methanosarcinales* was already shown in two other coal seams investigated recently (9, 34) and in comparable habitats, i.e. hydrocarbon-contaminated aquifers (5). However, in coal mine water and drainages, hydrogenotrophic methanogens were prevailing (7, 35, 29). Hydrogenotrophic methanogenesis dominated in the drainage water of coal reservoirs. In the floating systems, easy degradable substrates might be released that lead to intermediate hydrogen formation.

Methane release via acetoclastic methanogenesis

The fact that *Methanosarcina* spp. were responsible for methane production in our enrichments would be in agreement with our earlier studies showing that the *Methanosarcinales* are most abundant in the *in situ* coal and timber samples (2). Although, *Methanosarcina* spp.are known to use both, hydrogen or acetate, those identified here seem to be strictly adapted to the conditions in this habitat. In the mines studied, acetate seems to be quantitatively more available for the *Methanosarcinales.* Hydrogen might be hardly formed at low metabolic rates and therefore not be available for methanogens. Even after an incubation time of six months with adequate supply of hydrogen, coal mine methanogens did to not make direct use of the hydrogen for methane production. Hydrogen rather appeared to be used by acetogens producing acetate which then, in turn, was utilized by the *Methanosarcinales*.

Active *Geobacteraceae* predominate in coal

Acetotrophic members of the *Geobacteraceae* were found to be labeled in the coal enrichments. Their abundance could be increased by amendment with acetate. However, they were also active in the hydrogen incubations. This could be explained by secondary cross-feeding processes since labeled acetate was formed. In our cultures, it is not clear which electron acceptor is used by the *Geobacteraceae* for the acetate oxidation. One possibility could be the utilization of electron acceptors directly from the coal like sulfur, since family members of the *Geobacteracea* are described to be elemental-sulfur reducers (11). Jones *et al.* (12) also obtained high numbers of *Geobacter* species from the coal, but none of the known electron acceptors was present, suggesting that *Geobacter* might be capable of coupling the degradation of organics to an electron- or H_2-accepting partner. This could also be proposed in our case. However, different from our strict anoxic enrichments, coal mines showed low concentration of oxygen in surface-near layers of coal. Corresponding to this, members of the genus *Geobacteraceae* constituted for the bulk part of the overall bacterial community in the original coal samples (Gründger, data unpublished).

Coal and timber: two substrates, two different active bacterial communities

The active bacterial communities differ in the coal and timber. The amendments with acetate or H_2+CO_2 did not have a strong effect on the active community composition. Besides the active *Geobacteraceae*, the coal samples comprised active members of *Pelobacter acetylenicus*, *Clostridium* and *Pseudomonas* species. In earlier studies, *Pseudomonas stutzeri* was already isolated from coal samples and is potentially able to utilize polycylic aromatic hydrocarbons (24). This suggestion can be supported by the fact that the first event of coal fragmentation is carried out via exoenzymatic hydrolysis into small PAHs (29). Moreover, *Pseudomonas stutzeri* is even more active when acetate is present as second electron donor (19), and that could be one reason for its predominance in the coal-enrichments amended with acetate. However, oxygen and nitrate were not available as electron acceptors.

In the H_2+ $^{13}CO_2$ cultures, the accumulation of labeled acetate was detected. Relatives of *Pelobacter* and *Clostridium* species might be involved in acetate formation, at least if acetylene is present in case of *Pelobacter acetylenicus* (23). Which other coal compounds could be feasible substrates for acetogenesis is still unknown.

The timber enrichments were predominated by active bacteria similar to *Hydrogenophaga* and *Clostridium* species, suggesting that they might use timber compounds or secondary fermentative products for metabolism. Only the H_2+$^{13}CO_2$ coal-cultures showed a distinct labelling of bacteria after 6 months. The slight heavy shift in the acetate coal-cultures might not be an indication of bacterial activity, but a result of an increase of bacterial DNA with a high GC-content like *Burkholderia* species (Fig. 3; Table 2). As in the coal cultures, the H_2+$^{13}CO_2$ timber-enrichments also showed acetate formation. Obviously, timber provides other acetogenic substrates than coal. However, *Hydrogenophaga* as well as *Clostridium* species were recently also detected in coal samples from another mine (12).

Acetate is the main precursor of methane

In connecting the results from our earlier investigations (14, 2) with our new findings in the present study, we have the following arguments supporting that acetoclastic methanogenesis is the main route for methane formation: First, natural isotopic signatures of methane indicated an acetoclastic origin, supported by the isotopic signatures of acetate that was formed from $^{13}CO_2$ (14). Second, highest methane formation rates were observed in the acetate-amended enrichments of coal and timber, while H_2 gave lower activities. Third, acetate was depleted in the acetate cultures but accumulated in the H_2+ CO_2 and BES-treated enrichments. Finally, DNA-SIP revealed that relatives of *Methanosarcina* spp. were responsible for methane production under both ^{13}C-acetate and H_2 + $^{13}CO_2$-amendment, the second coupled to the activity of bacteria related to acetogens.

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REFERENCES

1. **Altschul, S. F., T.L. Madden, A. A. Schaffer, J. H. Zhang, Z. Zhang, W. Miller, and D. J. Lipman.** 1997. Gapped BLAST and PSI-BLAST: a new generation of protein database search programs. Nucl. Acids Res. **25:**3389-3402.
2. **Beckmann, S., M. Krüger, B. Engelen, A. A. Gorbushina, and H. Cypionka.** 2011. Role of bacteria, archaea and fungi involved in methane release in abandoned coal mines. Geomicrobiol. J. **28**(4).
3. **Del Panno, M., I. Morelli, B. Engelen, and L. Berthe-Corti.** 2005. Effect of petrochemical sludge concentration on microbial communities during a soil bioremediation process. FEMS Microbiol. Ecol. **53:**305-316.
4. **Denman, K., G. Brasseur, A. Chidthaisong, P. Ciais, P. Co, R. Dickinson, and D. Hauglustaine.** 2007. Couplings between changes in the climate system and biogeochemistry. In: Solomon, S., Qin, D., Manning, M., Chen, Z., Marquis, M., Averyt, K.B., Tignor, M., Miller, H.L. (eds.), Climate Change 2007: The Physical Science Basis. Contribution of Working Group I to the Fourth Assessment Report of the Intergovernmental Panel on Climate Change. Cambridge and New York. Cambridge University Press.
5. **Dojka, M. A., P. Hugenholtz, S. K. Haack, and N. R. Pace.** 1998. Microbial diversity in a hydrocarbon- and chlorinated-solvent-contaminated aquifer undergoing intrinsic bioremediation. Appl. Environ. Microbiol. **64:**3869-3877.
6. **Engelen, B., K. Ziegelmüller, L. Wolf, B. Köpke, A. Gittel, H. Cypionka, T. Treude, S. Nakagawa, F. Inagaki, M. A. Lever, and O. Steinsbu.** 2008. Fluids from the oceanic crust support microbial activities within the deep biosphere. Geomicrobiol. J. **25:**56-66.
7. **Flores, R. M.** 2008. Microbes, methanogenesis, and microbial gas in coal. Int. J. Coal Geol. **76:** 1-2.
8. **Fry, C. J., B. Horsfield, R. Sykes, B. A. Cragg, C. Heywood, G. T. Kim, K. Mangelsdorf, D. C. Mildenhall, J. Rinna, A. Vieth, K.-G. Zink, H. Sass, A. J. Weightman, and R. J. Parkes.** 2009. Prokaryotic populations and activities in an interbedded coal deposit, including a previously deeply buried section (1.6-2.3 km) above ~150 Ma basement rock. Geomicrobiol. J.**26:**163-178.
9. **Green, M. S., Flanegan, K.C., and P.C. Gilcrease.** 2008. Characterization of a methanogenic consortium enriched from a coalbed methane well in the Powder River Basin, USA. Int. J. Coal Geol. **76:**34-45.
10. **Han, B., Y. Chen, G. Abell, H. Jiang, L. Bodrossy, J. Zhao, J. C. Murell, and X. H. Xing.** 2009. Diversity and activity of methanotrophs in alkaline soil from a Chinese coal mine. FEMS Microbiol. Ecol. **70:**196-207.
11. **Holmes, D. E., P. N. Kelly, and D. R. Lovley.** 2004. Comparison of 16S rRNA, *nifD*, *recA*, *gyrB*, *rpoB*, and *fusA* genes within the family *Geobacteraceae* fam. nov. Int. J. Syst. Evolut. Microbiol. **54:**1591-1599.
12. **Jones, E. J. P., M. A. Voytek, M. D. Corum, and W. H. Orem.** 2010. Stimulation of methane generation from nonproductive coal by addition of nutrients or a microbial consortium. Appl. Environ. Microbiol. **76:**7013-7022.
13. **Krüger M, Frenzel P, and R. Conrad.** 2001. Microbial processes influencing methane emission from rice fields. Global Change Biology **7:**49-63.
14. **Krüger, M., S. Beckmann, B. Engelen, T. Thielemann, B. Cramer, A. Schippers, and H. Cypionka.** 2008. Microbial methane formation from hard coal and timber in an abandoned coal mine. Geomicrobiol. J. **25:**315-321.

15. **Lanoil, B. D., and S.-K. Han.** 2002-2003. Identification of microbes responsible fro acetate consumption in soils under different wetting regimes. Kearney Foundation of Soil Science: Soil Carbon and California`s Terrestrial Ecosystems. Final Report: 2001028.
16. **Lu, Y., T. Lueders, M. W. Friedrich, and R. Conrad.** 2005. Detecting active methanogenic populations on rice roots using stable isotope probing. Environ. Microbiol. **7:**326-336.
17. **Lueders, T., B. Wagner, P. Claus, and M. W. Friedrich.** 2004a. Stable isotope probing of rRNA and DNA reveals a dynamic methylotroph community and trophic interactions with fungi and protozoa in oxic rice field soil. Environ. Microbiol. **6:**60-72.
18. **Lueders, T., M. Manefield, and M. W. Friedrich.** 2004b. Enhanced sensitivity of DNA-and rRNA-based stable isotope probing by fractionation and quantitative analysis of isopycnic centrifugation gradients. Environ. Microbiol. **6:**73-78.
19. **Mittal, M., and K. J. Rockne.** 2008. Indole production by Pseudomonas stutzeri strain NAP-3 during anaerobic naphthalene biodegradation in the presence of dimethyl formamide. J. Environ. Science Health Part A **43:**1027-1034.
20. **Muyzer, G., E. C. Dewaal, and A. G. Uitterlinden**. 1993. Profiling of complex microbial-populations by denaturing gradient gel-electrophoresis analysis of polymerase chain reaction-amplified genes-coding for 16S ribosomal-RNA. Appl. Environ. Microbiol. **59:**695-700.
21. **Neufeld, J. D., J. Vohra, M. G. Dumont, T. Lueders, M. Manefield, M. W. Friedrich, and J.C. Murrell.** 2007. DNA stable-isotope probing. Nat. Protoc. **2:**860-866.
22. **Radajewski, S., P. Ineson, N. R. Parekh, and J. C. Murell.** 2000. Stable-isotope probing as a tool in microbial ecology. Nat. **403:**646-649.
23. **Schink, B.** 1985. Fermentation of acetylene by an obligate anaerobe, *Pelobacter acetylenicus* sp. nov. Arch. Microbiol. **142:**295-301.
24. **Schreiber, A. F., and U. K. Winkler.** 1983. Transformation of tetralin by whole cells of *Pseudomonas stutzeri* AS39*. Eur. J. Appl. Microbiol. Biotechnol. **18:**6-10.
25. **Shimizu, S., M. Akiyama, T. Naganuma, M. Fujioka, M. Nako, and Y. Ishijima.** 2007. Molecular characterization of microbial communities in deep coal groundwater of northern Japan. Geobiol. **5:**423-433.
26. **Shin, S.G., Lee, C.S., Hwang, K., Ahn, J.H., S. Hwang.** 2008. Use of order-specific primers to investigate the methanogenic diversity in acetate enrichment system. J. of Industrial Microbiol. Biotechnol. **35:**1345-1352.
27. **Stams, A. J. M.** 1994. Metabolic interactions between anaerobic bacteria in methanogenic environments. Ant. van Leeuwenhoek **66:**271-294.
28. **Strapoc, D., M. Mastalerz, A. Schimmelmann, A. Drobniak, and S. Hedges.** 2008a. Variability of geochemical properties in a microbially dominated gas system from the eastern margin of the Illinois Basin, USA. Intern. J.of Coal Geol. **76:**98-110.
29. **Strapoc, D., F. W. Picardal, C. Turich, I. Schaperdoth, J. L. Macalady, J. S. Lipp, Y.-S. Lin, T. F. Ertefai, F. Schubotz, K.-U. Hinrichs, M. Mastalerz, and A. Schimmelmann.** 2008b. Methane-producing microbial community in a coal bed of the Illinois Basin. Appl. Environ. Microbiol. **74:**2424-2432.
30. **Süss, J., K. Schubert, H. Sass, H. Cypionka, J. Overmann, and B. Engelen.** 2006. Widespread distribution and high abundance of *Rhizobium radiobacter* within Mediterranean subsurface sediments. Environ. Microbiol. **8:**1753-1763.
31. **Tao, M. X., B. G. Shi, J. Y. Li, W. C. Wang, X. B. Li, and B. Gao.** 2007. Secondary biological coalbed gas in the Xinji area, Anhui province, China: Evidence from the geochemical features and secondary changes. Int. J. Coal Geol. **71:**358-370.

32. **Thauer, R. K., D. Möller-Zinkhan, and A. M. Spormann.** 1989. Biochemistry of acetate catabolism in anaerobic chemotrophic bacteria. Ann. Rev.Microbiol. **43:**43-67.

33. **Thielemann, T., B. Cramer, and A. Schippers.** 2004. Coalbed methane in the Ruhr Basins, Germany: a renewable energy resource? Org. Geochemistry **35:**1537-1549.

34. **Ulrich, G., and S. Bower.** 2008. Active methanogenesis and acetate utilization in Powder River Basin coals, United States. Int. J. Coal Geol. **76:**25-33.

35. **Warwick, P. D., F. C. Breland, and P. C. Hackley.** 2008. Biogenic origin of coalbed gas in the northern Gulf of Mexico Coastal Plain, USA. Int. J. Coal Geol. **76:**119-137.

36. **Widdel, F., and F. Bak.** 1992. Gram-negative mesophilic sulfate-reducing bacteria. In: Balows, A., Dworkin, M., Harder, W., Schleifer, K.H. (eds.). The Prokaryotes. 36. New York: Springer-Verlag, pp. 3352-3378.

37. **Wilms, R., H. Sass, B. Köpke, H. Cypionka, and B. Engelen.** 2007. Methane and sulfate profiles within the subsurface of a tidal flat are reflected by the distribution of sulfate-reducing bacteria and methanogenic archaea. FEMS Microbiol. Ecol. **59:**611-621.

38. **Zinder, S. H.** 1993.Physiological ecology of methanogens. Ferry, J.C.(eds). Methanogenesis: ecology, physiology, biochemistry and genetics. New York. Chapman & Hall.

3 Discussion

In the present study, we have verified that a strong biogenic component of mine gas in abandoned coal mines has a recent origin. Besides hard coal, the conversion of mine timber is responsible for higher methane formation rates. The ongoing processes were unraveled and we demonstrated that acetate is the main precursor of methane. We have identified the complex prokaryotic community consisting of members of Fungi, Bacteria and Archaea. Furthermore, we have identified the active Archaea and the Bacteria involved in the processes leading to methane formation.

3.1 Ongoing biogenic methane formation

The natural stable-isotope analyses indicated that the biogenically produced methane is formed via acetoclastic methanogenesis. This was confirmed by the fast stimulation of methane formation in acetate-amended enrichment cultures. The respective acetoclastic archaea were identified by the detailed microscopical and molecular analyses. The predominance of these archaea and the ongoing methane formation in the unamended hard coal and mine timber incubations furthermore indicated that acetoclastic methanogenesis is an important process *in situ*.

Since hard coal is effectively sterilized due to the high temperatures during its formation, the best explanation for the presence of these diverse microbial communities is a recolonisation introduced by the anthropogenic mining activities. Possibly, also the transport of microbes via water in faults might provide a further source of microbial life, as postulated e.g. for gold mines (Lollar *et al.* 2006).

3.2 Mine timber as the major source for biogenic methane

Besides hard coal, mine timber is a possible source for biogenic methane formation providing the main precursors of methane. Wood-degrading fungi were detected, that mediate complete oxidation of their substrates in an aerobic environment. However, underneath the thick fungal mats, oxygen depletion might occur and create anoxic conditions, particularly at lowered atmospheric oxygen concentrations. Under these conditions, fungi might perform incomplete oxidation and release reduced substrates which can be channeled into methanogenesis.

3.3 Active acetoclastic *Methanosarcinales* release the methane

Acetoclastic *Methanosarcinales* are most abundant in the *in situ* coal and timber samples and responsible for the methane production in the enrichments. Although *Methanosarcinales* are known to use both, hydrogen as well as acetate, those identified here seem to be adapted to the utilization of acetate. In the mines studied, the acetate seems to be quantitatively more available for the *Methanosarcinales.* Hydrogen might be hardly formed at low metabolic rates and therefore might not be available for methanogens. Even after an incubation time of six months with an adequate supply of hydrogen, the methanogens did not take energetical benefit by using the hydrogen for methane production. The hydrogen rather appeared to be used by acetogens producing the acetate for the *Methanosarcinales*.

3.4 Coal and timber: two substrates, two different active bacterial communities

The active bacterial communities differ in the coal and timber. The amendments with acetate or H_2+CO_2 did not have a strong effect on the active community composition.

3.4.1 Active *Geobacteraceae* predominate in coal

Acetotrophic members of the *Geobacteraceae* were found to be labeled in the coal enrichments. Their abundance could be increased by amendment with acetate. However, they were also active in the hydrogen incubations. This could be explained by secondary cross-feeding processes since labeled acetate was formed. In our cultures, it is not clear which electron acceptor is used by the *Geobacteraceae* for the acetate oxidation. One possibility could be the utilization of electron acceptors directly from the coal like sulfur, since family members of the *Geobacteracea* are described to be elemental-sulfur reducers (Holmes *et al.* 2004). Jones *et al.* (2010) also obtained high numbers of *Geobacter* species from the coal, but none of the known electron acceptors was present, suggesting that *Geobacter* might be capable of coupling the degradation of organics to an electron- or H_2-accepting partner. This could also be proposed in our case. However, different from our strict anoxic enrichments, coal mines showed low concentration of oxygen in surface-near layers of coal. Corresponding

to this, members of the genus *Geobacteraceae* constituted for the bulk part of the overall bacterial community in the original coal samples (Gründger, data unpublished).

Besides the active *Geobacteraceae*, the coal samples comprised active members of *Pelobacter acetylenicus*, *Clostridium* and *Pseudomonas* species. In earlier studies, *Pseudomonas stutzeri* was already isolated from coal samples and is potentially able to utilize polycylic aromatic hydrocarbons (Schreiber *et al.* 1983). This suggestion can be supported by the fact that the first event of coal fragmentation is carried out via exoenzymatic hydrolysis into small PAHs (Strapoc *et al.* 2008b). Moreover, *Pseudomonas stutzeri* is even more active when acetate is present as second electron donor (Mittal *et al.* 2008), and that could be one reason for its predominance in the coal-enrichments amended with acetate. However, oxygen and nitrate were not available as electron acceptors.

3.4.2 Active bacteria with potential acetogenic features

In the H_2+ $^{13}CO_2$ coal-enrichments, the accumulation of labeled acetate was detected. Relatives of *Pelobacter* and *Clostridium* species might be involved in acetate formation, at least if acetylene is present in case of *Pelobacter acetylenicus* (Schink 1985). However, relatives of *Clostridium* species are known to be acetogenic using hydrogen as electron donor (Küsel *et al*. 2000). Which other coal compounds could be feasible substrates for acetogenesis is still unknown. The presence of the acetogens might explain the low abundance of hydrogenotrophic methanogens, and thus favouring acetoclastic methanogenesis as the dominant process.

The timber enrichments were predominated by active bacteria similar to *Hydrogenophaga* and *Clostridium* species, suggesting that they might use timber compounds or secondary fermentative products for metabolism. Only the H_2+$^{13}CO_2$ coal-cultures showed a distinct labelling of bacteria after 6 months. The slight heavy shift in the acetate coal-cultures might not be an indication of bacterial activity, but a result of an increase of bacterial DNA with a high GC-content like *Burkholderia* species (Fig. 3, Table 2). As in the coal cultures, the H_2+$^{13}CO_2$ timber-enrichments also showed acetate formation. Obviously, timber provides other acetogenic substrates than coal. However, *Hydrogenophaga* as well as *Clostridium* species were recently also detected in coal samples from another mine (Jones *et al*. 2010).

3.5 Different coal deposits, different methanogenic pathways

Within the last few years, methane release was also observed and studied in other coal mine reservoirs, worldwide. Several of the community members we have detected in our samples have been found previsouly in other coal mine deposits (especially *Pelobacter acetylenicus*, *Clostridium* sp., Uncultured *Geobacteraceae*, *Methanosarcinales* sp.) indicating their potential role in the process of methane release (Shimizu *et al*. 2007, Shin *et al.* 2008, Fry *et al.* 2009, Jones *et al.* 2010). A predominance of acetoclastic *Methanosarcinales* was already shown in two other coal seams and in deep coal deposits investigated recently (Green *et al*. 2008; Ulrich and Bower 2008, Fry et al. 2009) and in comparable habitats, i.e. hydrocarbon-contaminated aquifers (Dojka *et al*. 1998). However, in coal mine water and drainages, hydrogenotrophic methanogens were prevailing (Flores 2008, Warwick *et al*. 2008, Strapoc *et al.* 2008a). Hydrogenotrophic methanogenesis dominated in the drainage water of coal reservoirs. In the floating systems, easy degradable substrates might be released that lead to intermediate hydrogen formation. Furthermore, methylotrophic methanogenesis appears to play a role in coal seam groundwaters investigated by Shimizu *et al.* (2007). We selectively enriched methylotrophic methanogens (with methanol or trimethylamine), but did not find a quantitative impact on the *in situ* processes. However, in the coal mines investigated here, acetoclastic methanogenesis is responsible for the methane production.

3.6 Acetate is the main precursor of methane

In conclusion, we have the following arguments supporting that acetoclastic methanogenesis is the main route for methane formation:

1. Natural isotopic signatures of methane indicated an acetoclastic origin, supported by the isotopic signatures of acetate that was formed from $^{13}CO_2$.
2. Highest methane formation rates were observed in the acetate-amended enrichments of coal and timber, while H_2 gave lower activities.
3. Acetate was depleted in the acetate cultures but accumulated in the H_2+ CO_2 and BES-treated enrichments.
4. DNA-SIP revealed that relatives of *Methanosarcina* spp. were responsible for methane production under both ^{13}C-acetate and H_2+$^{13}CO_2$-amendment, the second coupled to the activity of bacteria related to acetogens.

3.7 An overview: From coal and timber to methane

In the sealed compartments of the coal mines, a complex community of fungi, bacteria and archaea is involved in methane formation, inhabiting very distinct ecological niches. Finally, the following overview (Fig. 1) regards the environmental situation and the fact that acetate is the precursor of methane.

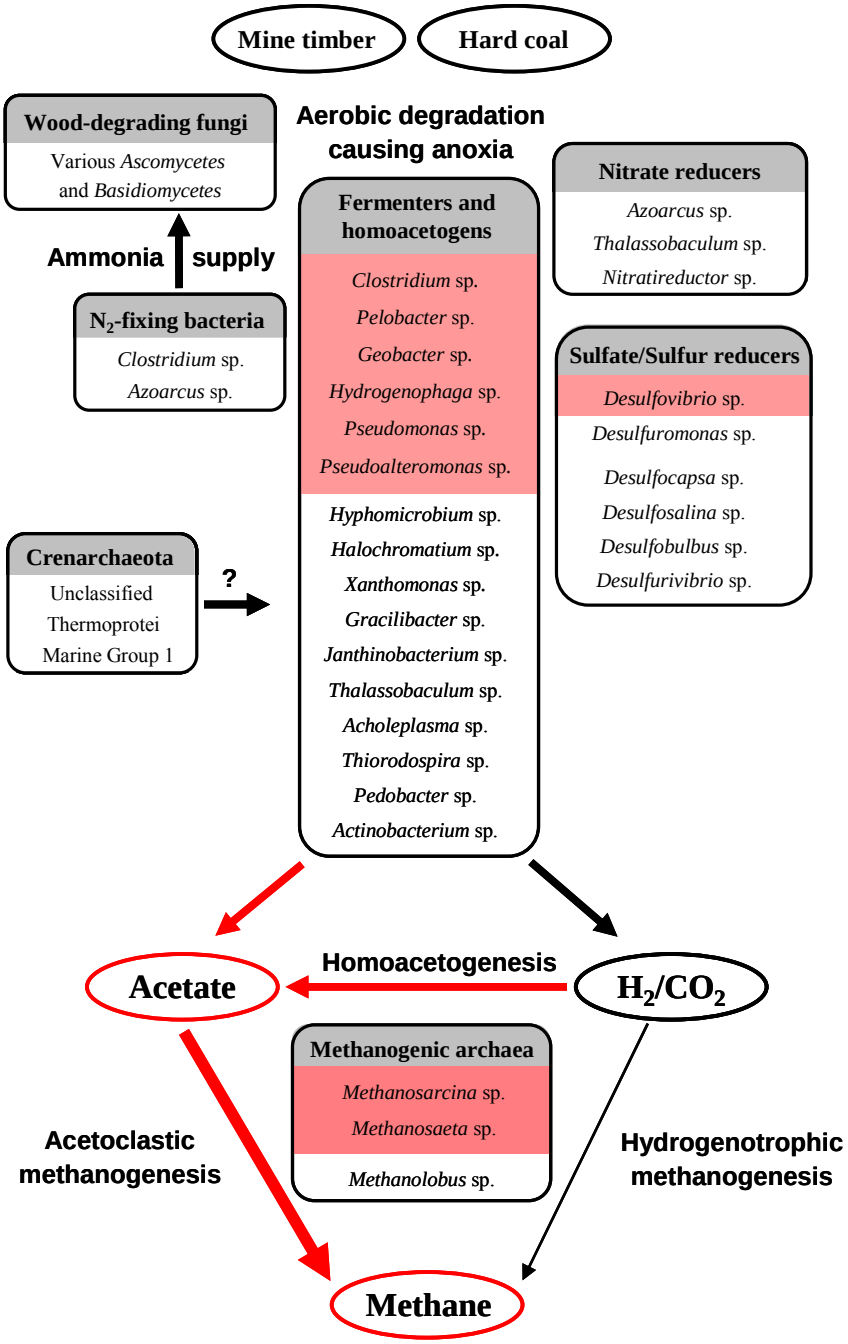

Figure 1: Overview of the main organism groups and their involvement in the degradation of hard coal and mine timber in two investigated coal mines. Active bacteria and archaea as well as the main pathways to methane formation are highlighted in red

3.8 References

Dojka MA, Hugenholtz P, Haack SK, Pace NR. 1998. Microbial diversity in a hydrocarbon- and chlorinated-solvent-contaminated aquifer undergoing intrinsic bioremediation. Appl. Environ. Microbiol. 64: 3869-3877.

Flores RM. 2008. Microbes, methanogenesis, and microbial gas in coal. Int. J. Coal Geol.76: 1-2.

Fry CJ, Horsfield B, Sykes R, Cragg BA, Heywood C, Kim GT, Mangelsdorf K, Mildenhall DC, Rinna J, Vieth A, Zink KG, Sass H, Weightman AJ, Parkes RJ. 2009. Prokaryotic populations and activities in an interbedded coal deposit, including a previously deeply buried section (1.6-2.3 km) above ~150 Ma basement rock. Geomicrobiol. J. 26: 163-178.

Green MS, Flanegan KC, Gilcrease PC. 2008. Characterization of a methanogenic consortium enriched from a coalbed methane well in the Powder River Basin, USA. Int. J. Coal Geol. 76: 34-45.

Holmes DE, Kelly PN, Lovley DR. 2004. Comparison of 16S rRNA, *nifD*, *recA*, *gyrB*, *rpoB*, and *fusA* genes within the family *Geobacteraceae* fam. nov. Int. J. Syst. Evolut. Microbiol. 54:1591-1599.

Jones EJP, Voytek MA, Corum MD, Orem WH. 2010. Stimulation of methane generation from nonproductive coal by addition of nutrients or a microbial consortium. Appl. Environ. Microbiol. 76:7013-7022.

Küsel K, Dorsch T, Acker G, Stackebrandt E, Drake HL. 2000. *Clostridium scatologenes* strain SL1 isolated as an acetogenic bacterium from acidic sediments. Int. J. Syst. Evolut. Microbiol. 50:537-546.

Lollar BS, Lacrampe-Couloume G, Slater GF, Ward J, Moser DP, Gihring TM, Lin LH, Onstott TC. 2006. Unravelling abiogenic and biogenic sources of methane in the Earth`s deep subsurface. Chem. Geol. 226:328-339.

Mittal M, Rockne KJ. 2008. Indole production by *Pseudomonas stutzeri* strain NAP-3 during anaerobic naphthalene biodegradation in the presence of dimethyl formamide. J. Environ. Science Health Part A 43:1027-1034.

Schink B. 1985. Fermentation of acetylene by an obligate anaerobe, *Pelobacter acetylenicus* sp. nov. Arch. Microbiol. 142:295-301.

Schreiber AF, Winkler UK. 1983. Transformation of tetralin by whole cells of *Pseudomonas stutzeri* AS39*. Eur. J. Appl. Microbiol. Biotechnol. 18:6-10.

Shimizu S, Akiyama M, Naganuma T, Fujioka M, Nako M, Ishijima Y. 2007. Molecular characterization of microbial communities in deep coal groundwater of northern Japan. Geobiol. 5: 423-433.

Shin SG, Lee CS, Hwang K, Ahn JH, Hwang S. 2008. Use of order-specific primers to investigate the methanogenic diversity in acetate enrichment system. J. Ind. Microbiol. Biotechnol. 35: 1345-1352.

Strapoc D, Mastalerz M, Schimmelmann A, Drobniak A, Hedges S. 2008a. Variability of geochemical properties in a microbially dominated gas system from the eastern margin of the Illinois Basin, USA. Int. J. Coal Geol. 76: 98-110.

Strapoc D, Picardal FW, Turich C, Schaperdoth I, Macalady JL, Lipp JS, Lin Y-S, Ertefai TF, Schubotz F, Hinrichs K-U, Mastalerz M, Schimmelmann A. 2008b. Methane-producing microbial community in a coal bed of the Illinois Basin. Appl. Environ. Microbiol. 74:2424-2432.

Ulrich G, Bower S. 2008. Active methanogenesis and acetate utilization in Powder River Basin coals, United States. Int. J. Coal Geol. 76: 25-33.

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Erklärung

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

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