Carbon isotope fractionation during the anaerobic degradation of acetate

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1. **Goevert, D. and R. Conrad (2008)** Carbon isotope fractionation during acetoclastic methanogenesis by *Methanosarcina barkeri* and *Methanosarcina acetivorans* (in preparation)

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4. **Goevert, D. and R. Conrad (2008)** Effects of the competition for acetate between methanogens and sulfate reducers on carbon isotope fractionation (in preparation)

This work is dedicated to two beloved people, who passed away during my PhD,

my mother Birgitt Gövert

and

my former colleague John Morton who called himself just a ‘dirty microbiologist’.

Contents

ABBREVIATIONS 7

ZUSAMMENFASSUNG 8

SUMMARY 9

I. INTRODUCTION 10

I.1 Anaerobic degradation of organic matter 10
I.2 Utilization of acetate among methanogens, sulfate reducers, and sulfur reducers 11
I.3 Principles of stable carbon isotope fractionation 13
I.4 Objectives of this study 15

II. MATERIALS AND METHODS 16

II.1 Sterilization practices 16
II.2 Chemicals and gases 16
II.3 Cultures 16
II.4 Growth conditions 16
II.4.1 Growth of sulfate-reducing bacteria 20
II.4.2 Growth of methanogenic archaea 21
II.4.3 Growth of sulfur-reducing bacteria 21
II.5 Incubation of rice field soil 22
II.6 Chemical analyses 23
II.6.1 Quantitative chromatographic analyses 23
II.6.1.1 Analysis of CH_4 and CO_2 23
II.6.1.2 Analysis of acetate 23
II.6.1.3 Analysis of sulfate 23
II.6.2 Determination of stable carbon isotope ratios 24
II.6.2.1 CH_4 and CO_2 24
II.6.2.2 Acetate 25
II.6.2.3 Methyl group of acetate (off-line pyrolysis and GC-C–IRMS) 26
II.6.2.4 Biomass (EA-IRMS) 26
II.6.3 Determination of sulfide 27
II.6.4 Determination of pH and optical density 27
II.6.5 Radiotracer experiments 27
II.6.6 Calculations 28
II.6.6.1 Moles of gases 28
II.6.6.2 Moles of inorganic carbon 28

II.6.6.3 Isotope fractionation 28
II.6.6.4 Carbon isotope signature of total inorganic carbon....29
II.7 Molecular analyses 30
II.7.1 DNA extraction 30
II.7.2 DNA amplification by PCR....30
II.7.3 T-RFLP analysis....32

III. RESULTS 33

III.1 Carbon isotope fractionation during acetoclastic methanogenesis by *Methanosarcina barkeri* and *Methanosarcina acetivorans* 33
III.2 Carbon isotope fractionation by sulfate-reducing bacteria using different pathways for the oxidation of acetate 47
III.3 Stable carbon isotope fractionation by acetotrophic sulfur reducers 58
III.4 Effects of the competition for acetate between methanogens and sulfate reducers on carbon isotope fractionation 64

IV. GENERAL DISCUSSION 78

IV.1 Acetoclastic methanogenesis 78
IV.2 Dissimilatory sulfate reduction 80
IV.3 Acetotrophic reduction of sulfur 82
IV.4 Conclusions and outlook 84

VI. LITERATURE.... 87

VII. APPENDIX 93

Index of figures 93
Index of tables 94
Curriculum Vitae 95
Contribution to national and international conferences 96
Acknowledgments....97
Erklärung 98

Abbreviations

bp	base pairs
BSA	Bovine serum albumin
DES	DNA elution solution – ultra pure water
EA	Elemental analyzer
EIE	Equilibrium isotope effect
FAM	Carboxyfluoresceine
FID	Flame ionization detector
GC	Gas chromatography
HPLC	High performance liquid chromatography
IC	Ion chromatography
i.d.	inner diameter
IPCC	Intergovernmental Panel on Climate Change
IRMS	Isotope ratio mass spectrometer
KIE	Kinetic isotope effect
OD	Optical density
o.d.	outer diameter
P	Product
p.A.	pro analysi
PCR	Polymerase chain reaction
ppmv	Parts per million per volume
R	Isotope ratio $^{13}C/^{12}C$
RI	Respiratory index
rpm	Rounds per minute
S	Substrate
SRB	Sulfate-reducing bacteria
TCA	Tricarboxylic acid
TIC	Total inorganic carbon
T-RF	Terminal restriction fragment
T-RFLP	Terminal restriction fragment length polymorphism
v/v	volume per volume
w/v	weight per volume
$\delta^{13}C$	Stable carbon isotope ratio relative to the international standard
δ_{ac}	$\delta^{13}C$ of acetate
ε und α	Isotope fractionation factors (defined in material and methods section)

Zusammenfassung

Acetat ist das wichtigste Zwischenprodukt der mikrobiellen Methanogenese. Unter anoxischen Bedingungen resultieren etwa 70% der gesamten CH_4-Produktion aus der Umsetzung von Acetat. Da Methan ein sehr bedeutendes Treibhausgas ist, wird es immer wichtiger die natürlichen Prozesse zu verstehen, die zur Methanbildung führen. Im Allgemeinen kann die Kohlenstoffisotopensignatur herangezogen werden, um biochemische Stoffwechselwege zu quantifizieren, wenn die Isotopensignaturen ($\delta^{13}C$) und Fraktionierungsfaktoren (α and ε) der beteiligten Substrate und Produkte bekannt sind. Daher wurden Isotopenfraktionierungsfaktoren während des anaeroben Abbaus von Acetat für die bedeutensten mikrobiellen Gruppen bestimmt, die Acetat verwerten können. Hierbei handelt es sich u.a. um methanogene Archaea, sowie sulfat- und schwefelreduzierende Bakterien.

In methanogenen Habitaten sind zwei acetatverwertende Familien der Archaea verantwortlich für die Produktion der Treibhausgase CH_4 und CO_2, *Methanosarcinaceae* und *Methanosaetaceae*. Es ist bekannt, dass sich diese beiden Familien in ihrer Isotopenfraktionierung bedeutend unterscheiden. Bislang wurde angenommen, dass die für *Methanosarcinaceae* in Reinkulturen bestimmten Fraktionierungsfaktoren auf Umweltsysteme übertragen werden können. Die vorliegende Arbeit zeigt jedoch, dass sich die Isotopensignaturen nicht nur innerhalb der Gattung *Methanosarcina* geringfügig unterscheiden, sondern auch Unterschiede der Isotopenverteilung im Vergleich zu Habitaten auftreten, in denen *Methanosarcina* der dominante Methanogene ist.

Durch Bestimmungen von Isotopensignaturen in acetotrophen, sulfatreduzierenden Bakterien wurden Unterschiede in der Kohlenstoffisotopenfraktionierung zwischen Sulfatreduzierern festgestellt, die verschiedene Stoffwechselwege für die Acetatoxidation benutzen. Denn interessanterweise zeigten Sulfatreduzierer, die den Citrat-Zyklus verwenden, keine Diskriminierung gegenüber ^{13}C und drückten eine inverse Fraktionierung aus, bei der das schwerere Isotop bevorzugt wird. Demzufolge geben diese Isotopendaten einen Hinweis darüber, über welchen Stoffwechselweg die Acetatoxidation verlief. Desweiteren wurden Kohlenstoffisotopeneffekte während der Acetatoxidation durch die Schwefelreduzierer *Desulfuromonas acetoxidans* und *Desulfurella acetivorans* untersucht. Es wurde herausgefunden, dass sich die Diskriminierung gegen ^{13}C im Acetat um bis zu 6‰ unterschied. Dies wurde mit den verschiedenen Mechanismen der Acetataktivierung begründet. Daher scheint es möglich, Isotopeneffekte von Acetat (ε_{ac}) zur Bestimmung des ersten biochemischen Schrittes der Acetatoxidation bei Schwefelreduzierern zu benutzen.

Weiterhin wurde der Einfluss der Kompetition um Acetat zwischen Sulfatreduzierern und acetoklastischen Methanogenen auf die Fraktionierung von stabilem Kohlenstoff in konkurrierenden Kokulturen und im Reisfeldboden untersucht. Die Ergebnisse können dazu beitragen, die anaeroben Stoffwechselwege von Kohlenstoff via Acetat in methanogenen und sulfidogenen Umweltbereichen einzugrenzen. Die Messung der natürlichen Isotopensignaturen von ^{13}C ist dabei ein wichtiges Hilfsmittel.

Summary

Acetate is the most important precursor of microbial methanogenesis. Under anoxic conditions about 70% of the total CH_4 production results from the consumption of acetate. Since CH_4 is a very important greenhouse gas it is necessary to understand the natural processes which lead to its production. Generally, stable carbon isotope signatures can be used to quantify biochemical pathways if isotope signatures ($\delta^{13}C$) and fractionation factors (α and ε) of the involved substrates and products are known. Therefore, isotope fractionation factors during the anaerobic degradation of acetate were determined for methanogenic archaea, sulfate-reducing bacteria, and sulfur-reducing bacteria, which are the most important microbial groups among others that are capable of utilizing acetate.

In methanogenic environments two acetate-consuming families of archaea, *Methanosarcinaceae* and *Methanosaetaceae*, are responsible for the formation of the greenhouse gases CH_4 and CO_2. It is known that the two archaeal families differ significantly in their isotope fractionation. Until now it was believed that the fractionation factors determined for pure cultures of *Methanosarcina* spp. could also be used for environmental systems. This study showed for the first time that not only isotope signatures differ slightly within the genus *Methanosarcina* but also that differences occur in the isotopic distribution compared to environmental samples where *Methanosarcina* is the most abundant methanogen.

By studying isotopic signatures in acetotrophic sulfate-reducing bacteria, differences in carbon isotope fractionation between sulfate reducers which oxidize acetate via the acetyl-CoA pathway and sulfate reducers using the TCA cycle were observed. Interestingly, the latter did not discriminate against ^{13}C and expressed an inverse fractionation where the heavier isotope is preferably consumed. Hence, isotopic data may be used as indication for which acetate oxidation pathway has been operative. The carbon isotope effects associated with the oxidation of acetate were also examined for the sulfur reducers *Desulfuromonas acetoxidans* and *Desulfurella acetivorans*. It was found that the discrimination against ^{13}C in acetate differed by about 6‰. It is suggested that the two organisms differ in isotope fractionation because they have different mechanisms for the activation of acetate. Thus, it may be possible to use isotope effects of acetate (ε_{ac}) to determine the first biochemical step during acetate oxidation in sulfur reducing bacteria.

Finally, the effects of the competition for acetate between sulfate reducers and acetoclastic methanogens on the fractionation of stable carbon were investigated in competing co-cultures and in rice field soil. The results will help to constrain the paths of anaerobic carbon flow via acetate in methanogenic and sulfidogenic environments by measuring natural ^{13}C isotope signatures.

I. Introduction

I.1 Anaerobic degradation of organic matter

At low availability of alternative electron acceptors such as nitrate, sulfate, oxidized iron, or manganese, complex organic matter is in anoxic environments degraded to methane (CH_4) and carbon dioxide (CO_2). Characteristic environmental systems for this degradation process are soils and freshwater sediments, in which peatbogs and flooded rice field soils are of particular importance, since they possess organic matter to a great extent. Compared with other oxidative processes, like aerobic degradation or alternative anaerobic respirations, the anaerobic degradation of organic matter to CH_4 is the lowest exergonic process and hence, has the fewest release of energy. In natural habitats, at least four functionally different groups of microorganisms participate in this degradation (Schink, 1997). This includes primary fermenters, secondary fermenters, as well as two types of methanogens (Figure I-1). Starting with polymers (polysaccharides, proteins, nucleic acids, and lipids), the respective previous processes provide the substrate for subsequent reactions.

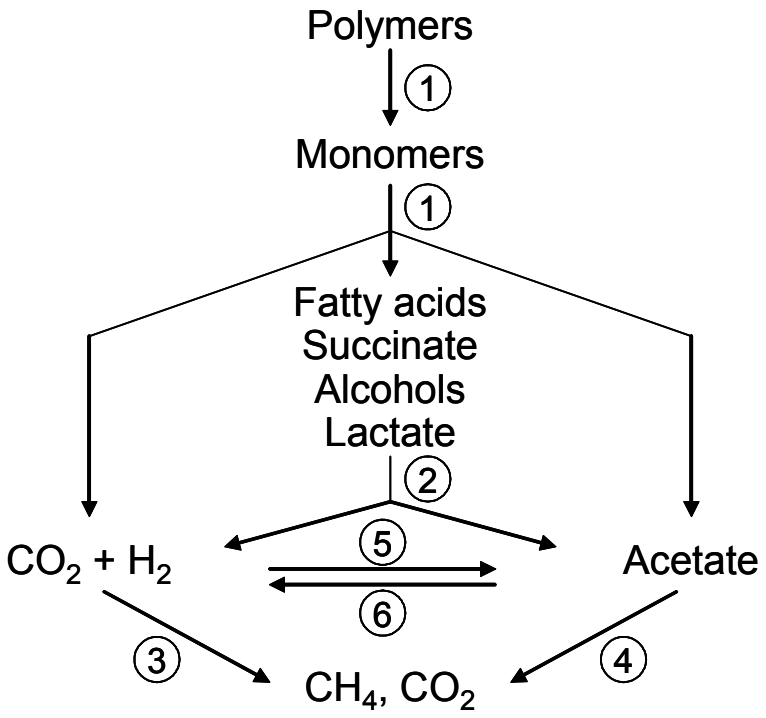

Figure I-1: Pathway of anaerobic degradation of organic matter by different groups of microorganisms. (1) Primary fermenters; (2) Secondary fermenters; (3) Hydrogenotrophic, methanogenic archaea; (4) Acetoclastic, methanogenic archaea; (5) Homoacetogenic bacteria; (6) Syntrophic acetate-oxidizing bacteria (Schink, 1997; modified).

At the beginning, primary fermenters hydrolyze polymers to oligomers and monomers (sugars, amino acids, purines, pyrimidines, fatty acids, and glycerine) and catabolize the resulting monomers to alcohols, fatty acids, and H_2. Some of these products of fermentation, in particular acetate, H_2, CO_2 and other C_1-compounds, can be converted directly to CH_4 and CO_2 by methanogenic archaea. From the remaining intermediates secondary fermenters produce precursors for methanogenesis.

In anoxic sulfate-rich environments, such as sea sediments, the situation seems to be different. The first steps of the degradation process are also carried out by primary fermenters which form the same fermentation products as mentioned above. However, in contrast to methanogenic archaea, sulfate-reducing bacteria (SRB) are metabolically versatile. Thus, an extensive community of sulfate reducers can utilize most products of primary fermentation and further oxidize these to CO_2 during a simultaneous reduction of sulfate to sulfide. Hence, SRB are able to compete with methanogens for H_2 and acetate (Figure I-2).

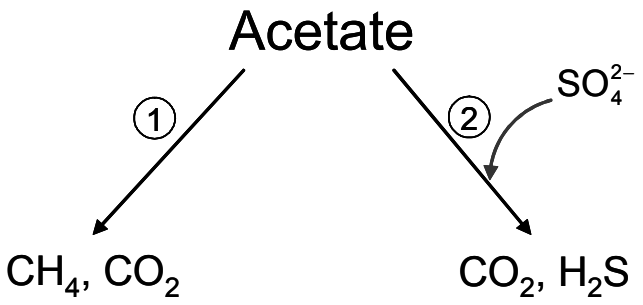

Figure I-2: Competition for acetate between acetoclastic methanogens (1) and acetotrophic sulfate reducers (2).

I.2 Utilization of acetate among methanogens, sulfate reducers, and sulfur reducers

Acetate plays a major role in the anaerobic degradation of organic matter. The most important utilizers of acetate among others (such as iron and nitrate reducers) are methanogenic archaea, acetotrophic SRB, and sulfur-reducing bacteria. Theoretically, acetate is the precursor of about 70% of biological methanogenesis. Methane is the most abundant organic gas in the earth's atmosphere (Cicerone and Oremland, 1988) and a relatively potent greenhouse gas with a high global warming potential (approx. 25 times higher than carbon dioxide; IPCC, 2001). The most important natural methane sources are wetlands, in particular flooded rice fields, ruminants, and termites. Only recently it has been shown that even terrestrial plants may produce CH_4 (Keppler et al., 2006). Nevertheless, the significance of this observation is highly controversial among the scientific community.

In methanogenic environments, the ability to disproportionate acetate to CH_4 and CO_2 is restricted to the genera *Methanosarcina* and *Methanosaeta* (Boone et al., 1993):

$$CH_3COO^- + H^+ \rightarrow CH_4 + CO_2 \quad \text{(I-1)}$$

While *Methanosarcina* is metabolically versatile and can use a wide range of substrates besides acetate such as H_2/CO_2, methanol, methylamines, and methylated sulfides, *Methanosaeta* only uses acetate. Among methanogens, *Methanosarcina* also displays the largest environmental diversity. *Methanosarcinaceae* can be found in freshwater sediments and soil, marine habitats, landfills, and animal gastrointestinal tracts (Zinder, 1993). Due to their higher affinity for acetate, *Methanosaetaceae* dominate in the low acetate environments of rice field soil and anaerobic waste digesters.

Acetate is also the main electron donor for dissimilatory sulfate reduction which plays a major role in the sulfur cycle in nature. SRB are widespread in marine and terrestrial aquatic environments. They can be found in flooded soils such as rice paddies and technical aqueous systems like sludge digesters and oil tanks. Acetotrophic sulfate reducers live at the expense of acetate oxidation to CO_2 with sulfate:

$$CH_3COO^- + SO_4^{2-} + 3H^+ \rightarrow 2CO_2 + H_2S + 2H_2O \quad \text{(I-2)}$$

During this degradation process acetate can be oxidized via the acetyl-CoA/carbon monoxide dehydrogenase pathway or the tricarboxylic acid (TCA) cycle (Thauer et al., 1989).

Another group of bacteria capable of utilizing acetate are sulfur reducers which can grow on acetate and sulfur as sole energy source:

$$CH_3COO^- + H^+ + 4S^0 + 2H_2O \rightarrow 2CO_2 + 4H_2S \quad \text{(I-3)}$$

The rotten egg odor of sulfide is often a marker for the presence of sulfur-reducing bacteria and SRB in nature. Sulfide causes several problems during oil production as it is responsible for the biocorrosion of ferrous metals and reduces the oil quality by souring oil and gas (Cord-Ruwisch et al., 1987). In higher concentrations sulfide is very toxic because it binds to heavy metals. Thus, it inactivates enzymes which contain heavy metals as cofactors. E.g., binding of sulfide to the iron in hemoglobin occupies the site for oxygen which can result in suffocation.

I.3 Principles of stable carbon isotope fractionation

Isotope fractionation terms the shift of a relative occurrence of isotopes of an element, such as hydrogen, carbon, nitrogen, and oxygen which are expressed by physical and chemical processes. In the mentioned elements, with the exception of oxygen, the lighter isotope can be found more often than the isotope one mass unit heavier. In the following only the element carbon will be considered. Stable carbon isotopes differ strongly in their occurrence, in nature the isotope ^{13}C accounts for 1.1% of total carbon and the rest is related to the lighter ^{12}C. Isotopes of an element have very similar chemical properties but, however, they are not identical. The differences in the mass are responsible for slightly changed binding and vibration energies (de Vries, 2005) which cause two isotope effects. In equilibrium reactions the equilibrium isotope effect (EIE) induces a non-statistical distribution of the isotopes on products and reactants due to different binding energies. Usually, the highest abundance of the heavy isotope can be found in the dense phase (liquid rather than the vapor phase) or in the compound having the largest molecular mass. The second isotope effect, called kinetic isotope effect (KIE), is primarily determined by the binding energies of the original compounds because during physical processes isotopically lighter molecules have higher velocities and smaller binding energies. Because in chemical processes the lighter isotope reacts faster than the heavy one, products in irreversible reactions are usually enriched in ^{12}C.

Isotope effects are physical phenomena which can not be observed directly. However, isotope effects cause isotope fractionation which has a characteristic value for a given reaction that can be measured. This value is described as the fractionation factor α:

$$\alpha_{S/P} = \frac{R_S}{R_P} \tag{I-4}$$

with S being the substrate, P the product of a reaction, and R the isotope ratio $^{13}C/^{12}C$. A reaction without any fractionation yields α = 1. But if the heavy isotope is discriminated during the reaction (and the light isotope preferred), α becomes > 1 and vice versa. Such a carbon isotope fractionation is considered as normal and, during unbranched irreversible reactions, the rule due to the above mentioned principles. The isotope fractionation of a KIE during an irreversible reaction S → P can always be observed in a closed system (Figure I-3). As the reaction proceeds (irreversibly) from onset to the complete conversion of the substrate, the fractional yield of P increases from 0 to 1 and the isotope composition of both, substrate and product, varies continuously. At a quantitative conversion of the substrate (yield = 1), the isotope ratio of the pooled product (represented by curve P) conforms with the initial isotope ratio of the substrate. During the reaction the preferred consumption of the ^{12}C-substrate causes an enrichment of the heavier isotope ^{13}C in the residual substrate (see curve S). The isotope fractionation between the increment of product forming at any instant (red-dashed

line) and the residual substrate is fixed by the magnitude of the isotope effect. Thus, the curve denoting the isotopic composition of successfully formed product increments (P') is separated from curve S by a constant difference, whereas the difference between S and the pooled product P increases continuously (Hayes, 2002).

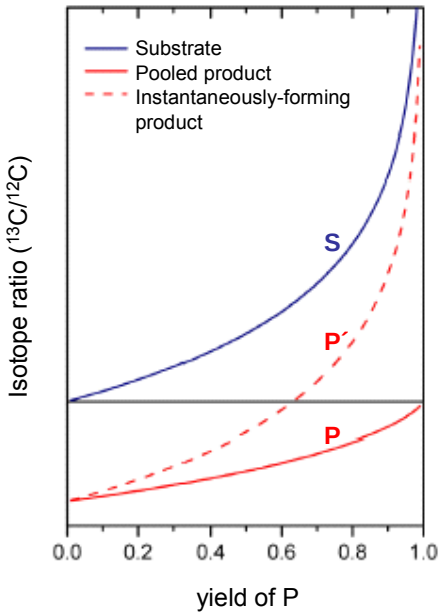

Figure I-3: Schematic representation of kinetic isotope fractionation in a closed system as a reaction proceeds to completion.

As the changes of isotope ratios during a reaction are very small, natural isotope ratios are expressed in the delta notation:

$$\delta^{13}C = [(R_{sample} / R_{standard}) - 1] \times 10^3 \text{ [‰]} \quad \text{(I-5)}$$

where R is the isotope ratio $^{13}C/^{12}C$ of a sample or of the used standard. For carbon, first a belemnite of a Pee Dee formation, a limestone in the USA, was used as reference material but because this material became exhausted, a new, artificial standard was introduced, the V-PDB (Vienna Pee Dee Belemnite). An increase of the δ-value corresponds to an increase of the heavy isotope and vice versa.

I.4 Objectives of this study

It is suggested that the contribution of CH_4 to the greenhouse effect will even increase in future. This has made it necessary and more urgent to understand the natural processes which lead to the production of CH_4. Major sources for the production of climate relevant greenhouse gases are terrestrial anoxic habitats. During the anaerobic degradation of organic matter to CH_4 and CO_2, acetate is the most important substrate since it contributes to bacterial methanogenesis to about 70%. Acetate is also the main electron donor for sulfate reduction and hence, a competitive substrate for acetoclastic methanogens and acetotrophic sulfate reducers. Generally, stable carbon isotope signatures can be used to quantify biochemical pathways if isotope signatures and fractionation factors of the involved substrates and products are known. Nevertheless, the effect of the competition for acetate on carbon isotope fractionation is unknown and only little isotopic data are available for the organisms participating this competition. Therefore, the aim of this study was to investigate stable carbon isotope fractionation for archaeal and bacterial groups which utilize acetate. The following tasks were examined:

- Literature data on acetoclastic methanogens show significant differences in carbon isotope fractionation between *Methanosarcina* and *Methanosaeta*. Are there also differences within the genus *Methanosarcina* observable?
- Do sulfate reducers show a different carbon isotope fractionation of acetate when they have different metabolic pathways for the acetate oxidation?
- Do sulfur-reducing bacteria discriminate differently against ^{13}C when they use different mechanisms for the activation of acetate?
- How does the competition for acetate between methane-producing archaea and sulfate-reducing bacteria effect the carbon isotope fractionation?

II. Materials and methods

II.1 Sterilization practices

Media, buffers, and solutions were prepared with demineralized water and sterilized by autoclaving (30 min at 121°C and 1 bar overpressure) unless otherwise noted. Non-autoclavable components were filtered (0.2 µm pore size, No. FP 30/0.2 CA-S, Schleicher und Schuell GmbH, Dassel, Germany) and supplemented under sterile conditions. All glass bottles and butyl rubber stoppers for the incubation of cultures and rice field soil were autoclaved before use (30 min at 121°C and 1 bar overpressure).

II.2 Chemicals and gases

All chemicals, unless otherwise noted, were purchased in p.A. quality from the following suppliers: Amersham Pharmacia Biotech (Freiburg, Germany), Eppendorf AG (Hamburg, Germany), Merck (Darmstadt, Germany), Roth (Karlsruhe, Germany), and Sigma-Aldrich (Taufkirchen, Germany). The following technical gases, all purchased from Air Liquide (Duesseldorf, Germany), were used in this study: For the calibration of measurements by gas chromatography a mixture of CH_4 and CO_2 in N_2 was used. N_2 and a mixture of N_2/CO_2 (80/20%) served for gassing samples and cultures.

II.3 Cultures

The following pure cultures were used in this study: *Desulfobacca acetoxidans* strain ASRB2 (DSM 11109), *Desulfobacter hydrogenophilus* strain AcRS1 (DSM 3380), *Desulfobacter postgatei* strain 2ac9 (DSM 2034), *Desulfurella acetivorans* strain A63 (DSM 5264), *Desulfuromonas acetoxidans* strain 11070 (DSM 684), *Methanosarcina acetivorans* strain C2A (DSM 2834), and *Methanosarcina barkeri* strain Fusaro (DSM 804), were obtained from the Deutsche Sammlung von Mikroorganismen und Zellkulturen (Braunschweig, Germany).

II.4 Growth conditions

All species were grown in bicarbonate-buffered mineral medium, based on the salt concentrations (Table II-2) and stock solutions (Table II-3) described below. For the preparation of media, stock solutions of salts (5×) were prepared and mixed with appropriate volumes (see following chapters) of the stock solutions 1 – 5. Resazurine (1 mg/l) served as indicator for anoxic conditions. For cultivation and experiments, serum bottles were filled with medium to a maximum of 50% and closed with butyl rubber stoppers and aluminum or stainless steel caps. The headspace was replaced against N_2/CO_2 (80/20%) by repeatedly flushing and evacuating for 10 min. The final over pressure was set to 0.7 bar. After addition of solutions 6 – 8 the medium was autoclaved. Solution 9, containing a mixture of vitamins

and $CaCl_2 \times 2\ H_2O$, was added sterile prior to inoculation. For experiments, inocula of cultures in the late exponential phase were transferred into glass bottles (500 ml, Ochs, Bovenden-Lenglern, Germany) resulting in a final volume of 250 ml. The different organisms were cultivated under the following growth conditions:

Table II-1: Growth conditions for microorganisms used in this study

Microorganism	Electron acceptor	Electron donor	Temp. [°C]	Shaking	Inoculum
Desulfobacter hydrogenophilus	Sulfate (20 mM)	Acetate (20mM)	30	–	10%
Desulfobacter postgatei	Sulfate (20 mM)	Acetate (20 mM)	30	–	10%
Desulfobacca acetoxidans	Sulfate (20 mM)	Acetate (20 mM)	37	–	1%
Methanosarcina barkeri	Acetate (20 mM)	Acetate[a] (20mM)	30	–	10%
Methanosarcina acetivorans	Acetate (20 mM)	Acetate[a] (20mM)	37	–	10%
Desulfurella acetivorans	Sulfur (6.2 mM)	Acetate (3.7 mM)	55	–	10%
Desulfuromonas acetoxidans	Sulfur (6.2 mM)	Acetate (3.7 mM)	30	120 rpm	10%

[a] Acetate serves as both, electron donor and electron acceptor for the methanogenic process

Table II-2: Different salt concentrations for culture media (in g/l)

	Solution				
Compound	No. 1[a]	No. 2[b]	No. 3[c]	No. 4[d]	No. 5[e]
KH_2PO_4	0.2	0.68	0.4	0.33	1.0
$Na_2HPO_4 \times 2\ H_2O$	–	–	0.5	–	–
NH_4Cl	0.3	1.0	0.3	0.33	0.3
NaCl	7	23.3	0.3	–	20
$MgCl_2 \times 6\ H_2O$	1.3	11	0.1	0.33	2
$MgSO_4 \times 7\ H_2O$	–	–	–	–	1.0
KCl	0.5	1.0	–	0.33	–
$CaCl_2 \times 2\ H_2O$	–	–	–	0.18	0.1

[a] for low salt medium
[b] for high salt medium
[c] for anaerobic multipurpose medium
[d] for *Desulfurella* medium
[e] for *Desulfuromonas* medium

Table II-3: Stock solutions for media

Solution	Compound	Amount
(1) Acid trace element solution (1000×)	HCl (25%)	10 ml
	$FeCl_2 \times 4\ H_2O$	1.50 g
	$ZnCl_2$	70 mg
	$MnCl_2 \times 4\ H_2O$	100 mg
	H_3BO_3	6 mg
	$CoCl_2 \times 6\ H_2O$	190 mg
	$CuCl_2$	1.6 mg
	$NiCl_2 \times 6\ H_2O$	24 mg
	$Na_2MoO_4 \times 2\ H_2O$	36 mg
	H_2O	ad 1l
(2) Neutral trace element solution (100×)	Titriplex I	1.5 g
	→ dissolved in 500 ml H_2O, adjusted to pH 6.5 with KOH	
	$FeSO_4 \times 7\ H_2O$	0.1 g
	$CoCl_2 \times 6\ H_2O$	0.1 g
	$ZnSO_4 \times 7\ H_2O$	0.1 g
	$CuSO_4 \times 5\ H_2O$	0.0087 g
	$AlCl_3 \times 6\ H_2O$	0.01g
	$Na_2MoO_4 \times 2\ H_2O$	0.01 g
	$NiCl_2 \times 6\ H_2O$	0.03 g
	Na_2O_4Se	0.019 g
	H_2O	ad 1l
	→ adjusted to pH 7.0 with KOH	
(3) Alkaline trace element solution (1000×)	NaOH	0.4 g
	$Na_2Se_3 \times 5\ H_2O$	0.015 g
	$Na_2WO_4 \times 2\ H_2O$	0.033 g
	$Na_2MoO_4 \times 2\ H_2O$	0.012 g
	H_2O	ad 1l
(4) Acetate solution	$NaCH_3COO \times 3\ H_2O$	277.8 g
	H_2O	ad 1l
(5) Sulfate solution	Na_2SO_4	66.7 g
	H_2O	ad 1l

(6) Bicarbonate solution	$NaHCO_3$	94.5 g
	H_2O	ad 1l
(7) Cysteine-HCl solution	Cysteine-HCl × H_2O	5.0 g
	Anoxic H_2O	100 ml
→ Stored under N_2 atmosphere and wrapped in aluminum foil.		
(8) Sulfide solution	Na_2S × 9 H_2O	12 g
	Anoxic H_2O	60 ml
→ Stored under N_2 atmosphere at 4°C and wrapped in aluminum foil.		
(9) Vitamin calcium chloride solution (after DSM-Medium 141)	a) Vitamin solution	
	Biotin	20 mg
	Folic acid	20 mg
	Pyridoxine-HCl	100 mg
	Thiamine-HCl	50 mg
	Riboflavin	50 mg
	Nicotinate	50 mg
	Pantothenic acid	50 mg
	Vitamin B_{12} (10 mg ml^{-1})	1 ml
	p-aminobenzoic acid	50 mg
	Liponat	50 mg
	H_2O	ad 1l
	b) Calcium chloride solution	
	$CaCl_2$ × 2 H_2O	15[a] / 30[b] g
	H_2O	ad 100 ml
→ Aliquots of vitamin and calcium chloride solution 1:1 (v/v) were stored at –20°C. Before use, 50 ml of the mixed solution were filtered into a sterile and anoxic 120 ml serum bottle, wrapped in aluminum foil, and stored at 4°C.		

[a] referred to as solution 9a
[b] referred to as solution 9b

II.4.1 Growth of sulfate-reducing bacteria

For pure culture studies of the two *Desulfobacter* spp., *D. postgatei* and *D. hydrogenophilus*, cultures were grown in both, a low salt and a high salt medium, which had the following compositions:

Table II-4: Compositions of low and high salt medium (in ml/l)

Component	Low salt medium	High salt medium
Salt solution[a]	200, No. 1	200, No. 2
Acid trace element solution (1)[b]	0.5	0.5
Neutral trace element solution (2)[b]	5	5
Acetate solution (4)[b]	10.8	10.8
Sulfate solution (5)[b]	46.5	46.5
Bicarbonate solution (6)[b]	40	40
Cysteine-HCl solution (7)[b]	10	10
Sulfide solution (8)[b]	1.8	1.8
Vitamin calcium chloride solution (9b)[b]	2	2
H_2O	683.4	683.4

[a] for compositions of salt solutions see Table II-2
[b] for compositions of stock solutions see Table II-3

For growth of *Desulfobacca acetoxidans* an anaerobic multipurpose medium described by Stams et al. (1993) was used which contained the following:

Table II-5: Composition of medium for growth of *Desulfobacca acetoxidans* (in ml/l)

Component	Anaerobic multipurpose medium
Salt solution[a]	200, No. 3
Acid trace element solution (1)[b]	1.0
Alkaline trace element solution (3)[b]	1.0
Acetate solution (4)[b]	10.8
Sulfate solution (5)[b]	46.5
Bicarbonate solution (6)[b]	40
Sulfide solution (8)[b]	2.5
Vitamin calcium chloride solution (9a)[b]	2
H_2O	696.2

[a] for compositions of salt solutions see Table II-2
[b] for compositions of stock solutions see Table II-3

Desulfobacca acetoxidans could also be grown on the low salt medium, as described above, when grown as a competing co-culture with *Methanosarcina barkeri*.

II.4.2 Growth of methanogenic archaea

The two acetoclastic *Methanosarcina* spp., *M. barkeri* and *M. acetivorans*, were both grown as single cells (Sowers et al., 1993) in HS medium (Metcalf et al., 1996) which was composed of the following:

Table II-6: Composition of HS medium for growth of *Methanosarcina* spp. (in ml/l)

Component	HS medium
Salt solution[a]	200, No. 2
Neutral trace element solution (2)[b]	10
Acetate solution (4)[b]	10.8
Bicarbonate solution (6)[b]	40
Cysteine-HCl solution (7)[b]	10
Sulfide solution (8)[b]	1.8
Vitamin calcium chloride solution (9b)[b]	0.25
H_2O	727.2

[a] for compositions of salt solutions see Table II-2
[b] for compositions of stock solutions see Table II-3

For competition experiments with *Desulfobacca acetoxidans*, *M. barkeri* was additionally grown on the low salt medium, as described above. Both *Methanosarcina* spp. also grew on the high salt medium described above.

II.4.3 Growth of sulfur-reducing bacteria

The two sulfur reducers that were used in this study, *Desulfurella acetivorans* and *Desulfuromonas acetoxidans*, were both grown with 3.7 mM acetate as electron donor and 6.2 mM sulfur (subl., purum, purchased from Fluka, Buchs, Switzerland) as electron acceptor. Culture vessels for growth of *Desulfuromonas acetoxidans* were provided with three glass beads and shaken on a rotary shaker (120 rpm) to improve growth (Pfennig and Biebl, 1976). Media containing sulfur were autoclaved for 30 min at 115°C. The compositions of the media for *Desulfurella acetivorans* and *Desulfuromonas acetoxidans* were as follows:

Table II-7: Compositions of media for growth of *Desulfurella* and *Desulfuromonas* (in ml/l)

Component	*Desulfurella* medium	*Desulfuromonas* medium
Salt solution[a]	200, No. 4	200, No. 5
Acid trace element solution (1)[b]	1.0	1.0
Acetate solution (4)[b]	2.0	2.0
Bicarbonate solution (6)[b]	20	20
Sulfide solution (8)[b]	2.5	1.5
Vitamin calcium chloride solution (9a)[b]	2	2
H_2O	772.5	773.5

[a] for compositions of salt solutions see Table II-2
[b] for compositions of stock solutions see Table II-3

II.5 Incubation of rice field soil

Rice field soil was collected in 2006 from rice paddies of the Italian Rice Research Institute near Vercelli in the valley of the river Po, Italy. The characteristics and cultivation were described by Schuetz *et al*. (1989a; 1989b). The soil was air-dried and stored in polyethylene vats at room temperature. Afterwards, residues of straw and roots were hackled using a jaw crusher (Retsch, Dietz-Motoren GmbH & Co. KG, Dettingen and Teck, Germany) and the rice field soil was sieved (≤ 1 mm mesh size). For experiments, rice field slurries (soil and demineralized water, 1:1) amended with rice straw (1 g per kg slurry; chaffed with an A11 Basic Analytical Mill, IKA Werke, Staufen, Germany) were preincubated at 25°C for at least four weeks to reduce alternative electron acceptors such as iron, sulfate, or nitrate. The rice slurry was distributed into 27 ml pressure tubes (Ochs, Bovenden-Lenglern, Germany), each tube was filled with 10 g slurry and sealed with a butyl rubber stopper. Thereafter, the incubation vessels were repeatedly flushed and evacuated with N_2 for 10 min and a final over pressure of 0.5 bar was adjusted inside the tubes. After this the substrates (acetate and sulfate) were added under sterile conditions and the incubation was started at 25°C in the dark. At each sampling day three tubes were harvested to determine the concentrations and isotope ratios of substrates and products and for DNA extraction.

II.6 Chemical analyses

II.6.1 Quantitative chromatographic analyses

Prior to the analysis of gases, cultures were shortly shaken by hand to obtain an equilibrium between medium and headspace. Gas samples were taken directly before the analysis using a 0.25 ml pressure lock syringe (VICI, Baton Rouge, LA, USA) and sampling through the septum. The sample volume was 0.2 ml.

II.6.1.1 Analysis of CH_4 and CO_2

CH_4 and CO_2 were analyzed by gas chromatography (GC-8A, Shimadzu, Kyoto, Japan) using a flame ionization detector (FID). To detect oxidized gases, such as CO_2, a methanizer was used which e.g., reduced CO_2 to CH_4. A mixture of CH_4 (995 ppmv) and CO_2 (1000 ppmv) in nitrogen served as calibration gas.

Table II-8: Operating data for gas chromatograph

Column	2 m stainless steel column, i.d. 1/8”; carrier material: Poropack QS 50/100 mesh
Carrier gas	Hydrogen 5.0
Detector	Flame ionization detector (FID); fuel gas: Hydrogen und FID-gas; Quenching gas: Nitrogen 5.0
Temperature	Injector, detector: 160°C; column: 120°C
Integration	Integrator C-R A6 (Shimadzu)
Methanizer	Self-construction, column: NiCr-Ni catalyst (Chrompack, Middelburg, Netherlands), 20 cm stainless steel column, i.d. 1/8”, operating temperature: 350°C
Detection limit	1 ppmv CH_4

II.6.1.2 Analysis of acetate

See II.6.2.2

II.6.1.3 Analysis of sulfate

Sulfate was analyzed using ion chromatography (IC). Liquid samples were centrifuged in a microcentrifuge (Eppendorf, 5415 C) for 10 min at maximum speed (14,000 rpm) and subsequent, the supernatant was filtered (REZIST 13/0.2 PTFE, Schleicher und Schuell, Dassel, Germany) and stored at –20°C until analysis. A solution of 1 mM sulfate served as calibration standard.

Table II-9: Operating data for ion chromatograph

IC system	Solvent delivery system S1121, column oven S4260B, suppressor unit S4260A (all from Sykam, Fuerstenfeldbruck, Germany), sample injector S5200 (Schambeck SFD, Bad Honnef, Germany)
Column	6 cm stainless steel column, i.d. 4.6 mm
Eluant	5 mM Na_2CO_3, 1 ml l^{-1} modifier[a]; flow rate: 1.5 ml min^{-1}
Detector	Conductivity detector S3111
Oven temperature	70°C
Integration	Program Peak Simple (SRI-Instruments, Torrence, USA)
Detection limit	approx. 5 µM

[a] 1 g 4-hydroxybenzonitrile in 50 ml methanol

II.6.2 Determination of stable carbon isotope ratios

Isotope ratios of stable carbon are, in this study, reported in the common delta notation. The isotope ratio ($^{13}C/^{12}C$) of a sample is compared to the isotope ratio of an international standard, the Vienna Pee Dee Belemnite (V-PDB) with $R_{standard} = 11180.2 \pm 2.8 \times 10^{-6}$ (see equation I-5). Units for δ and for measured isotope effects are parts per thousand, termed per mill, and assigned the symbol ‰.

II.6.2.1 CH_4 and CO_2

Stable isotope analysis of $^{13}C/^{12}C$ in gas samples was performed using a gas chromatograph combustion isotope ratio mass spectrometer (GC-C–IRMS) system that was purchased from Finnigan (Thermo Fisher Scientific, Bremen, Germany). The principle operation was described by Brand (1996). The isotope reference gas was CO_2 (99.998% purity; Air Liquide, Duesseldorf, Germany), calibrated with the working standard methylstearate (Merck). The latter was intercalibrated at the Max Planck Institute for Biogeochemistry, Jena, Germany (courtesy of Dr. W.A. Brand) against NBS 22 and USGS 24. The precision of repeated analysis of 1.3 nmol CH_4 was ± 0.2‰.

Table II-10: Operating data for GC-C–IRMS

GC	Hewlett Packard 6890 (Waldbronn, Germany)
Injector	Split ratio 1:10; operating temperature: 150°C
Column	27.5 m Pora PLOT Q, i.d. 0.32 mm, 10 µm film thickness (Chrompack, Frankfurt, Germany)
Carrier gas	Helium 5.0; flow rate: 2.6 ml min^{-1}
GC/C-Interface	Standard GC Combustion Interface III (Thermo Electron, Bremen, Germany), oxidation reactor at 940°C, reduction reactor at 650°C
Detector	IRMS: Finnigan MAT delta plus (Thermo Electron)
Oven temperature	30°C
Integration	ISODAT™ NT 2.0 (Thermo Electron)

II.6.2.2 Acetate

Isotopic measurements and quantification of acetate were performed on a HPLC system. The carbon compounds of liquid samples were first separated via HPLC and subsequently completely oxidized to CO_2 by using sodium persulfate (0.42 M; Fluka) and phosphoric acid (1.35 M; Merck) at 99.9°C. CO_2 was transferred into a helium flow via a membrane and transported to the IRMS. The principle was described by Krummen et al. (2004). Isotope reference gas was CO_2 calibrated as described above.

Table II-11: Operating data for HPLC-IRMS

HPLC system	Pump Spectra System P1000 (Thermo Finnigan, Jan Jose, CA, USA), column oven Mistral (Spark, Emmen, Netherlands)
Sample injector	HTC Pal (CTC Analysis, Zwingen, Switzerland)
Column	30 cm stainless steel column, i.d. 7.8 mm
Carrier material	Sulfurized divinylbenzol styrene (Aminex HPX-87-H, BioRad)
Eluant	1 mM sulfuric acid, flow rate: 0.3 ml min^{-1}
Oxidation reagents	Sodium persulfate and phosphoric acid, flow rate: 50 µl min^{-1} each
Interface	Finnigan LC IsoLink (Thermo Electron)
Detector	IRMS: Finnigan MAT delta plus advantage (Thermo Electron)
Oven temperature	35°C
Integration	ISODAT™ NT 2.0 (Thermo Electron)

II.6.2.3 Methyl group of acetate (off-line pyrolysis and GC-C–IRMS)

An off-line pyrolysis was conducted to determine $\delta^{13}C$ of the methyl group of acetate ($\delta_{ac\text{-}methyl}$). Liquid samples were filtered (REZIST 13/0.2 PTFE, Schleicher und Schuell), adjusted to pH > 10 with NaOH, and dried (DNA-SpeedVac, DNA 110, Savant Instruments). The samples were dissolved in 45 µl deionized water and purified by using HPLC equipped with a manual injector (S5110, Sykam, Fuerstenfeldbruck, Germany). The purified sample was collected in a 1.5 ml tube, which contained 20 µl 5 N NaOH, and subsequently dried. The sample was, once again, dissolved in 45 µl deionized water, added to a strong NaOH solution (approx. 100fold the amount of acetate), and dried in a Pyrex tube (o.d. 6 mm) under vacuum at 60°C in a water bath. The dried reactants were pyrolyzed under vacuum at 400°C, converting the carboxyl carbon to CO_2 and the methyl carbon to CH_4 (Blair et al., 1985) as confirmed by mass balances:

$$Na^*CH_3COO + NaOH \rightarrow {}^*CH_4 + Na_2CO_3 \qquad \text{(II-1)}$$

Gas samples were taken and then analyzed by GC-C–IRMS as described in II.6.2.1. Two, in their intramolecular isotopy different acetate compounds, were set as standard and intercalibrated with other laboratories (thanks to Dr. Roland Werner und Dr. Stan C. Tyler) because no international standards are available.

II.6.2.4 Biomass (EA-IRMS)

The bacterial suspension was centrifuged for 15 min at 4°C and 26.000 × g (RC 5B Plus, Rotor SS34; Sorvall, Langenselbold, Germany). The supernatant was discarded, the settled cells resuspended in 25 mM phosphate buffer (pH 6.8), and centrifuged again as described above (this step was performed twice). Finally, the supernatant was discarded and the cells were dried in an oven at 105°C. The dried biomass (approx. 1 mg) was weight in tin capsules (IVA, Meerbusch, Germany). The analysis of $\delta^{13}C$ of biomass was carried out at the Centre for Stable Isotope Research & Analysis (KOSI) at Goettingen University, Germany (thanks to Reinhard Langel), with the following EA-IRMS system:

Table II-12: Operating data for EA-IRMS

Elemental analyzer:	NA 2500 (CE Instruments, Rodano, Italy)
Carrier gas:	Helium, flow rate: 90 ml min^{-1}
Interface:	Finnigan ConFlo III (Thermo Electron)
Detector:	IRMS: Finnigan MAT delta plus (Thermo Electron)
Reference compound:	Acetanilide

The precision of repeated analysis was ± 0.18‰ when 0.4–1.5 mg acetanilide was injected.

II.6.3 Determination of sulfide

Sulfide (H_2S) was determined photometrically after reaction with $CuSO_4$ to form CuS as described by Cord-Ruwisch (1985). For this purpose 100 µl of culture fluid was removed by a syringe from the culture vessel and rapidly injected into 8 ml copper reagent which was posed on a whirl mixer (900 rpm). Immediately after mixing for 5 s, the absorbance was measured at 480 nm in a spectronic photometer (Hitachi U-1100, Berlin, Germany). HCl (50 mM) served as blank. A dilution series with defined concentrations of H_2S resulted in a proportional regression ($r^2 = 0.996$) in the range of 0.2 to 25 mM.

II.6.4 Determination of pH and optical density

A digital pH meter (Microprocessor pH meter 539, Wissenschaftlich-Technische Werkstätten GmbH, Weilheim, Germany) with an InLab Semi-Micro pH electrode (pH 0 to 12, Mettler Toledo, Gießen, Germany) was used to determine pH-values. For media and culture fluids which contained H_2S a sulfide-resistant pH electrode was used (InLab Solids, pH 1 to 11, Mettler Toledo).

The optical density of microbial suspensions was measured at 578 nm in a spectronic photometer (Hitachi U-1100, Berlin, Germany). The corresponding media served as blank.

II.6.5 Radiotracer experiments

Radiotracer experiments were done during growth of acetoclastic methanogens to determine the fraction of CH_4 and CO_2 produced from the methyl group of acetate. For that, 10 µCi/ml (0.37 MBq/ml) of Na-[2-^{14}C]acetate (Amersham, Braunschweig, Germany) was added in a volume of 1.0 ml to 120 ml bottles (Ochs, Bovenden-Lenglern, Germany) which were filled with 50 ml culture liquid. The origin, specific radioactivity and the quantity of the added tracer were 2.1 GBq mmol^{-1} and 3.7 MBq, respectively. The ^{14}C-labeled acetate was added after CH_4 production was observed. Total and radioactive CH_4 and CO_2 were analyzed in a gas chromatograph equipped with a flame ionization detector, reduction column and a RAGA radioactivity detector (Conrad et al., 1989). Total and radioactive acetate were analyzed in the liquid phase in a HPLC system equipped with a refraction index detector and a RAMONA radioactivity detector (Krumböck and Conrad, 1991). The respiratory index (RI) was determined at the end of the incubation after addition of 2.0 ml 2.5 M H_2SO_4 per bottle to liberate CO_2 (CO_2 + bicarbonate):

$$RI = {}^{14}CO_2 / ({}^{14}CO_2 + {}^{14}CH_4) \qquad \text{(II-2)}$$

II.6.6 Calculations

II.6.6.1 Moles of gases

The measured concentrations (ppmv) of gases were converted into moles (mmol) by using the ideal gas law:

$$n = \frac{p \times V}{R \times T} \qquad \text{(II-3)}$$

n: Moles of gases [mol]
p: Partial pressure of measured gas in [bar] (1 ppmv = 10^{-6} bar)
V: Volume of headspace inside reaction vessel [l]
R: Gas constant (0.083144 [l bar K^{-1} mol^{-1}])
T: Temperature [K]

II.6.6.2 Moles of inorganic carbon

Total oxidized carbon was distributed among the following different carbon species: gaseous CO_2 ($CO_{2(g)}$), dissolved CO_2 ($CO_{2(d)}$; contains dissolved CO_2 and carbonic acid), HCO_3^-, and CO_3^{2-}. To determine the total amount of inorganic carbon (TIC; equation II-7), the distribution of carbon among these species was calculated using solubility and equilibrium constants (Stumm and Morgan, 1995; K-values are exemplary shown for 25°C):

$CO_{2(g)} \leftrightarrow CO_{2(d)}$ $\quad K_0 = 10^{-1.47}$ (II-4)

$CO_{2(d)} + H_2O \leftrightarrow H^+ + HCO_3^-$ $\quad K_1 = 10^{-6.35}$ (II-5)

$HCO_3^- \leftrightarrow H^+ + CO_3^{2-}$ $\quad K_2 = 10^{-10.33}$ (II-6)

$$n(TIC) = n(CO_{2(g)}) + n(CO_{2(d)}) + n(HCO_3^-) + n(CO_3^{2-}) \qquad \text{(II-7)}$$

II.6.6.3 Isotope fractionation

Fractionation factors for a reaction A → B are defined after Hayes (1993) as follows (by analogy to equation I-4 but now using the delta notation instead of isotope ratios):

$$\alpha_{A/B} = (\delta_A + 1000)/(\delta_B + 1000) \qquad \text{(II-8)}$$

where $\delta = \delta^{13}C$ of a reactant A or a product B. Isotope fractionation is also expressed as $\varepsilon \equiv 10^3 (1 - \alpha)$. Equation II-8 can only be used to calculate isotope fractionation by assuming an unlimited substrate reservoir or a dynamic equilibrium. In closed systems (cp. Figure I-2) the fractionation factor ε can be determined as described by Mariotti et al. (1981), which is

based on a Rayleigh distillation, from the residual reactant (equation II-9) and the product formed (equation II-10):

$$\delta_r = \delta_{ri} + \varepsilon[\ln(1-f)] \quad \text{(II-9)}$$

$$\delta_p = \delta_{ri} - \varepsilon(1-f)[\ln(1-f)]/f \quad \text{(II-10)}$$

where δ_{ri} is the isotope composition of the reactant (either ac or ac-methyl) at the beginning (f = 0), δ_r and δ_p are the isotope compositions of the residual ac and the pooled CH_4, respectively, at the instant when f was determined, and f is the fractional yield of the products based on the consumption of ac ($0 < f < 1$). Linear regression of δ_r against $\ln(1 - f)$ and of δ_p against $(1 - f)[\ln(1 - f)]/f$ gives ε as the slope of best-fit lines.

To analyze ε of the carboxyl group of acetate ($\varepsilon_{ac\text{-}carboxyl}$), values for $\delta_{ac\text{-}carboxyl}$ were calculated using the following equation:

$$\delta_{ac\text{-}carboxyl} = 2\delta_{ac} - \delta_{ac\text{-}methyl} \quad \text{(II-11)}$$

II.6.6.4 Carbon isotope signature of total inorganic carbon

The calculated moles of the different carbon species (II.6.6.2) and their isotopic compositions were used to determine the isotopic signature of TIC:

$$\delta_{TIC} = X_g\delta_g + X_d\delta_d + X_b\delta_b + X_c\delta_c \quad \text{(II-12)}$$

where X = mole fraction and δ = isotopic composition of the C of g = gaseous CO_2, d = dissolved CO_2, b = HCO_3^-, and c = CO_3^{2-}. δ_g was measured directly, the remaining isotopic compositions were calculated from the relevant equilibrium isotope fractionation factors at different temperatures (Deines and Langmuir, 1974; Mook et al., 1974):

$$\delta_d = \alpha_{d/g}\delta_g + (\alpha_{d/g} - 1)1000 \quad \text{(II-13)}$$

$$\delta_b = \alpha_{b/g}\delta_g + (\alpha_{b/g} - 1)1000 \quad \text{(II-14)}$$

$$\delta_c = \alpha_{c/g}\delta_g + (\alpha_{c/g} - 1)1000 \quad \text{(II-15)}$$

II.7 Molecular analyses

II.7.1 DNA extraction

DNA extraction from rice field soil was performed using the 'FastDNA®SPIN Kit for Soil' (Qbiogene, Heidelberg, Germany) following the manufacturer's instructions. In-between, the following treatment was carried out after precipitation of proteins, cell components, and other impurities and after binding of DNA, to remove humic acids. 1 ml of a 5.5 M guanidine thiocyanate solution was added and gently mixed. After short centrifugation for 5 s (14,000 rpm) the supernatant was discarded. This washing procedure was repeated twice. After the last washing step the binding matrix was resuspended in 600 µl guanidine thiocyanate and 600 µl of the resulting mixture was transferred to a spin filter and centrifuged for 1 min (14,000 rpm). The catch tube was emptied and the remaining supernatant added to the spin filter and spinned again. After that several washing steps were performed according to the instructions of the manufacturer. Finally, the DNA was dissolved in 100 µl DES (DNase/Pyrogen free water) which was now ready for further application. The DNA concentration was determined at 260 nm with a biophotometer (BioPhotometer, Eppendorf).

II.7.2 DNA amplification by PCR

DNA fragments were amplified using PCR (polymerase chain reaction) and the resulting products were used for T-RFLP (terminal restriction fragment length polymorphism) analysis (II.7.3) to analyze the archaeal diversity. The used primers are listed in Table II-13. For every amplification a negative control was performed by adding the appropriate amount of water instead of DNA template. DNA containing the target molecule for amplification served as positive control.

Table II-13: Oligonucleotide primers used for T-RFLP analysis of partial archaeal 16S rRNA genes

Oligonuc.[a]	Sequence (5' to 3')	Target site	Reference
A109f	ACKGCTCAGTAACACGT	Archaea	(Grosskopf et al., 1998)
A915r[b]	GTGCTCCCCCGCCAATTCCT	Archaea	(Stahl and Amann, 1991)

[a] Oligonuc. = Oligonucleotide
[b] FAM labeled

The PCR reactions were performed in a thermocycler (Primus, MWG Biotech, Ebersberg, Germany) using the reaction listed in Table II-14. Amplifications of PCR products for T-RFLP analysis were carried out with the primers A109f and A915r, the latter was carboxyfluoresceine (=FAM) labeled (5' end). The PCR conditions are listed in Table II-15.

Table II-14: PCR reaction to amplify 16S rRNA genes from rice field soil

Component	Concentration of stock solution	Added volume [µl]	Final concentration
Forward and reverse primer	33 µM	0.5	0.33 µM
dNTPs	2 mM	5	200 µM
$MgCl_2$	25 mM	3	1.5 mM
Green Go *Taq* Flexi Buffer (Promega)	5×	10	1×
Go *Taq* DNA Polymerase	5 U/µl	0.2	1 U
BSA	20 mg/ml	0.5	10 µg
DNA template		1	
H_2O		ad 50	

Table II-15: PCR conditions for analysis of the microbial diversity of 16S rRNA genes

Temperature	Duration	Number of cycles	Function
94°C	3 min		Denaturation
94°C	45 s		Denaturation
52°C	45 s	29	Annealing
72°C	90 s		Elongation
72°C	5 min		Final DNA synthesis
4°C	∞		Cooling phase

Gel electrophoresis was carried out as a visual control for a successful amplification. For that, 5 µl of PCR product was loaded onto a 1.5% (w/v) 1× TAE agarose gel (SeaKem LE, Biozym; in TAE-buffer) and separated for 25 min at 120V. Afterwards, the 'GenElute™ PCR Clean-Up Kit' (Sigma-Aldrich, Taufkirchen, Germany) was used to purify the PCR product by following the preparation instructions of the manufacturer. The DNA was finally eluted in 25 µl elution solution and stored at –20°C or immediately digested for 3 h at 65°C using 5 µl of the eluted DNA. Further components of the restriction batch were 1 µl incubation buffer and 0.5 µl *Taq*I (10 U μl^{-1}, Fermentas) as restriction enzyme (5'-TCGA-3'). The batch was filled up with sterile H_2O to a total volume of 10 µl. The restriction digestion was purified via the 'Sigma Spin™ Post Reaction Clean-Up Columns Kit' according to the preparation instructions.

II.7.3 T-RFLP analysis

To prepare the samples for the T-RFLP analysis, 3 µl of the purified restriction digestions (II.7.2.1) were mixed with 0.3 µl of an internal lane standard (MapMarker® 1000, 50 to 1000 bp, x-rhodamine labeled, BioVentures Inc., USA) and 11 µl HiDi™ formamide (Applied Biosystems, Weiterstadt, Germany) and denatured for 3 min at 95°C. The analysis of the digested PCR products was performed by separation using capillary electrophoresis with an automatic sequencer (3130 Genetic Analyzer, Applied Biosystems) for 50 min at 15 kV and 9 µA. The injection time per sample was 6 s. After capillary electrophoresis, the length of the fluorescently labeled T-RF's were identified by comparison to the internal standard using the GeneMapper software (version 4.0, Applied Biosystems). The areas of the measured peaks were used to determine the relative abundances of the terminal restriction fragments.

III. Results

III.1 Carbon isotope fractionation during acetoclastic methanogenesis by *Methanosarcina barkeri* and *Methanosarcina acetivorans*

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Abstract

In methanogenic environments *Methanosarcinaceae* are beside *Methanosaetaceae* the only acetate-consuming family of archaea and thus important contributors to the formation of the greenhouse gases methane and carbon dioxide. In this study, the carbon isotope fractionation during this process was determined for two species of the *Methanosarcinaceae* family, *M. barkeri* and *M. acetivorans*. The calculated isotope enrichment factors (ε) associated with acetoclastic methanogenesis differed slightly compared to literature data for *M. barkeri* where ε_{ac} and ε_{CH4} typically range from –21 and –27‰. Our experiments showed partially stronger fractionation yielding ε_{ac} = –30.5‰ and ε_{CH4} = –27.4‰ for *M. barkeri* and ε_{ac} = –36.9‰ and ε_{CH4} = –23.8‰ for *M. acetivorans*. Since fractionation varied during the catabolism of acetate a new approach is shown which allows to differentiate isotope fractionation at different stages of acetate consumption.

Introduction

Methane (CH_4) is the most abundant organic gas in the earth's atmosphere (Cicerone and Oremland, 1988) and an important greenhouse gas with a high global warming potential (approx. 25 times higher than carbon dioxide; IPCC, 2001). It is suggested that the contribution of CH_4 to the greenhouse effect will even increase in future. This has made it necessary and more urgent to understand natural processes which lead to the production of CH_4.

Methanogenesis, the microbial formation of CH_4, is the final step in the degradation of organic matter in anoxic environments like natural wetlands, lake sediments, and flooded rice fields. The most important precursors for the production of CH_4 are acetate (Eqn. 1) and CO_2 (Eqn. 2) with the following reactions (Conrad, 1989):

$$CH_3COOH \rightarrow CO_2 + CH_4 \quad (1)$$

$$CO_2 + 4H_2 \rightarrow 2H_2O + CH_4 \quad (2)$$

Acetate is the most important substrate since it contributes to microbial methanogenesis to about 70%. In methanogenic environments only two genera of archaea, *Methanosarcina* and *Methanosaeta*, are capable of using acetate (Boone et al., 1993). While *Methanosarcina* can use a wide range of substrates besides acetate like methanol, methylamines, and methylated sulfides, *Methanosaeta* can be considered a specialist who only uses acetate. Among methanogens, *Methanosarcina* also displays the largest environmental diversity. *Methanosarcinaceae* can be found in freshwater sediments and soil, marine habitats, landfills, and animal gastrointestinal tracts (Zinder, 1993).

Additionally, differences between *Methanosarcina* and *Methanosaeta* were found for isotope fractionation of stable carbon. The fractionation factor (α) during acetoclastic methanogenesis in *Methanosarcina* spp. typically ranges from 1.021 to 1.027 (Krzycki et al., 1987; Zyakun et al., 1988; Gelwicks et al., 1994), whereas isotope fractionation in *Methanosaeta* spp. is apparently weaker, ranging between 1.007 for *Methanosaeta thermophila* (Valentine et al., 2004) and 1.010 for *Methanosaeta concilii* (Penning et al., 2006a). It is suggested that the two archaeal genera differ in isotope fractionation due to differences in biochemical activation of acetate to acetyl coenzyme A (Penning et al., 2006a). We found out that even within the genus *Methanosarcina* differences in isotope fractionation occur. We determined isotope ratios of stable carbon for the acetoclastic species *M. barkeri* and *M. acetivorans* which were grown anaerobically under defined conditions. Furthermore, a new approach to determine carbon isotope fractionation during the course of acetate consumption will be discussed.

Material and Methods

Cultures and growth conditions

Methanosarcina barkeri (DSM 804) and *Methanosarcina acetivorans* (DSM 2834) were obtained from the Deutsche Sammlung von Mikroorganismen und Zellkulturen (Braunschweig, Germany). Both species were grown under N_2/CO_2 (80:20) as single cells (Sowers et al., 1993) in HS medium (Metcalf et al., 1996) with 20 mM acetate as electron acceptor. *M. barkeri* and *M. acetivorans* were incubated in glass bottles (500 ml, Ochs, Bovenden-Lenglern, Germany), without shaking, at 30°C and 37°C, respectively. For experiments 10% inocula of cultures in the late exponential phase were transferred resulting in a final volume of 250 ml. Samples from the headspace and the liquid phase were removed to determine pH, concentrations, and the carbon isotope compositions of acetate, methane and carbon dioxide. All experiments were performed in triplicates.

Chemical and isotopic analyses

CH_4 and CO_2 were analyzed by gas chromatography using a flame ionization detector (Shimadzu, Kyoto, Japan). CO_2 was detected after conversion to CH_4 with a methanizer (Ni-catalyst at 350°C, Chrompack, Middelburg, Netherlands).

Stable isotope analysis of $^{13}C/^{12}C$ in gas samples was performed using a gas chromatograph combustion isotope ratio mass spectrometer (GC-C–IRMS) system that was purchased from Finnigan (Thermo Fisher Scientific, Bremen, Germany). The principle operation was described by Brand (1996). The CH_4 and CO_2 in the gas samples (30–400 µl) were first separated in a Hewlett Packard 6890 gas chromatograph using a Pora Plot Q column (27.5 m length, 0.32 mm i.d.; 10 µm film thickness; Chrompack, Frankfurt, Germany) at 30°C and He (99.996% purity; 2.6 ml/min) as carrier gas. After conversion of CH_4 to CO_2 in the Finnigan Standard GC Combustion Interface III the isotope ratio of $^{13}C/^{12}C$ was analyzed in the IRMS (Finnigan MAT Delta^{plus}). The isotope reference gas was CO_2 (99.998% purity; Air Liquide, Düsseldorf, Germany), calibrated with the working standard methylstearate (Merck). The latter was intercalibrated at the Max Planck Institute for Biogeochemistry, Jena, Germany (courtesy of Dr W.A. Brand) against NBS 22 and USGS 24, and reported in the delta notation vs. V-PDB:

$$\delta^{13}C = 10^3 \left(R_{sa} / R_{st} - 1\right) \qquad (3)$$

with R = $^{13}C/^{12}C$ of sample (sa) and standard (st), respectively. The precision of repeated analysis was ± 0.2‰ when 1.3 nmol CH_4 was injected.

Isotopic measurements and quantification of acetate were performed on a HPLC system (Spectra System P1000, Thermo Fisher Scientific, San Jose, CA, USA; Mistral, Spark, Emmen, the Netherlands) equipped with an ion-exclusion column (Aminex HPX-87-H, BioRad, München, Germany) and coupled to Finnigan LC IsoLink (Thermo Fisher Scientific, Bremen, Germany) as described (Krummen et al., 2004). Isotope ratios were detected on an IRMS (Finnigan MAT Delta^{plus} Advantage). Isotope reference gas was CO_2 calibrated as described above.

Off-line pyrolysis was performed to determine $\delta^{13}C$ of the methyl group of acetate ($\delta_{ac\text{-}methyl}$). Acetate in the liquid sample was purified using HPLC by collecting the acetate fraction from each run. The purified sample was added to a strong NaOH solution and dried in a Pyrex tube under vacuum. The dried reactants were pyrolysed under vacuum at 400°C, converting the carboxyl carbon to CO_2 and the methyl carbon to CH_4 (Blair et al., 1985). Gas samples were taken and analyzed by GC-C–IRMS as described above.

The analysis of $\delta^{13}C$ of biomass was carried out at the Centre for Stable Isotope Research & Analysis (KOSI) at Goettingen University, Germany, with an EA-IRMS system consisting of an elemental analyzer (NA 2500, CE Instruments, Rodano, Italy) and an IRMS (Finnigan MAT Delta^{plus}), coupled via an interface (ConFlo III; Thermo Fisher Scientific). The samples and the laboratory reference compound acetanilide were applied as solid samples in tin capsules (IVA, Meerbusch, Germany). The standardisation scheme of the EA-IRMS measurements as well as the measurement strategy and the calculations for assigning the

final $\delta^{13}C$-values on the V-PDB scale were analogous to those described by Werner and Brand (2001) on an elemental-analyzer-IRMS. The precision of repeated analysis was ± 0.18‰ when 0.4–1.5 mg acetanilide was injected.

Radiotracer experiments were done to determine the fraction of CH_4 and CO_2 produced from the methyl group of acetate. For that, 10 µCi/ml (0.37 MBq/ml) of Na-[2-^{14}C]acetate (Amersham, Braunschweig, Germany) were added in a volume of 1.0 ml to 120 ml bottles (Ochs, Bovenden-Lenglern, Germany) which were filled with 50 ml culture liquid. The origin, specific radioactivity and the quantity of the added tracer were 2.1 GBq mmol^{-1} and 3.7 MBq, respectively. The ^{14}C-labeled acetate was added after CH_4 production was observed. Total and radioactive CH_4 and CO_2 were analyzed in a gas chromatograph equipped with a flame ionization detector, reduction column and a RAGA radioactivity detector (Conrad et al., 1989). Total and radioactive acetate were analyzed in the liquid phase in a HPLC system equipped with a refraction index detector and a RAMONA radioactivity detector (Krumböck and Conrad, 1991). The respiratory index (RI) was determined at the end of the incubation after addition of 2.0 ml 2.5 M H_2SO_4 per bottle to liberate CO_2 (CO_2 + bicarbonate):

$$RI = {}^{14}CO_2 / \left({}^{14}CO_2 + {}^{14}CH_4\right) \qquad (4)$$

Calculations

Fractionation factors for a reaction A → B are defined after Hayes (1993) as:

$$\alpha_{A/B} = \left(\delta_A + 1000\right)/\left(\delta_B + 1000\right) \qquad (5)$$

also expressed as $\varepsilon \equiv 10^3 (1 - \alpha)$. The isotope enrichment factor ε associated with acetoclastic methanogenesis was determined as described by Mariotti et al. (1981) from the residual reactant

$$\delta_r = \delta_{ri} + \varepsilon\left[\ln\left(1-f\right)\right] \qquad (6)$$

and from the product formed

$$\delta_p = \delta_{ri} - \varepsilon\left(1-f\right)\left[\ln\left(1-f\right)\right]/f \qquad (7)$$

where δ_{ri} is the isotope composition of the reactant (either ac or ac-methyl) at the beginning, δ_r and δ_p are the isotope compositions of the residual ac and the pooled CH_4, respectively, at the instant when *f* was determined, and *f* is the fractional yield of the products based on the consumption of ac ($0 < f < 1$). Linear regression of δ_r against $\ln(1 - f)$ and of δ_p against $(1 - f)[\ln(1 - f)]/f$ gives ε as the slope of best-fit lines. To analyze ε of the carboxyl group of acetate ($\varepsilon_{ac\text{-}carboxyl}$), values for $\delta_{ac\text{-}carboxyl}$ were calculated using the following equation:

$$\delta_{ac-carboxyl} = 2\delta_{ac} - \delta_{ac-methyl} \qquad (8)$$

Because total oxidized carbon was distributed among different carbon species (gaseous CO_2, dissolved CO_2, HCO_3^-, and CO_3^{2-}), $\delta^{13}C$ of total inorganic carbon (δ_{TIC}) could not be determined directly. This value was calculated by the following mass-balance equation:

$$\delta_{TIC} = X_g\delta_g + X_d\delta_d + X_b\delta_b + X_c\delta_c \qquad (9)$$

where X = mole fraction and δ = isotopic composition of the C of *g* = gaseous CO_2, *d* = dissolved CO_2, *b* = HCO_3^-, and *c* = CO_3^{2-}. The distribution of carbon among these species was calculated using solubility and equilibrium constants (Stumm and Morgan, 1995). δ_g was measured directly, the remaining isotopic compositions were calculated from the relevant equilibrium isotope fractionation factors at 30°C and 37°C (Deines and Langmuir, 1974; Mook et al., 1974):

$$\delta_d = \alpha_{d/g}\delta_g + (\alpha_{d/g} - 1)1000 \quad (10)$$

$$\delta_b = \alpha_{b/g}\delta_g + (\alpha_{b/g} - 1)1000 \quad (11)$$

$$\delta_c = \alpha_{c/g}\delta_g + (\alpha_{c/g} - 1)1000 \quad (12)$$

Results

Methane production by *M. barkeri* was observed after nine days of incubation (Fig. 1A) and started immediately after inoculation in *M. acetivorans* (Fig. 3A). Acetate was consumed completely, leading to an increase of pH. Concentrations of CO_2 are not shown, since the high background level of the used bicarbonate buffer interfered with accurate measurements of CO_2. During the fermentation the preferred consumption of ^{12}C-acetate caused an enrichment of the heavier isotope ^{13}C in the remaining acetate (Figs.1B and 3B).

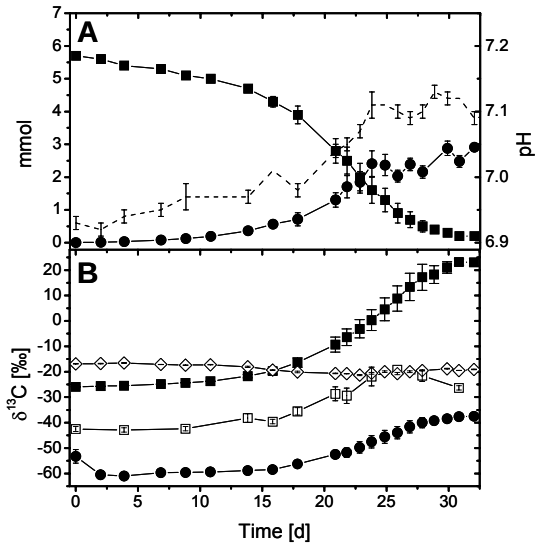

Figure 1: Catabolism of acetate in a pure culture of *Methanosarcina barkeri*. (A) Acetate consumption, CH_4 production, and pH. (B) Isotope signatures of total acetate, ac-methyl, CH_4, and CO_2 (illustrated as TIC, total inorganic carbon). ■, ac; □, ac-methyl; ●, CH_4; ◇ TIC, line without symbols, pH. The values are means ± standard errors (*n* = 3).

Likewise this led to an increased production of ^{13}C-CH_4. The initial high $\delta^{13}C$ value of CH_4 in *M. barkeri* resulted from the transfer of dissolved CH_4 during inoculation. $\delta^{13}C_{CO2}$ (illustrated as TIC, total inorganic carbon) was slightly depleted in ^{13}C with time but was not used for determination of isotope fractionation, since, as mentioned above, the high background of bicarbonate did not allow precise quantification of $\delta^{13}C$ of the newly formed TIC. Carbon isotope fractionation during acetoclastic methanogenesis was determined for total acetate (both carbon atoms), ac-methyl, ac-carboxyl, and CH_4 using equations 6 and 7, based on Rayleigh distillation (Figs. 2 and 4, Table 1).

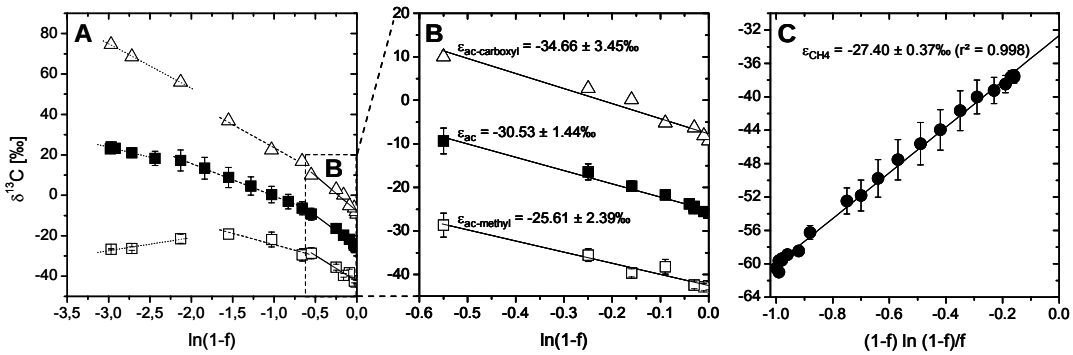

Figure 2: Carbon isotope fractionation during the conversion of acetate to CH_4 by *Methanosarcina barkeri*. Equations derived by Mariotti et al. (1981) have been used to calculate ε from fractional yields and isotope compositions of total acetate ■, ac-methyl □, ac-carboxyl △ (A,B), and CH_4 ● (C). Panel B shows a magnification of the framed segment in A. Isotope enrichment in acetate, ac-methyl, and ac-carboxyl was calculated for three different phases of acetate consumption (A): solid lines show substrate levels between 0 and –0.6 on the ln(1–*f*) scale (corresponding to up to 50% acetate consumption), dashed lines show levels between –0.6 and –1.6 (50 to 80%), and dotted lines levels between –1.6 and –3.0 (from 80% to maximum consumption of acetate). The values are means ± standard errors (*n* = 3).

The isotope enrichment factors for acetate, ac-methyl, and ac-carboxyl were calculated for three different phases of acetate consumption: until 50% of acetate was consumed (ln(1–*f*) values between 0 and –0.6), between 50 and 80% (–0.6 to –1.6), and from 80% to maximum consumption of acetate (–1.6 to –3.0). As carbon isotope fractionation decreased after the first phase, ε-values for the range 0 to –0.6 were finally used to determine and compare isotope fractionation (Figs. 2B and 4B). Isotope fractionation in CH_4 was linear during the complete experiments (Figs. 2C and 4C). As expected, isotope enrichment in ac-methyl ($\varepsilon_{ac\text{-}methyl}$) (Figs. 2B and 4B) and CH_4 (ε_{CH4}) (Figs. 2C and 4C) agreed within error, since the major part of the methyl group of acetate was converted to CH_4 (see below).

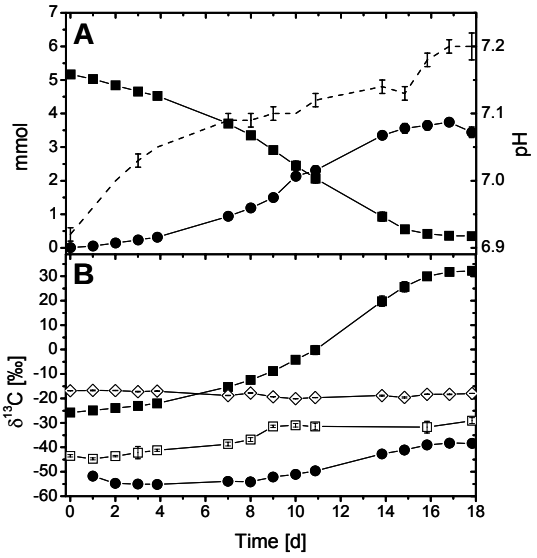

Figure 3: Batch culture of *Methanosarcina acetivorans* growing on 20 mM acetate as sole substrate. (A) Acetate consumption, CH_4 production, and pH. (B) Isotope signatures of total acetate, ac-methyl, CH_4, and CO_2 (illustrated as TIC, total inorganic carbon). ■, ac; □, ac-methyl; ●, CH_4; ◇ TIC, line without symbols, pH. The values are means ± standard errors (*n* = 3).

Isotope signatures of total acetate, ac-methyl, CH_4, and CO_2 observed during the catabolism of acetate in *M. barkeri* and *M. acetivorans* followed similar trends. In both methanogens the continuous preferential consumption of ^{12}C-acetate caused an enrichment of ^{13}C in the remaining acetate and in CH_4, as expected for a closed system. Nevertheless, differences between the two archaeal species occurred in isotope fractionation of stable carbon (Table 1). The fractionation of acetate (ε_{ac}) and ac-carboxyl ($\varepsilon_{ac\text{-}carboxyl}$) was stronger in *M. acetivorans* than in *M. barkeri* by 6.4‰ and 14.0‰, respectively, and lighter for CH_4 (ε_{CH4}) by 3.6‰ during the first phase of acetate consumption (ln(1–*f*) values between 0 and –0.6). Isotope enrichment in ac-methyl ($\varepsilon_{ac\text{-}methyl}$) was identical for both cultures.

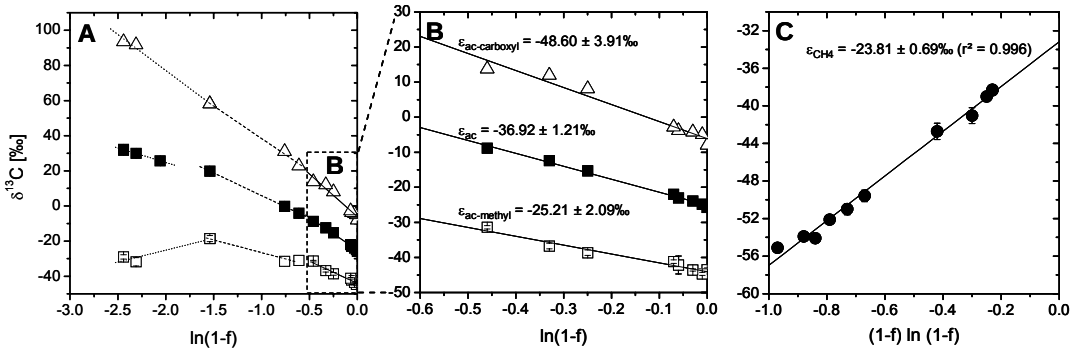

Figure 4: Isotope enrichment during acetoclastic methanogenesis by *Methanosarcina acetivorans*. The plots are based on equations derived by Mariotti et al. (1981). Total acetate ■, ac-methyl □, ac-carboxyl △ (A,B), and CH_4 ● (C). Panel B shows a magnification of the framed segment in A. Isotope enrichment in acetate, ac-methyl, and ac-carboxyl was calculated for three different phases of acetate consumption (A): solid lines show ln(1–*f*) values between 0 and –0.6 (corresponding to up to 50% acetate consumption), dashed lines show values between –0.6 and –1.6 (50 to 80%), and dotted lines values between –1.6 and –3.0 (from 80% to maximum consumption of acetate). The values are means ± standard errors (*n* = 3).

Also $\delta^{13}C$ of the biomass was determined at the end of the experiments and was found to be slightly ^{13}C enriched in both, *M. barkeri* ($\delta^{13}C_{biomass}$ = –19.1 ± 0.1‰) and *M. acetivorans* ($\delta^{13}C_{biomass}$ = –18.5 ± 0.8‰), compared to the initial $\delta^{13}C_{ac}$ values (–25.9 ± 0.1‰ and –25.7 ± 0.3‰, respectively).

Radiotracer experiments with [2-^{14}C]acetate were carried out to determine the fraction of CH_4 and CO_2 produced from the methyl group of acetate. Confirming literature data (Weimer and Zeikus, 1978), incubations with radiolabeled acetate resulted in a production of mostly $^{14}CH_4$ for both *Methanosarcina* strains. Nevertheless, also $^{14}CO_2$ was produced from ac-methyl. For both strains the calculated respiratory index (RI) was 0.11, according to a $^{14}CO_2$ production of 11%.

Table 1: Isotope enrichment factors for acetate, ac-methyl, ac-carboxyl, and CH_4 during acetoclastic methanogenesis by *M. barkeri* and *M. acetivorans* (values are means of triplicates).

Organisms	ln(1 – *f*)	ε_{ac}	$\varepsilon_{ac\text{-}methyl}$	$\varepsilon_{ac\text{-}carboxyl}$ *	ε_{CH4} †
M. barkeri	0 < *f* < -0.6	-30.53 ± 1.44‰	-25.61 ± 2.39‰	-34.66 ± 3.45‰	-27.40 ± 0.37‰
	-0.6 < *f* < -1.6	-16.08 ± 0.35‰	-11.10 ± 4.25‰	-22.90 ± 3.29‰	
	-1.6 < *f* < -3.0	-9.86 ± 0.92‰	6.46 ± 1.60‰	-21.99 ± 0.64‰	
M. acetivorans	0 < *f* < -0.6	-36.92 ± 1.21‰	-25.21 ± 2.09‰	-48.60 ± 3.91‰	-23.81 ± 0.69‰
	-0.6 < *f* < -1.6	-25.70 ± 0.09‰	-14.40 ± 2.66‰	-37.00 ± 2.83‰	
	-1.6 < *f* < -3.0	-16.59 ± 0.78‰	13.56 ± 4.64‰	-40.77 ± 3.54‰	

* δac-carboxyl = 2δac – δac-methyl

† Mariotti plots were linear during acetate consumption

Discussion

Carbon isotope fractionation during acetoclastic methanogenesis by *Methanosarcina barkeri*

Our results for isotope fractionation of stable carbon by *Methanosarcina* spp. agree with previous data (Krzycki et al., 1987; Zyakun et al., 1988; Gelwicks et al., 1994), ranging between α = 1.021 and 1.027, equivalent to ε-values between –21 and –27‰. However, our isotopic data during consumption of acetate by *Methanosarcina barkeri* yield ε_{ac} = –30.5‰ and $\varepsilon_{ac\text{-}methyl}$ = –25.2‰ and therewith differ from the data presented by Krzycki et al. (1987) and Gelwicks et al. (1994), who calculated ε_{ac}-values between –23.1 and –24.5‰ and $\varepsilon_{ac\text{-}methyl}$-values between –21.2 and –24.0‰. These differences can be related e.g., to the dissimilar strains that were used in each study. Compared to *M. barkeri* strain Fusaro in this study (DSM 804), Krzycki et al. (1987) worked with *M. barkeri* MS (DSM 800) and Gelwicks et al. (1994) with *M. barkeri* 228 (DSM 1538). These dissimilar strains may express different carbon isotope fractionation. Nevertheless, there is another aspect which could explain the differences of our results compared to previous. As the work of Krzycki et al. (1987) was based on initial and endpoint measurements, the authors were not able to determine fractionation factors during the course of acetate consumption. Also the determinations of ε by Gelwicks et al. (1994) depended only on few data points. Consequently, it was not possible to differentiate fractionation in ac and ac-methyl between varying stages of acetate consumption. We divided these data points into three sections (Figs. 2A and 4A) because strong changes in isotope enrichment occurred during the degradation process. Fractionation factors near the end of the incubation were smaller than the corresponding fractionation determined using data collected earlier in the experiment. These discontinuities, particularly at substrate levels below –2.0 on the ln(1 – *f*) scale, were observed recently (Kinnaman et al., 2007). A potential cause for this variable isotope fractionation could be transport limitation. This situation occurs when a cell changes from a bi-directional substrate flux (e.g. substrate goes into and out of the cell because of biochemical activation) to a uni-directional substrate flux (e.g. substrate entering the cell is consumed only). In the latter case the only fractionation expressed is during substrate transport into the cell, whereas in bi-directional flow concentrations and isotopic compositions of the substrate are also affected by the fractionation expressed during transport out of the cell. As the isotope fractionation associated with the biological process only appears during bi-directional fluxes, we suggest using the early stages of acetate consumption to determine isotope fractionation.

However, isotope fractionation only varied in acetate and not in methane. We think that these discontinuities were not observed in methane because by measuring $\delta^{13}C_{CH4}$ at low substrate concentrations the isotopic background of the pooled CH_4 at that time was very high compared to $\delta^{13}C$ of the instantaneously-forming CH_4. Hence, determinations of $\delta^{13}C_{CH4}$ were only sensitive during early stages of acetate consumption and the newly-formed CH_4 was not perceived as the degradation proceeded.

Differences in isotope fractionation within the genus *Methanosarcina*

Previous studies have shown that differences in carbon isotope fractionation between two acetoclastic genera, *Methanosarcina* and *Methanosaeta*, occur (for a review see Conrad, 2005). In this study we even observed differences within the genus *Methanosarcina*. While the enrichment factors for ac-methyl in *M. barkeri* and *M. acetivorans* (–25.61‰ and –25.21‰, respectively) were nearly identical, the values for ε_{ac} (–30.53‰ and –36.92‰) and ε_{CH4} (–27.40‰ and –23.81‰) disagreed to some extent. During the acetoclastic pathway both methanogens use the same enzymes for acetate activation, acetate kinase and phosphotransacetylase, but nevertheless two differences during the utilization of acetate were reported which may explain the dissimilar fractionation. Guss et al. (2005) reported that the Ech hydrogenase is essential for the growth on acetate in *M. barkeri*. Interestingly, this enzyme can not be found in *M. acetivorans*. Instead it is suggested that this archaeon connects ferredoxin with methanophenazine to conserve energy. These biochemical differences might cause a different isotope fractionation. Furthermore it has been observed that *M. barkeri* lacks genes encoding an acetyl-coenzyme A (CoA) synthetase that is found in *M. acetivorans* (Maeder et al., 2006). Consequentially, it can not be ruled out that in *M. acetivorans* other pathways for the conversion of acetate are involved which might also express isotope fractionation. A possible exchange reaction between ac-methyl and CO_2 can not explain this dissimilar fractionation because our radiotracer experiments with [2-^{14}C]acetate showed that *M. barkeri* and *M. acetivorans* perform this reaction to the same extent.

As expected for a closed system, the isotopic composition of the pooled product ($\delta^{13}C_{CH4}$) at completion almost agreed with the initial substrate ($\delta^{13}C_{ac\text{-}methyl}$) in both *Methanosarcina* spp. (Figs. 1B and 3B). Minor deviations could be theoretically caused by assimilation of acetate because a branching of the carbon flow occurs, when acetate is converted into biomass instead of CH_4. In our experiments the $\delta^{13}C$ of the biomass was slightly enriched in ^{13}C compared to the initial $\delta^{13}C_{ac}$ for both methanogens ($\delta^{13}C_{biomass}$ = –18.5 to –19.0‰). Consequently, this might have resulted in a slightly stronger depletion of ^{13}C in CH_4 than in acetate, which was not the case in our experiments. However, because of the relatively low level of biomass formation in anaerobic metabolism, we assume that assimilation had no significant influence on fractionation.

Different fractionation in ac-methyl and total acetate

Results of the experiments with both methanogens indicate a stronger fractionation of ^{13}C in total acetate than at methyl carbon at all phases of acetate consumption, as observed previously in *M. barkeri* (Gelwicks et al., 1994) and *Methanosaeta concilii* (Penning et al., 2006a). A theoretical explanation for the greater signification of ac-carboxyl could be a reversible exchange of the carbonyl carbon of acetyl-CoA with the ^{13}C-enriched CO_2 in the growth medium, a reaction which was observed in *M. barkeri* (Fischer and Thauer, 1990). However, if we consider such an exchange, with the example of the experiment with

M. barkeri, using a $\delta^{13}C_{ac\text{-}carboxyl}$ of –9.3‰ of the initial acetate and a $\delta^{13}C_{CO2}$ value of –20.8‰ (equivalent to $\delta^{13}C_{TIC}$ = –16.9‰, Fig. 1B), we would expect ^{13}C depletion leading to a weaker fractionation in $\delta^{13}C_{ac\text{-}carboxyl}$, which was not the case. Thus, we suppose that in both methanogens, *M. barkeri* and *M. acetivorans*, this exchange reaction was catalyzed to only a minor extend. Therefore, we suggest alternative explanations. For instance, depletion of ^{13}C in the carbonyl carbon of acetaldehyde, which has been shown by DeNiro and Epstein (1977), might cause heavier, residual acetate. As suggested by Zyakun (1996), the carbon isotope fractionation of the carbonyl carbon of acetate can be expressed during the bonding of acetate and HS-CoA. Thus, the carbonyl group of the resulting H_3C-CoS-CoA would be depleted in ^{13}C relative to the carboxyl group of the residual acetate.

Another explanation can be found in the acetyl-CoA pathway which is used for cleavage of acetate by methanogenic archaea (Fischer and Thauer, 1988; Grahame, 1991; Ferry, 1992). The main reaction during this pathway involves the multienzyme CO dehydrogenase/acetyl-CoA synthase complex, which catalyzes cleavage of acetyl-CoA, the oxidation of the carbonyl group to CO_2, and transfer of the methyl group of acetate to an acceptor to finally be transferred to CH_3-S-CoM (Grahame, 1991; Ferry, 1992). In agreement with Gelwicks et al. (1994) we assume that this multienzyme complex is the rate-limiting step of the overall fractionation and therefore is responsible for the observed fractionation. As stable carbon isotope fractionation during methanogenesis from acetate indicate that fractionation occurs prior to the formation of methyl-CoM (Krzycki et al., 1987), this argues for our assumption.

Conclusions

The calculated fractionation factors we observed during consumption of acetate by *M. barkeri* and *M. acetivorans* ranged at the more negative end of previous literature data which showed ε-values between –21 and –27‰. Thus, the differences between the two acetoclastic genera *Methanosarcina* and *Methanosaeta* are even bigger than observed before. Our data strengthen the assumption that under methanogenic conditions ε_{ac}-values of ≥ –20‰ indicate a predominant abundance of *Methanosarcinaceae*. Consequently, the determination of ε_{ac} can help to give a prediction which methanogenic genus is active. By selectively inhibition of acetoclastic methanogenesis using methyl fluoride (Conrad and Klose, 2000) this approach can also be used in environmental systems to determine ε_{CH4} produced from ac-methyl. For this purpose, the measured values of CH_4 produced from acetate are subtracted from CH_4-values resulting from both, acetoclastic and hydrogenotrophic methanogenesis to finally get the amount of CH_4 produced from H_2/CO_2 (Eqn. 2). These calculations have to be done since no selectively inhibitor of hydrogenotrophic methanogenesis is known.

In case of variable fractionation, we suggest using the first stage of substrate consumption (e.g., until 50% substrate has been consumed) to determine isotope

fractionation. Our data indicate that the isotope fractionation associated with the biological process only appears during this period where transport limitation is negligible. Furthermore we recommend that ε_{CH4} is more suitable to follow isotope fractionation during CH_4 production than ε_{ac} or $\varepsilon_{ac\text{-}methyl}$ because CH_4 fractionates more continuously than acetate. This is because transport limitation only affects the fractionation of substrates due to biochemical activation. Also the obvious differences between the fractionation of total acetate and the methyl or carboxyl group of acetate illustrate the disadvantages of using acetate as a criterion for CH_4 production compared to CH_4.

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III.2 Carbon isotope fractionation by sulfate-reducing bacteria using different pathways for the oxidation of acetate

(submitted to Environmental Science & Technology)

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Abstract

Acetate is a key intermediate in the anaerobic degradation of organic matter. In anoxic environments, available acetate is a competitive substrate for sulfate-reducing bacteria (SRB) and methane-producing archaea. Little is known about the fractionation of stable carbon by sulfate reducers. Therefore, we determined stable carbon isotope signatures in cultures of three acetate-utilizing SRB, *Desulfobacter postgatei*, *Desulfobacter hydrogenophilus*, and *Desulfobacca acetoxidans*. We found that these species showed strong differences in their isotope enrichment factors (ε) of acetate. During the consumption of acetate and sulfate, acetate was enriched in ^{13}C by 19.3‰ in *Desulfobacca acetoxidans*. By contrast, both *D. postgatei and D. hydrogenophilus* showed a slight depletion of ^{13}C. We suggest that the different isotope fractionation is due to the different metabolic pathways for acetate oxidation. The strongly fractionating *Desulfobacca acetoxidans* uses the acetyl-CoA/carbon monoxide dehydrogenase pathway, which is also used by acetoclastic methanogens that show a similar fractionation of acetate. In contrast, *Desulfobacter* spp. oxidize acetate to CO_2 via the tricarboxylic acid (TCA) cycle, which apparently does not discriminate against ^{13}C. Our results suggest that carbon isotope fractionation in environments with sulfate reduction will strongly depend on the composition of the sulfate-reducing bacterial community oxidizing acetate.

Introduction

Dissimilatory sulfate reduction plays a major role in the sulfur cycle in nature. The global emission of sulfur reached a maximum of 74 Tg a^{-1} in 1989 (Stern, 2005) with sulfate reduction being the main contributor (approx. 25%). Sulfate-reducing bacteria (SRB) are widespread in marine and terrestrial aquatic environments. They can be found in flooded soils such as rice paddies and technical aqueous systems like sludge digesters and oil tanks. Their ability to adapt to extreme physical and chemical conditions enables them to play an important role in global geochemical cycles (Jorgensen et al., 1990). Dissimilatory sulfate reducers use sulfate mainly as an electron acceptor during the anaerobic oxidation of

inorganic or organic substrates like H_2, acetate, lactate, and propionate. Former scientists doubted for a long time that sulfate reducers were able to oxidize acetate until Widdel and Pfennig isolated the first acetate-oxidizing SRB, *Desulfotomaculum acetoxidans* and *Desulfobacter postgatei* (1977; 1981). In the nineties, many pure cultures of SRB were isolated that could completely oxidize various substrates like alkanes (Aeckersberg et al., 1991; Coates et al., 1997; Caldwell et al., 1998), toluene (Rabus et al., 1993; Beller et al., 1996), xylenes (Harms et al., 1999), or naphthalene (Galushko et al., 1999) . Furthermore, it was demonstrated that SRB could grow with crude oil as substrate helping to explain sulfide production in oil reservoirs and oil production plants (Rueter et al., 1994).

We were interested in sulfate reduction, since in anoxic environments SRB compete with acetoclastic methanogens for available acetate (Blair and Carter, 1992). Sulfate reducers are believed to successfully compete with methanogens because of their higher affinity for acetate (e.g. Schoenheit et al., 1982). But while in the last few years much research has been done on isotope fractionation by methanogens (for review see reference Conrad, 2005), still very little is known about the fractionation of stable carbon by sulfate reducers. Generally, stable carbon isotope signatures can be used to quantify biochemical pathways if isotope signatures and fractionation factors of the involved substrates and products are known. To our knowledge, the only study on carbon isotope fractionation by SRB was published by Londry and Des Marais who investigated carbon isotope discrimination during heterotrophic and lithotrophic growth of SRB (2003). However, their work was based on initial and endpoint measurements and did not determine isotope fractionation during the course of acetate consumption. Since fractionation of stable isotopes can vary during the degradation processes (Kinnaman et al., 2007, this study), we followed the isotopic signature in the acetate as it was consumed and thus were able to determine fractionation factors.

In this study we focused on the question whether sulfate reducers show a different carbon isotope fractionation of acetate when they have different metabolic pathways for the acetate oxidation. Therefore, we determined isotope ratios of stable carbon for three acetate-utilizing SRB, *Desulfobacter postgatei*, *Desulfobacter hydrogenophilus*, and *Desulfobacca acetoxidans*. The *Desulfobacter* spp. oxidize acetate via the tricarboxylic acid (TCA) cycle (Gebhardt et al., 1983; Brandis-Heep et al., 1983), whereas *Desulfobacca acetoxidans* uses the acetyl-CoA/carbon monoxide dehydrogenase pathway (Oude Elferink et al., 1999). We found a very strong difference in the isotope fractionation depending on the pathway for acetate oxidation. This result implies that the community composition of SRB may have a strong effect on carbon isotope values in environments with sulfate reduction.

Materials and methods

Cultures and Growth Conditions

The following pure cultures were used in this study: *Desulfobacter postgatei* strain 2ac9 (DSM 2034), *Desulfobacter hydrogenophilus* strain AcRS1 (DSM 3380), and *Desulfobacca acetoxidans* strain ASRB2 (DSM 11109) were obtained from the Deutsche Sammlung von Mikroorganismen und Zellkulturen (Braunschweig, Germany). All species were grown in bicarbonate-buffered mineral medium under N_2/CO_2 (80:20). The composition of the medium used for the *Desulfobacter* strains was (in g/l unless otherwise noted): KH_2PO_4, 0.68; NH_4Cl, 1.0; NaCl, 23.3; $MgCl_2 \cdot 6H_2O$, 11.0; KCl, 1.0; $CaCl_2 \cdot 2H_2O$, 0.3; $NaHCO_3$, 3.78; cysteine-$HCl \cdot H_2O$, 0.5; $Na_2S \cdot 9H_2O$, 0.4; trace element solution, 0.5 ml (Wolin et al., 1963); trace element solution SL-10, 0.5 ml (Chin et al., 1998); vitamin solution, 1 ml (Wolin et al., 1963); and resazurine at 0.1% (wt/vol), 1 ml. For growth of *Desulfobacca acetoxidans* the medium described by Stams et al. (1993) was used which contained the following (in g/l unless otherwise noted): $Na_2HPO_4 \cdot 2H_2O$, 0.53; KH_2PO_4, 0.41; NH_4Cl, 0.3; $CaCl_2 \cdot 2H_2O$, 0.11; $MgCl_2 \cdot 6H_2O$, 0.10; NaCl, 0.3; $NaHCO_3$, 4.0; $Na_2S \cdot 9H_2O$, 0.48; acid and alkaline trace elements (each, 1 ml); vitamin solution, 1 ml (Wolin et al., 1963); and resazurine at 0.1% (wt/vol), 0.5 ml. The acid trace element solution was composed of the following (in mM): $FeCl_2$, 7.5; H_3BO_4, 1; $ZnCl_2$, 0.5; $CuCl_2$, 0.1; $MnCl_2$, 0.5; $CoCl_2$, 0.5; $NiCl_2$ 0.1; and HCl, 50. The alkaline trace element solution contained the following (in mM): Na_2SeO_3, 0.1; Na_2WO_4, 0.1; Na_2MoO_4, 0.1; and NaOH, 10. The vitamin solution had the following composition (in g/l): biotin, 0.02; niacin, 0.2; pyridoxine, 0.5; riboflavin, 0.1; thiamine, 0.2; cyanocobalamin, 0.1; p-aminobenzoic acid, 0.1; and pantothenic acid, 0.1. All microorganisms were grown with 20 mM acetate as electron donor and 20 mM sulfate as electron acceptor. The cultures (250 ml) were incubated in glass bottles (500 ml, Ochs, Bovenden-Lenglern, Germany) without shaking. Experiments with *Desulfobacter* spp. were inoculated with 10% of a culture in the late exponential phase and incubated at 30°C. *Desulfobacca acetoxidans* was grown at 37°C using 1% inoculum. Several samples from the headspace and the liquid phase were removed to determine pH, concentration of acetate, sulfate, sulfide, carbon dioxide, and carbon isotope composition of acetate and carbon dioxide. All experiments were performed in triplicates.

Chemical and Isotopic Analyses

Sulfate was analyzed by ion chromatography with an IC system, a LCA A14 column, and a S3111 conductivity detector (all from Sykam, Fuerstenfeldbruck, Germany). The eluant was 5 mM Na_2CO_3 plus 1 ml/l modifier (1 g 4-hydroxybenzonitrile in 50 ml methanol), with a flow rate of 1.5 ml/min. Sulfide was determined photometrically after reaction with $CuSO_4$ to form CuS as described by Cord-Ruwisch (1985). CO_2 was analyzed by gas chromatography using a flame ionization detector (Shimadzu, Kyoto, Japan) after conversion to CH_4 with a methanizer (Ni-catalyst at 350°C, Chrompack, Middelburg, Netherlands).

Stable isotope analysis of $^{13}C/^{12}C$ in gas samples was performed using a gas chromatograph combustion isotope ratio mass spectrometer (GC-C–IRMS) system that was purchased from Finnigan (Thermo Fisher Scientific, Bremen, Germany). The principle operation was described by Brand (1996). The CO_2 in the gas samples (30–400 µl) was first separated in a Hewlett Packard 6890 gas chromatograph using a Pora Plot Q column (27.5 m length, 0.32 mm i.d.; 10 µm film thickness; Chrompack, Frankfurt, Germany) at 30°C and He (99.996% purity; 2.6 ml/min) as carrier gas. Subsequent, the isotope ratio of $^{13}C/^{12}C$ was analyzed in the IRMS (Finnigan MAT Delta^{plus}). The isotope reference gas was CO_2 (99.998% purity; Air Liquide, Duesseldorf, Germany), calibrated with the working standard methylstearate (Merck). The latter was intercalibrated at the Max Planck Institute for Biogeochemistry, Jena, Germany (courtesy of Dr W.A. Brand) against NBS 22 and USGS 24, and reported in the delta notation vs. V-PDB:

$$\delta^{13}C = 10^3 \left(R_{sa} / R_{st} - 1\right) \quad (1)$$

with R = $^{13}C/^{12}C$ of sample (sa) and standard (st), respectively.

Isotopic measurements and quantification of acetate were performed on a HPLC system (Spectra System P1000, Thermo Fisher Scientific, San Jose, CA, USA; Mistral, Spark, Emmen, the Netherlands) equipped with an ion-exclusion column (Aminex HPX-87-H, BioRad, Muenchen, Germany) and coupled to Finnigan LC IsoLink (Thermo Fisher Scientific, Bremen, Germany) as described (Krummen et al., 2004). Isotope ratios were detected on an IRMS (Finnigan MAT Delta^{plus} Advantage). Isotope reference gas was CO_2 calibrated as described above.

Calculations

Fractionation factors for a reaction A → B are defined after Hayes (1993) as:

$$\alpha_{A/B} = \left(\delta_A + 1000\right)/\left(\delta_B + 1000\right) \quad (2)$$

also expressed as $\varepsilon \equiv 10^3 (1 - \alpha)$. The isotope enrichment factor ε associated with acetate oxidation was determined as described by Mariotti et al. (1981) from the residual reactant

$$\delta_r = \delta_{ri} + \varepsilon\left[\ln(1-f)\right] \quad (3)$$

where δ_{ri} is the isotope composition of the reactant (acetate) at the beginning, δ_r is the isotope composition of the residual acetate, and *f* is the fractional yield of the product based on the consumption of acetate ($0 < f < 1$). Linear regression of δ_r against $\ln(1 - f)$ gives ε as the slope of best-fit lines.

Because total oxidized carbon was distributed among different carbon species (gaseous CO_2, dissolved CO_2, HCO_3^-, and CO_3^{2-}), $\delta^{13}C$ of total inorganic carbon (δ_{TIC}) could not be determined directly. This value was calculated by the following mass-balance equation:

$$\delta_{TIC} = X_g\delta_g + X_d\delta_d + X_b\delta_b + X_c\delta_c \quad (4)$$

where X = mole fraction and δ = isotopic composition of the C of *g* = gaseous CO_2, *d* = dissolved CO_2, *b* = HCO_3^-, and *c* = CO_3^{2-}. The distribution of carbon among these species was calculated using solubility and equilibrium constants (Stumm and Morgan, 1995). δ_g was

measured directly, the remaining isotopic compositions were calculated from the relevant equilibrium isotope fractionation factors at 30°C and 37°C (Deines and Langmuir, 1974; Mook et al., 1974):

$$\delta_d = \alpha_{d/g}\delta_g + (\alpha_{d/g} - 1)1000 \quad (5)$$

$$\delta_b = \alpha_{b/g}\delta_g + (\alpha_{b/g} - 1)1000 \quad (6)$$

$$\delta_c = \alpha_{c/g}\delta_g + (\alpha_{c/g} - 1)1000 \quad (7)$$

Results and discussion

Acetate oxidation via the acetyl-CoA/carbon monoxide dehydrogenase pathway

Desulfobacca acetoxidans was grown to study carbon isotope fractionation during acetate oxidation via the acetyl-CoA/CODH pathway. The substance conversions followed the known stoichiometry (Thauer et al., 1989):

$$CH_3COO^- + SO_4^{2-} + 3H^+ \rightarrow 2CO_2 + H_2S + 2H_2O \quad (8)$$

At the end of the experiments, acetate was consumed below the detection limit of approx. 20 µM, leading to an increase of pH and production of sulfide (Figure 1A). Concentrations of the second product, CO_2, are not shown, since the high background level of the used bicarbonate-buffered medium interfered with accurate measurement of CO_2. During sulfate reduction the preferred consumption of the lighter isotope of acetate (^{12}C) caused an enrichment of the heavier isotope (^{13}C) in the remaining acetate (Figure 1B). $\delta^{13}C_{CO2}$ (illustrated as TIC, total inorganic carbon) was low at first and later, starting after 60 days of incubation, increased. However, $\delta^{13}C_{CO2}$ was not used for determination of isotope fractionation, since, as mentioned above, the high background of bicarbonate did not allow precise quantification of $\delta^{13}C$ of the newly formed TIC. The carbon isotope enrichment factor for acetate (ε_{ac}) was calculated from the temporal change of $\delta^{13}C$ of acetate using equation 3 (Figure 1C). Data points at substrate levels below –2.0 on the ln(1 – *f*) scale (i.e., 86% of acetate consumed) were not used for the calculation of ε since acetate might have become limiting and thus no longer being fractionated during consumption. Indeed, a strong change in isotope enrichment occurred during this growth phase in most of our experiments. Such a discontinuity was observed before (Kinnaman et al., 2007) and may result from low substrate concentrations at this phase. The average value of isotope fractionation of three experiments of acetate consumption amounted to ε_{ac} = –19.30 ± 0.30‰. Literature data on acetoclastic methanogens from the genus *Methanosarcina*, which also use the acetyl-CoA pathway, show a similar fractionation. During the methanogenic conversion of acetate fractionation factors (α) typically range from 1.021 to 1.027 (Krzycki et al., 1987; Zyakun et al., 1988; Gelwicks et al., 1994), equivalent to ε-values between –21 and –27‰. The similarity of ε-values between acetotrophic methanogens and *D. acetoxidans* indicates that microorganisms that are using the same biochemical pathway have similar isotope

fractionation. To investigate this further for sulfate reduction, ε_{ac} was also determined for SRB which use a different pathway for the oxidation of acetate, the TCA cycle.

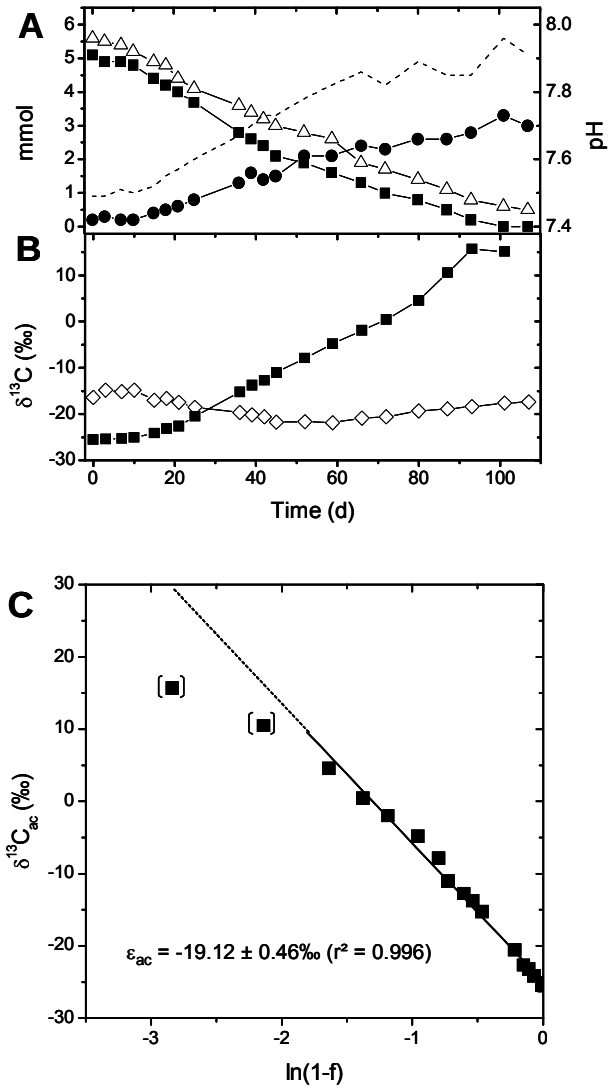

Figure 1: Acetate oxidation by *Desulfobacca acetoxidans* using the acetyl-CoA/CODH pathway. (A) Acetate (■) and sulfate (△) consumption, H_2S production (●), and pH (dashed line without symbols). (B) Isotope signatures of acetate (■) and CO_2 (◇, illustrated as TIC, total inorganic carbon). (C) The plot is based on equation (3); data points in parentheses were not used for the calculation of ε; regression lines drawn beyond used data points are dotted. The results of one out of three different experiments are shown. The equation within the graph expresses ε_{ac} of this experiment ± the standard error of the regression line.

Acetate oxidation via the TCA cycle

Two *Desulfobacter* species, *D. postgatei* and *D. hydrogenophilus*, were grown to determine the fractionation of stable carbon during the anaerobic degradation of acetate via the TCA cycle. Both microorganisms started growth directly after inoculation and consumed acetate completely (Figures 2A and 2D), showing the expected stoichiometry of acetate and

sulfate consumption at a ratio of 1:1. Different to *Desulfobacca acetoxidans*, both *Desulfobacter* species showed a depletion of ^{13}C in the residual acetate (Figures 2B and 2E) indicating a preferred consumption of the heavier ^{13}C-acetate. This is very interesting as, from a closed system approach, the opposite ratio of isotopes would have been expected if a kinetic isotope effect (KIE) was present. However, the observed isotopic composition required an inverse fractionation and positive fractionation factors, which were very similar for *D. postgatei* and *D. hydrogenophilus* (Figures 2C and 2F). In an inverse reaction, the heavy isotope reacts faster than the light isotope, in apparent violation of the laws of kinetic isotope effects. Consequently, the product of oxidation, acetate, is enriched in ^{13}C rather than depleted.

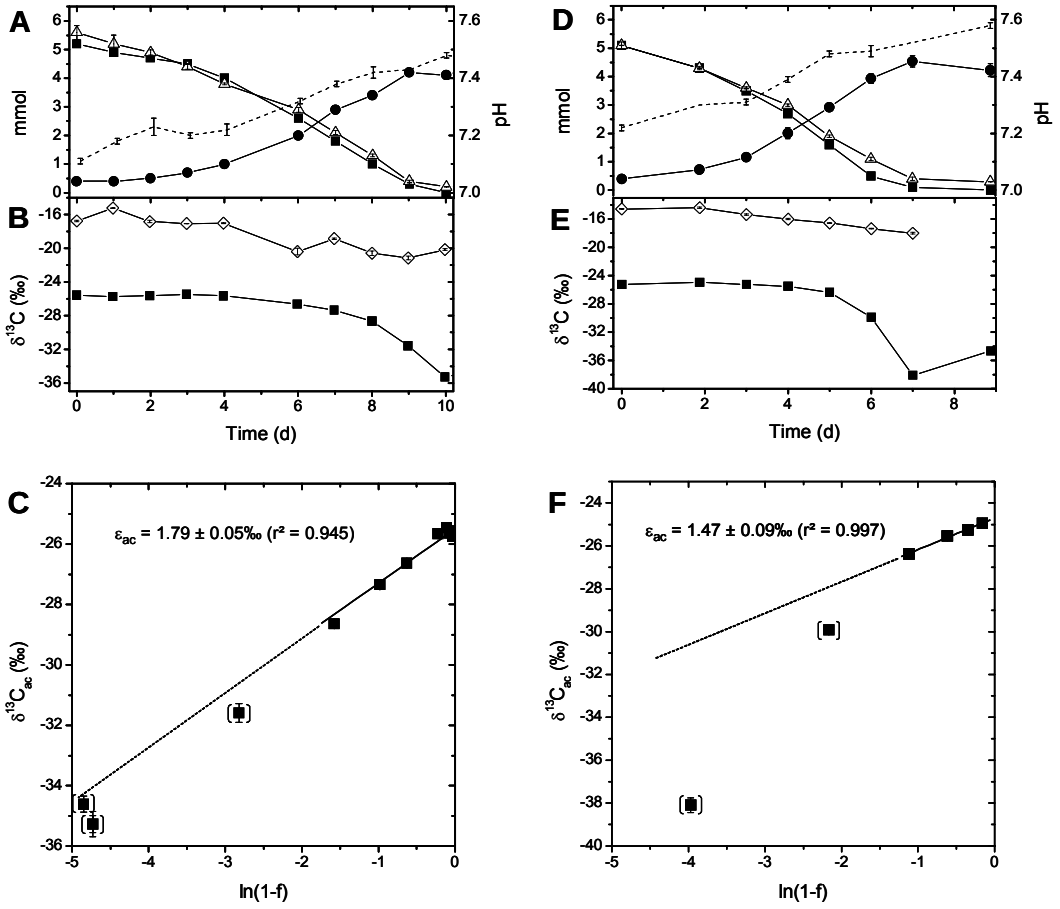

Figure 2: Acetate oxidation by sulfate-reducing bacteria using the tricarboxylic acid cycle. The figure shows substance conversions and isotope fractionations during the catabolism of acetate in pure cultures of *Desulfobacter postgatei* (A-C) and *Desulfobacter hydrogenophilus* (D-F). (A and D) Acetate (■) and sulfate (△) consumption, H_2S production (●), and pH (dashed line without symbols). (B and E) Isotope signatures of acetate (■) and CO_2 (◇, illustrated as TIC, total inorganic carbon). (C and F) The plots are based on equation (3); data points in parentheses were not used for the calculation of ε; regression lines drawn beyond used data points are dotted. The values are means ± standard errors (*n* = 3). If no error bars are shown, the standard errors were too small to be illustrated. Equations within the graph express ε_{ac} as the slope of linear regression ± the standard error of the regression lines.

We suggest that the carbon isotope fractionation of acetate during the oxidation via the TCA cycle was mainly expressed by a small equilibrium isotope effect (EIE) rather than a KIE. For EIE the highest abundance of the heavy isotope is usually found in the compound having the largest molecular mass. With respect to acetate, we would expect to find relatively more ^{13}C in form of undissociated acetic acid rather than acetate. If we assume that acetate is taken up by the cells as acetic acid, the heavier isotope would be preferentially consumed according to the EIE between acetate and acetic acid. This would result in a depletion of ^{13}C in the remaining acetate, as observed, and hence explain the inverse fractionation of acetate in sulfate reducers. This interpretation assumes that acetate is primarily transported into the cells as acetic acid, which is reasonable, since the protonated form is freely diffusible through the cell membrane. The interpretation further assumes that the KIE during acetate degradation by the TCA cycle is close to zero.

In fact, transport of undissociated acetic acid through the cell membrane seems to be common in microorganisms (Fleit, 1995), and has, for example, also been demonstrated for *Desulfovibrio vulgaris* (Varma et al., 1983) and acetoclastic methanogens (Fukuzaki et al., 1990). Hence, the EIE of about 1–2‰ observed in the *Desulfobacter* spp. may be effective in both acetotrophic SRB and acetoclastic methanogens that use the acetyl-CoA pathway, so that the KIE during acetate consumption in these microorganisms may be larger than observed.

To summarize our findings, fractionation factors during the anaerobic oxidation of acetate significantly differed between SRB using the acetyl-CoA pathway and bacteria using the TCA cycle. The sulfate reducer *Desulfobacca acetoxidans* which uses the acetyl-CoA pathway showed a similar fractionation as acetoclastic methanogens using the same pathway. In contrast, two *Desulfobacter* strains using the TCA cycle yielded an inverse fractionation of acetate which, to our knowledge, has not been observed before. We conclude that an EIE during protonation of acetate and the uptake of acetic acid cause this unusual fractionation. Our data agree with Preuss et al. (1989) who studied carbon isotope fractionation by autotrophic bacteria. They found that during CO_2 fixation bacteria using the acetyl-CoA pathway also had a stronger fractionation ($\Delta\delta^{13}C$ = –36‰) than bacteria using the TCA cycle ($\Delta\delta^{13}C$ = –10‰).

In conclusion, if different pathways for the oxidation of acetate result in different carbon isotope fractionation, isotopic data may be used as indication for which acetate oxidation pathway has been operative. Our results also indicate that the carbon isotope fractionation of acetate in environments with sulfate reduction strongly depends on the composition of the sulfate-reducing bacterial community oxidizing acetate.

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III.3 Stable carbon isotope fractionation by acetotrophic sulfur reducers

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Abstract — The carbon isotope effects associated with the oxidation of acetate were examined for *Desulfuromonas acetoxidans* and *Desulfurella acetivorans*. It has been found that the discrimination against ^{13}C in acetate differed to about 6‰. It is suggested that the two sulfur-reducing bacteria differ in isotope fractionation because they have different mechanisms for the activation of acetate. Hence, it may be possible to use isotope effects of acetate (ε_{ac}) to determine the first biochemical step during acetate oxidation.

Acetate is a key intermediate during the anaerobic degradation of organic matter. It can be used by many different groups of microorganisms. E.g., about 70% of biological methanogenesis results from acetate consumption and some sulfate-reducing bacteria utilize acetate as sole electron donor. Also few sulfur-reducing bacteria are capable of oxidizing acetate by coupled reduction of elemental sulfur to sulfide. Two main pathways for the oxidation of acetate by anaerobic organisms are known: the acetyl-CoA/carbon monoxide dehydrogenase pathway and the tricarboxylic acid (TCA) cycle. Recently, it was observed that sulfate reducers show differences in the fractionation of stable carbon when they have different pathways for acetate oxidation (Goevert and Conrad, 2008). However, until now all isolated sulfur reducers which were found to be capable of utilizing acetate use the TCA cycle (Pfennig and Biebl, 1976; Bonch-Osmolovskaya et al., 1990; Galushko and Schink, 2000) for the following reaction:

$$CH_3COO^- + H^+ + 4S^0 + 2H_2O \rightarrow 2CO_2 + 4H_2S \quad (1)$$

Nevertheless, it was found that sulfur-reducing bacteria differ in their biochemical activation of acetate. *Desulfuromonas acetoxidans* activates acetate to acetyl-CoA via CoA transfer from succinyl-CoA (Gebhardt et al., 1985)

$$\text{acetate} + \text{succinyl-CoA} \rightarrow \text{acetyl-CoA} + \text{succinate} \quad (2)$$

catalyzed by a succinyl-CoA:acetate-CoA-transferase. Whereas for *Desulfurella acetivorans* the following reaction was reported (Schmitz et al., 1990):

$$\text{acetate} + \text{ATP} \rightarrow \text{acetyl-phosphate} + \text{ADP} \quad (3)$$

where acetate is activated by two enzymes, acetate kinase and phosphate acetyltransferase. Interestingly, in *Geobacter sulfurreducens* high activities of both enzyme systems, succinyl-CoA:acetate-CoA-transferase and acetate kinase plus phosphate acetyltransferase were detected (Galushko and Schink, 2000).

We determined carbon isotope ratios for *Desulfuromonas acetoxidans* strain 11070 (DSM 684) and *Desulfurella acetivorans* strain A63 (DSM 5264) to find out whether sulfur reducers show different carbon isotope fractionation when they activate acetate via different mechanisms. Both cultures were grown under N_2/CO_2 (80:20) in 500 ml glass bottles (Ochs, Bovenden-Lenglern, Germany) in bicarbonate-buffered mineral medium. Pure cultures of *Desulfuromonas acetoxidans* were incubated with shaking (120 rpm) at 30°C using DSM medium 95 (without yeast extract). *Desulfurella acetivorans* was grown without shaking at 55°C using DSM medium 480 (also without yeast extract). Both bacteria were grown with 3.7 mM acetate as electron donor and 6.2 mM sulfur (subl., purum, purchased from Fluka, Buchs, Switzerland) as electron acceptor. For experiments 10% of bacterial suspensions in the late exponential phase were inoculated (resulting in a final volume of 250 ml) and several samples from the headspace and the liquid phase were removed to determine pH, concentration of acetate, sulfide, carbon dioxide, and carbon isotope composition of acetate and carbon dioxide. All experiments were performed in triplicates.

Chemical and isotopic analysis were performed as described by Goevert and Conrad (2008). Sulfide was determined photometrically after reaction with $CuSO_4$ to form CuS as described by Cord-Ruwisch (1985). CO_2 was analyzed by gas chromatography using a flame ionization detector (Shimadzu, Kyoto, Japan) after conversion to CH_4 with a methanizer (Ni-catalyst at 350°C, Chrompack, Middelburg, Netherlands). Stable isotope analysis of $^{13}C/^{12}C$ in gas samples was performed using a gas chromatograph combustion isotope ratio mass spectrometer system (Thermo Fisher Scientific, Bremen, Germany). Isotopic measurements and quantification of acetate were performed on a high-performance liquid chromatography system coupled to Finnigan LC IsoLink (Thermo Fisher Scientific, Bremen, Germany) (Goevert and Conrad, 2008).

As total oxidized carbon was distributed among different carbon species, $\delta^{13}C$ of total inorganic carbon (δ_{TIC}) was calculated by a mass-balance equation (Goevert and Conrad, 2008). Concentrations of TIC are not shown, since the high background level of the used bicarbonate-buffered medium interfered with accurate measurement of CO_2. Therefore, $\delta^{13}C_{TIC}$ was not used for determination of isotope fractionation. The carbon isotope enrichment factor associated with acetate oxidation (ε_{ac}) was calculated from the temporal change of $\delta^{13}C$ of acetate as described by Mariotti et al. (1981) from the residual reactant

$$\delta_r = \delta_{ri} + \varepsilon[\ln(1 - f)] \quad (4)$$

where δ_{ri} and δ_r are the isotope compositions of the reactant (acetate) at the beginning and of the residual acetate, respectively, and f is the fractional yield of the product based on the consumption of acetate ($0 < f < 1$). Linear regression of δ_r against $\ln(1 - f)$ yields ε as the slope of best-fit lines. Isotope enrichment factors were converted to fractionation factors:

$$\varepsilon \equiv 10^3 (1 - \alpha) \quad (5)$$

Data points at substrate levels below –2.0 on the $\ln(1 - f)$ scale (i.e., 86% of acetate consumed) were not used for the calculation of fractionation factors since acetate might have become limiting and thus no longer been fractionated during consumption.

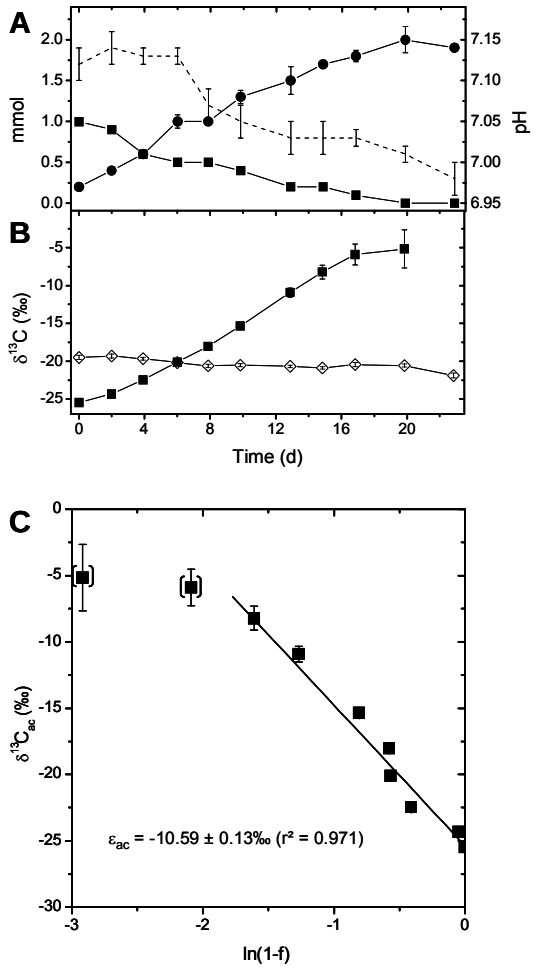

Figure 1: Acetate oxidation by *Desulfuromonas acetoxidans* activating acetate via succinyl-CoA:acetate-CoA-transferase. (A) Acetate consumption (■), H_2S production (●), and pH (dashed line without symbols). (B) Isotope signatures of acetate (■) and CO_2 (◇, illustrated as TIC, total inorganic carbon). (C) The plot is based on equation (4); data points in parentheses were not used for the calculation of ε; the equation within the graph expresses ε_{ac} ± the standard error of the regression line. Values are means ± standard errors (*n* = 3).

During growth of *Desulfuromonas acetoxidans* and *Desulfurella acetivorans* acetate was consumed below the detection limit (approx. 20 µM) and was converted to CO_2 and sulfide (Figs. 1A and 2A). While acetate was oxidized the preferred consumption of light ^{12}C-acetate caused an enrichment of heavy ^{13}C in the residual acetate (Figs. 1B and 2B), as expected for a closed system approach. Values of $\delta^{13}C_{TIC}$ remained almost unchanged during the experiments but were not used to determine isotope fractionation since, as mentioned above, the high background of bicarbonate did not allow precise quantification of $\delta^{13}C$ of the newly formed TIC. Carbon isotope fractionation associated with acetate oxidation was determined for acetate (ε_{ac}) using equation (4) (Figs. 1C and 2C).

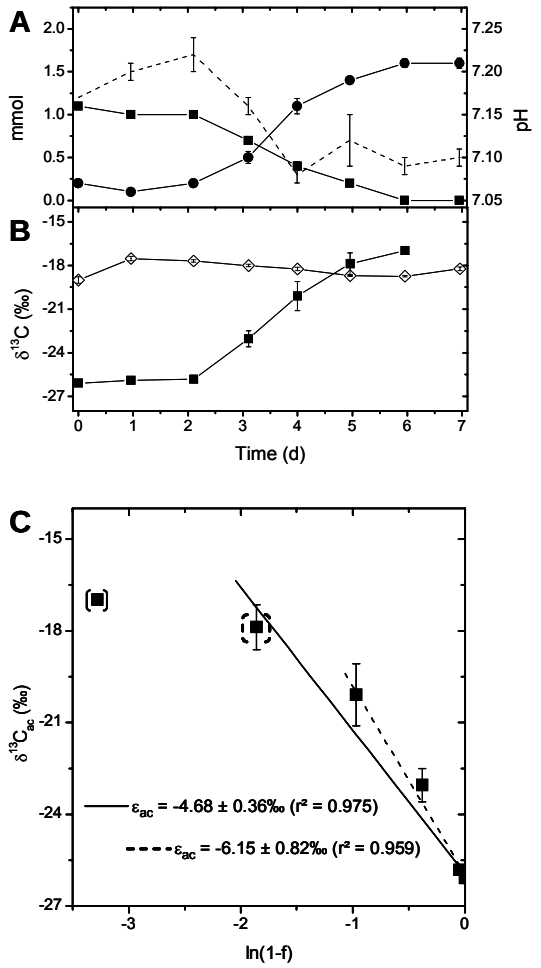

Figure 2: Acetate oxidation by *Desulfurella acetivorans* activating acetate using acetate kinase and phosphate acetyltransferase. (A) Acetate consumption (■), H_2S production (●), and pH (dashed line without symbols). (B) Isotope signatures of acetate (■) and CO_2 (◇, illustrated as TIC, total inorganic carbon). (C) The plot is based on equation (4); data points in parentheses were not used for the calculation of ε; equations within the graph express ε_{ac} ± the standard error of the regression line. Values are means ± standard errors (*n* = 3).

Our study shows that carbon isotope fractionation of acetate differs among acetotrophic sulfur-reducing bacteria by up to 6‰. Nevertheless, it seems that the linear regression of the Rayleigh distillation in *Desulfurella acetivorans* became limited earlier than in *Desulfuromonas acetoxidans*. If only data points at substrate levels below –1.0 (instead of –2.0) on the ln(1 – *f*) scale (i.e., 62% of acetate consumed) were used, the resulting ε_{ac} in *Desulfurella* would get bigger (Fig. 2C, dashed line), whereas in *Desulfuromonas* ε_{ac} remains unchanged (data not shown). Hence, the difference in isotope fractionation between the two sulfur reducers becomes smaller, being approx. 4‰. However, we think that these differences in isotopic discrimination may be related to the different mechanisms for the activation of

acetate. As mentioned above, two possibilities for the first step of acetate oxidation occur in sulfur reducers. Either, acetate is activated to acetyl-CoA catalyzed by succinyl-CoA:acetate-CoA-transferase as in *Desulfuromonas acetoxidans*, or via acetate kinase and phosphate acetyltransferase as in *Desulfurella acetivorans*. If our hypothesis is true, then the determination of ε_{ac} may help to identify the biochemical mechanism of acetate activation in acetotrophic sulfur reducers. However, one has to consider that other factors may affect isotope fractionation in sulfur-reducing bacteria such as temperature which has been previously shown to be a dominant factor controlling isotope fractionation (Zhang et al., 2001; Hoek et al., 2006). Generally, isotope effects tend to become smaller at higher temperatures. Thus, this may also explain the rather low fractionation in *Desulfurella acetivorans* (55°C) compared to *Desulfuromonas acetoxidans* (30°C). Since, to our knowledge, this is the first study on carbon isotope fractionation by sulfur-reducing bacteria more data need to be collected to explain the occurring differences in isotope effects.

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III.4 Effects of the competition for acetate between methanogens and sulfate reducers on carbon isotope fractionation

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Abstract

In anoxic environments, available acetate is a competitive substrate for sulfate-reducing bacteria and methane-producing archaea. Sulfate reducers are believed to successfully compete with methanogens because of their higher affinity for acetate. However, the effects of this competition on carbon isotope fractionation are unknown. Therefore we studied the competition for acetate between sulfate reduction and acetoclastic methanogenesis in co-cultures and in rice field soil. During growth of *Desulfobacter postgatei* and *Methanosarcina acetivorans* the sulfate reducer outcompeted its methanogenic component and expressed a similar isotope fractionation factor as when grown in pure culture. Incubations with rice field soil which were amended with acetate and sulfate showed only minor differences in fractionation compared to soil amended with acetate only. Thus, the competition for acetate seemed to have only little effect on carbon isotope fractionation in soil. Furthermore, we determined the abundance of individual archaeal populations by using analysis of terminal restriction fragment length polymorphism (T-RFLP) targeting 16S rRNA genes. These data showed that *Methanosarcina* spp. express different carbon isotope fractionation when grown as pure cultures or incubated in rice field soil. Here we present for the first time isotope ratios of stable carbon during the competition for acetate. The results will help to constrain the paths of anaerobic carbon flow via acetate in methanogenic and sulfidogenic environments by measuring natural ^{13}C isotope signatures.

Introduction

Competition is defined as a biological situation which can inhibit the growth of a population. In nature, it occurs between living organisms which coexist in the same environment and compete for the same substrate. For sulfate reducers it is believed that they can compete successfully with methanogens for H_2 and acetate. Indeed, it has been demonstrated that H_2-dependent methanogenesis is inhibited in the presence of sulfate (Robinson and Tiedje, 1984; Ward and Winfrey, 1985). However, the competition for acetate is less understood. During the last decades different hypotheses and theories were published to explain why sulfate reducers might be able to outcompete acetoclastic methanogens for acetate.

The competition for acetate between methanogenic archaea and sulfate-reducing bacteria (SRB) was first mentioned in 1974 by Cappenberg who suggested that the sulfide produced by SRB inhibits growth of methane-producing archaea (Cappenberg, 1974). In the same year Martens and Berner published that during their observations CH_4 was not produced before dissolved sulfate has been previously removed by SRB and they explained this with the competition for available hydrogen and different relative free energy yields (Martens and Berner, 1974). Three years later Winfrey and Zeikus mentioned that the competition for acetate is also a factor in the inhibition of methanogenesis by sulfate (Winfrey and Zeikus, 1977). However, scientists doubted for a long time that sulfate reducers are capable of utilizing acetate until Widdel and Pfennig isolated the first acetate-oxidizing SRB, *Desulfotomaculum acetoxidans* and *Desulfobacter postgatei* (Widdel and Pfennig, 1977; Widdel and Pfennig, 1981). Due to this new knowledge Schoenheit *et al.* determined K_s values for acetate which were 0.2 mM for *Desulfobacter postgatei* and 3.0 mM for *Methanosarcina barkeri* (Schoenheit et al., 1982). Thus, these differences in the substrate affinity could also influence the outcome of the competition for acetate. According to that, Lovley and Klug published in the following year that sulfate reduces can outcompete methanogens for acetate and substantiated this with the sulfate reducers lower half-saturation constant for acetate metabolism (Lovley and Klug, 1983). However, Oude Elferink *et al.* noted that additional factors might appear which influence the competition for acetate, such as adherence properties, mixed substrate utilization, affinity of sulfate reducers for sulfate, relative numbers of bacteria, or simply the reactor conditions like pH, temperature, and sulfide concentrations (Oude Elferink et al., 1994). Nevertheless, finally it was concluded that still little is known about the competition for acetate between methanogens and sulfate reducers (Scholten et al., 2002).

The effects of this competition on carbon isotope fractionation are absolutely unknown. Therefore, we determined isotope enrichment factors during the competition for acetate between sulfate-reducing bacteria and methanogenic archaea in co-cultures and in rice field soil. Furthermore, abundances of archaeal populations were monitored by terminal restriction length polymorphism (T-RFLP) analysis. This is the first study analyzing the effects of the competition for acetate on carbon isotope fractionation.

Material and methods

Growth conditions of cultures and soil incubations

Desulfobacter postgatei (DSM 2034) and *Methanosarcina acetivorans* (DSM 2834) were obtained from the Deutsche Sammlung von Mikroorganismen und Zellkulturen (Braunschweig, Germany). Both organisms were grown under N_2/CO_2 (80:20) at 30°C in glass bottles (500 ml, Ochs, Bovenden-Lenglern, Germany) without shaking using bicarbonate-buffered mineral medium. Acetate and sulfate (20 mM each) served as substrates. The composition of the medium was (in g/l unless otherwise noted): KH_2PO_4, 0.68; NH_4Cl, 1.0; NaCl, 23.3; $MgCl_2 \times 6H_2O$, 11.0; KCl, 1.0; $CaCl_2 \times 2H_2O$, 0.3; $NaHCO_3$, 3.78; cysteine-HCl×H_2O, 0.5; $Na_2S \times 9H_2O$, 0.4; trace element solution, 0.5 ml (Wolin et al., 1963); trace element solution SL-10, 0.5 ml (Chin et al., 1998); vitamin solution, 1 ml (Wolin et al., 1963); and resazurine at 0.1% (w/v), 1 ml. Competition experiments were performed by adding 10% bacterial suspension of *D. postgatei* in the late exponential phase into a growing culture of *M. acetivorans* in the early exponential phase (resulting in a final volume of 250 ml). Gas and liquid samples were taken and used for analysis of pH, concentration of acetate, CH_4, CO_2, sulfate, sulfide, and carbon isotope composition of acetate, CH_4, and CO_2.

Rice field soil samples were collected from an Italian rice field located near Vercelli in the valley of the river Po. Characteristics and cultivation of the soil have been described earlier (Schuetz et al., 1989a; Schuetz et al., 1989b). The soil was stored in dry lumps at room temperature. For experiments, slurries of anoxic paddy soil were prepared by suspending sieved (≤ 1 mm mesh size) soil in demineralized water (1 g dry weight soil plus 1 ml H_2O), amended with rice straw (1 g per kg slurry), and preincubated at 25°C for at least four weeks to reduce alternative electron acceptors such as iron, sulfate, or nitrate. The slurries were then filled into 27 ml pressure tubes (Ochs, Bovenden-Lenglern, Germany; each tube was filled with 10 g of slurry) which were flushed with N_2, closed with butyl rubber stoppers, and incubated at 25°C in the dark. Some tubes were amended with substrates (acetate and/or sulfate). For that, 100 µl of sterile stock solutions were added resulting in final concentrations of about 6 mM. At every sampling day three tubes were harvested for DNA extraction and to determine the concentrations and isotope ratios of substrates and products. All experiments were performed in triplicates.

Extraction of DNA and PCR amplification of 16S rRNA genes

DNA extraction from rice field soil was performed using the FastDNA spin kit for soil (Qbiogene, Heidelberg, Germany) according to the manufacturer's instructions. Archaeal 16S rRNA genes were amplified using the forward primer A109f (5'-ACKGCTCAGTAACACGT-3') (Grosskopf et al., 1998) and the 5-carboxyfluorescein-labeled (5'-terminal) backward primer A915r (5'-GTGCTCCCCCGCCAATTCCT-3') (Stahl and Amann, 1991). In a total volume of 50 µl, the PCR mixture contained 10 µl 5× Green Go *Taq* Flexi buffer (Promega, Hilden, Germany), 1 U of *Taq* DNA polymerase (Invitrogen GmbH,

Karlsruhe, Germany), 200 µM dNTPs (Amersham Pharmacia Biotech, Freiburg, Germany), 1.5 mM $MgCl_2$ (Promega), 10 µg of bovine serum albumin (Roche, Mannheim, Germany), and 3.3 nM of each primer (Sigma-Aldrich, Taufkirchen, Germany). A volume of 1 µl DNA solution was added as template. The amplification was performed using a Primus cycler (MWG Biotech, Ebersberg, Germany) with an initial denaturation step (3 min, 94°C) followed by 29 cycles of denaturation (45 s, 94°C), annealing (45 s, 52°C), and elongation (90 s, 72°C), and a terminal elongation step (5 min, 72°C).

Terminal restriction fragment length polymorphism analysis

The principle of the T-RFLP analysis has been described by Liu et al. (1997). Gel electrophoresis was carried out as a visual control for a successful amplification of 16S rRNA genes. Afterwards, fluorescently labeled 16S rRNA gene amplicons were purified by the use of the GenElute PCR clean-up kit (Sigma-Aldrich) according to the instructions of the manufacturer. DNA concentrations of purified 16S rRNA gene fragments were determined by standard UV photometry (Biophotometer; Eppendorf, Hamburg, Germany) at 260 nm. Restriction digestion was performed in a total volume of 10 µl containing approx. 80 ng of 16S rRNA gene amplicons. The latter were restricted with 5 U of enzyme *Taq*I (Fermentas, St. Leon-Rot, Germany) and 1 µl of the appropriate incubation buffer and incubated for 3 h at 65°C. The restriction digestion was purified using the spin post reaction clean-up columns kit (Sigma-Aldrich) according to the manufacturer's instructions. To prepare the samples for the T-RFLP analysis, 3 µl of the purified restriction digestions were mixed with 0.3 µl of an internal lane standard (MapMarker 1000, 50 to 1000 bp, x-rhodamine labeled, BioVentures Inc., USA) and 11 µl HiDi formamide (Applied Biosystems, Weiterstadt, Germany) and denatured for 3 min at 95°C. The analysis of the digested amplicons was performed by separation using capillary electrophoresis with an automatic sequencer (3130 Genetic Analyzer, Applied Biosystems) for 50 min at 15 kV and 9 µA. The injection time per sample was 6 s. After capillary electrophoresis, the length of the fluorescently labeled T-RF's were identified by comparison to the internal standard using the GeneMapper software (version 4.0, Applied Biosystems). The relative abundance of a detected terminal restriction fragment (T-RF) within a given T-RFLP pattern was calculated as the respective signal area of the peak divided by the peak area of all peaks of the T-RFLP pattern, starting from a fragment size of 50 bp to exclude T-RF's caused by primers.

Chemical and isotopic analyses

Sulfate was analyzed by ion chromatography with an IC system, a LCA A14 column, and a S3111 conductivity detector (all from Sykam, Fuerstenfeldbruck, Germany). The eluant was 5 mM Na_2CO_3 plus 1 ml/l modifier (1 g 4-hydroxybenzonitrile in 50 ml methanol), with a flow rate of 1.5 ml/min. Sulfide was determined photometrically after reaction with $CuSO_4$ to form CuS as described by Cord-Ruwisch (1985). CH_4 and CO_2 were analyzed by gas chromatography using a flame ionization detector (Shimadzu, Kyoto, Japan). CO_2 was

detected upon conversion to CH_4 with a methanizer (Ni-catalyst at 350°C, Chrompack, Middelburg, Netherlands). Stable isotope analysis of $^{13}C/^{12}C$ in gas samples was performed using a gas chromatograph combustion isotope ratio mass spectrometer (GC-C–IRMS) system (Thermo Fisher Scientific, Bremen, Germany). For principle operation see Brand (1996). Briefly, after conversion of CH_4 to CO_2 in the Finnigan Standard GC Combustion Interface III, isotope ratios were detected with the IRMS (Finnigan MAT Delta^{plus}). The reference gas was CO_2 (99.998% purity; Air Liquide, Düsseldorf, Germany), calibrated with the working standard methylstearate (Merck, Darmstadt, Germany). The latter was intercalibrated at the Max Planck Institute for Biogeochemistry, Jena, Germany (courtesy of Dr W.A. Brand) against NBS 22 and USGS 24, and reported in the delta notation vs. V-PDB:

$$\delta^{13}C = 10^3 \left(R_{sa} / R_{st} - 1\right) \quad (1)$$

with R = $^{13}C/^{12}C$ of sample (sa) and standard (st), respectively. The precision of repeated analysis was ± 0.2‰ when 1.3 nmol CH_4 was injected.

Isotopic measurements and quantification of acetate were performed on a HPLC system (Spectra System P1000, Thermo Fisher Scientific, San Jose, CA, USA; Mistral, Spark, Emmen, the Netherlands) equipped with an ion-exclusion column (Aminex HPX-87-H, BioRad Laboratories, Munich, Germany) and coupled to Finnigan LC IsoLink (Thermo Fisher Scientific, Bremen, Germany) as described by Krummen et al. (2004). Isotope ratios were detected on an IRMS (Finnigan MAT Delta^{plus} Advantage). Isotope reference gas was CO_2 calibrated as described above.

Calculations

Fractionation factors for a reaction A → B are defined after Hayes (1993) as:

$$\alpha_{A/B} = \left(\delta_A + 1000\right)/\left(\delta_B + 1000\right) \quad (2)$$

also expressed as $\varepsilon \equiv 10^3 (1 - \alpha)$. The isotope enrichment factor ε was determined as described by Mariotti et al. (1981) from the residual reactant

$$\delta_r = \delta_{ri} + \varepsilon\left[\ln\left(1-f\right)\right] \quad (3)$$

and from the product formed

$$\delta_p = \delta_{ri} - \varepsilon\left(1-f\right)\left[\ln\left(1-f\right)\right]/f \quad (4)$$

where δ_{ri} is the isotope composition of the reactant (acetate) at the beginning, δ_r and δ_p are the isotope compositions of the residual ac and the pooled CH_4, respectively, at the instant when *f* was determined, and *f* is the fractional yield of the products based on the consumption of ac ($0 < f < 1$). Linear regression of δ_r against $\ln(1 - f)$ and of δ_p against $(1 - f)[\ln(1 - f)]/f$ gives ε as the slope of best-fit lines.

Because total oxidized carbon was distributed among different carbon species (gaseous CO_2, dissolved CO_2, HCO_3^-, and CO_3^{2-}), $\delta^{13}C$ of total inorganic carbon (δ_{TIC}) could not be determined directly. This value was calculated by the following mass-balance equation:

$$\delta_{TIC} = X_g\delta_g + X_d\delta_d + X_b\delta_b + X_c\delta_c \quad (5)$$

where X = mole fraction and δ = isotopic composition of the C of *g* = gaseous CO_2, *d* = dissolved CO_2, *b* = HCO_3^-, and *c* = CO_3^{2-}. The distribution of carbon among these species was calculated using solubility and equilibrium constants (Stumm and Morgan, 1995). δ_g was measured directly, the remaining isotopic compositions were calculated from the relevant equilibrium isotope fractionation factors (Deines and Langmuir, 1974; Mook et al., 1974):

$$\delta_d = \alpha_{d/g}\delta_g + (\alpha_{d/g} - 1)1000 \quad (6)$$

$$\delta_b = \alpha_{b/g}\delta_g + (\alpha_{b/g} - 1)1000 \quad (7)$$

$$\delta_c = \alpha_{c/g}\delta_g + (\alpha_{c/g} - 1)1000 \quad (8)$$

Results

Anaerobic degradation of acetate in competing co-cultures

Pure cultures of *M. acetivorans* started to consume acetate directly after inoculation (Fig. 1A). Production of CH_4 and CO_2 were slow at the beginning but increased after 8 days. At the beginning of the exponential phase a pure culture of the sulfate-reducing acetate-oxidizing bacterium *D. postgatei* was added to create a competitive situation for acetate between those two organisms. Immediately, *D. postgatei* became the dominant competitor indicated by a rapid consumption of sulfate and a consequential production of sulfide. Also acetate was converted much faster now resulting in an increase of pH. During the first days of the competition (day 12 – 16) both processes, sulfate reduction and acetoclastic methanogenesis, were running simultaneously. Nevertheless, after day 16 no new CH_4 was formed whereas the production of sulfide and CO_2 increased largely indicating that sulfate reduction was now the dominant process and methanogenesis was inhibited. At the end of the experiment, acetate was consumed completely showing the expected stoichiometry during acetotrophic sulfate reduction of acetate and sulfate consumption at a ratio of 1:1.

The observations during substrate conversion were confirmed by the determinations of isotope ratios (Fig. 1B). During methanogenesis, the preferred consumption of ^{12}C-acetate caused an enrichment of the heavier isotope ^{13}C in the remaining acetate. Likewise this led to a slightly increased production of ^{13}C-CH_4. After addition of *D. postgatei* this trend first continued but again, after 16 days of incubation, the isotopic distribution among acetate changed and showed a depletion of ^{13}C in the residual acetate indicating a preferred consumption of the heavier ^{13}C-acetate. This, for a closed system approach, untypical distribution was observed previously in pure cultures of *Desulfobacter* spp. (Goevert and Conrad, 2008). In agreement with the interrupted production of CH_4, also the isotope ratios of CH_4 remained constant during the last sampling days. $\delta^{13}C_{CO2}$ (illustrated as TIC, total inorganic carbon) showed only slight changes during the competitive process and was not used for determination of isotope fractionation, since the high background of the bicarbonate buffered medium prevented a precise quantification of δ^{13}C of the newly formed TIC.

Isotope fractionations factors during the competition were calculated for acetate using equation (3). Isotope enrichment during acetoclastic methanogenesis and the beginning of the competition for acetate (Fig. 1C, dotted line) amounted –30.4 ± 3.3‰ and thus was similar compared to the corresponding fractionation factor determined for pure cultures of *M. acetivorans* being ε_{ac} = –36.9 ± 1.2‰ (see Results III.1, Table 1). Also the observed inverse fractionation (ε_{ac} = +1.9‰) during the second part of the experiment where sulfate reduction seemed to be the dominant process agreed with previously determined fractionation factors in *Desulfobacter* spp. (Goevert and Conrad, 2008) being ε_{ac} = +1.8‰.

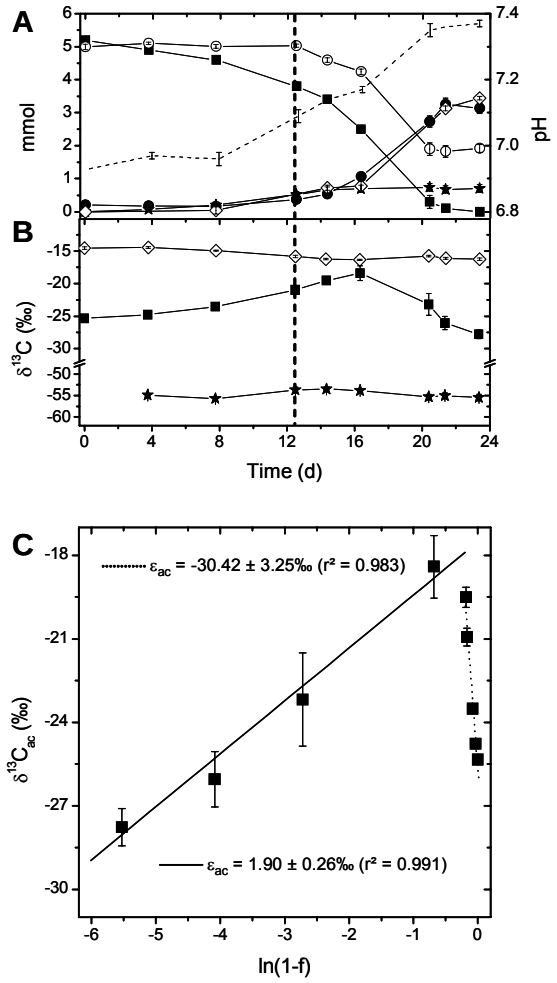

Figure 1: Competition between *Desulfobacter postgatei* and *Methanosarcina acetivorans* during the degradation of acetate. The vertical dashed line indicates the addition of *D. postgatei* to a growing culture of *M. acetivorans*. (A) Acetate (■) and sulfate (○) consumption, CH_4 (★), CO_2 (◇, illustrated as TIC, total inorganic carbon) and H_2S (●) production, and pH (dashed line without symbols). (B) Isotope signatures of acetate (■), CH_4 (★), and TIC (◇). (C) Isotope composition of acetate calculated using equation (3); during the competitive consumption of acetate two isotope fractionation factors were determined, illustrated as ε within the graph. The values are means ± standard errors (*n* = 3).

Competition for acetate in rice field soil

The competition for acetate and its effects on carbon isotope fractionation were also analyzed in anoxic rice field soil by amendment with acetate and sulfate. Addition of sulfate completely inhibited CH_4 production confirming literature data (Achtnich et al., 1995a; Achtnich et al., 1995b) and was accompanied by a decrease of sulfate concentrations (Fig. 2B). Presumably sulfate reducers outcompeted methanogens for H_2 since acetate concentrations remained unchanged. Partial pressures of H_2 were also measured but were very low in all treatments (≤ 0.5 Pa; data not shown).

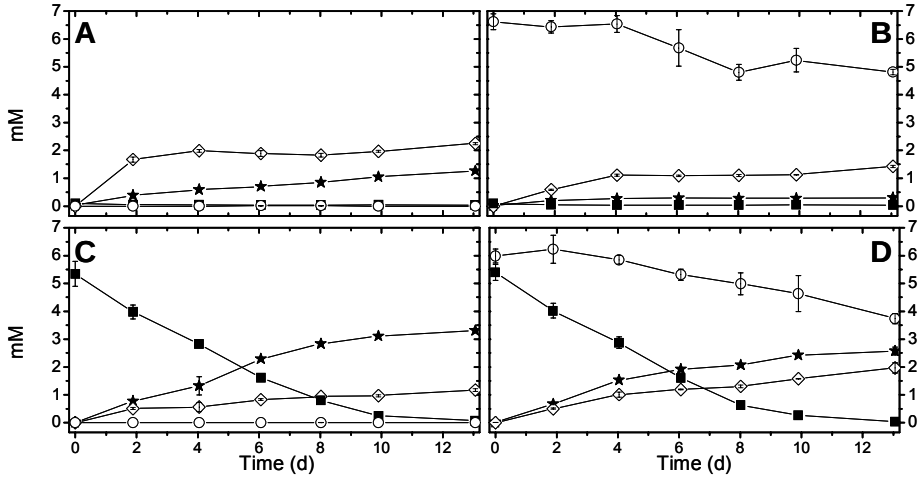

Figure 2: Substance conversions during anaerobic incubation of rice field soil. (A) Incubation of soil without any amendment as control, (B) Soil + approx. 6 mM sulfate, (C) Soil + approx. 6 mM acetate, (D) Soil + approx. 6 mM each, acetate and sulfate. Acetate (■) and sulfate (○) consumption, CH_4 (★) and CO_2 (◇) production. The values are means ± standard errors (*n* = 3).

Methanogenesis was stimulated by adding acetate which led to an increased production of CH_4 (Fig. 2C). Simultaneously, acetate was consumed completely indicating a high activity of acetoclastic methanogens in the paddy soil. Amendment of acetate and sulfate stimulated methanogens as well as sulfate reducers (Fig. 2D). Sulfate reduction was slightly stronger compared to the treatment where only sulfate was added (Fig. 2B), possibly indicating an activity of acetotrophic sulfate reducers. Accordingly, CH_4 production was lower than in the acetate treatment (Fig. 2C) which might be due to a competition for acetate between methanogens and sulfate reducers. The effect of the different amendments on concentrations of CO_2 were comparatively small (Fig. 2A–D). Likewise the carbon isotope fractionation of CO_2 showed no significant changes during amendments (Fig. 3B–D) compared with the control (Fig. 3A). During the incubation the lighter ^{12}C-acetate was preferably consumed resulting in an enrichment of the heavier isotope ^{13}C in the remaining acetate (Fig. 3C). Likewise this led to an increased production of heavy ^{13}C-CH_4. An

additional amendment of sulfate and the resulting assumed competition for acetate caused no significant variation of the isotopic distribution in acetate and CH_4 (Fig. 3D).

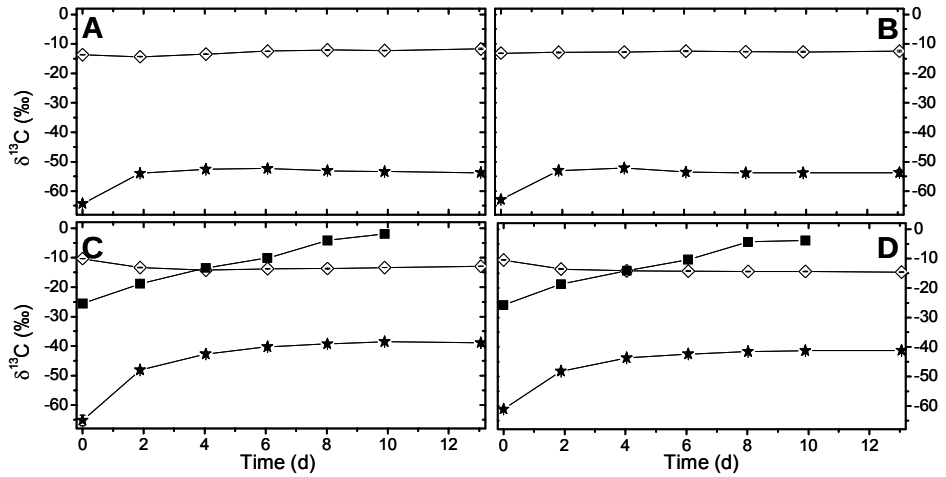

Figure 3: Carbon isotope ratios during anaerobic incubation of rice field soil with different amendments. (A) Soil only, (B) Soil + sulfate, (C) Soil + acetate, (D) Soil + acetate and sulfate. Acetate (■), CH_4 (★), and CO_2 (◇). The values are means ± standard errors (*n* = 3).

Isotope fractionation factors during treatments with acetate and acetate plus sulfate were calculated for acetate and CH_4 using equations (3) and (4), respectively (Fig. 4). Since fractionation varied during the experiments, isotope enrichment factors for acetate were calculated for three different phases of acetate consumption in agreement with pure culture studies (see Results III.1): until 50% of acetate was consumed (ln(1–*f*) values between 0 and –0.6), between 50 and 80% (–0.6 to –1.6), and from 80% to maximum consumption of acetate (–1.6 to –3.0). Fractionation in CH_4 was separated into two parts: until 50% of acetate consumption ((1–*f*) ln(1–*f*) values between –1.0 and –0.6) and from 50% to maximum acetate consumption ((1–*f*) ln(1–*f*) values between –0.6 and 0). Because carbon isotope fractionation in acetate and CH_4 decreased with time, ε-values determined at the early phases of acetate consumption were used to compare isotope fractionation between different amendments and between pure culture and environmental studies. As indicated by the isotope ratios, the simultaneous proceeding of sulfate reduction and methanogenesis did not, or only to a minor extent, effect the fractionation of stable isotopes — the calculated fractionation factors of CH_4 for the treatments with acetate (Fig. 4A) and with acetate + sulfate (Fig. 4C) were similar, amounting –35.9 and –32.4‰, respectively. Fractionation factors for acetate were nearly identically, ranging at –23.4‰ (Fig. 4B) and –23.5‰ (Fig. 4D), respectively.

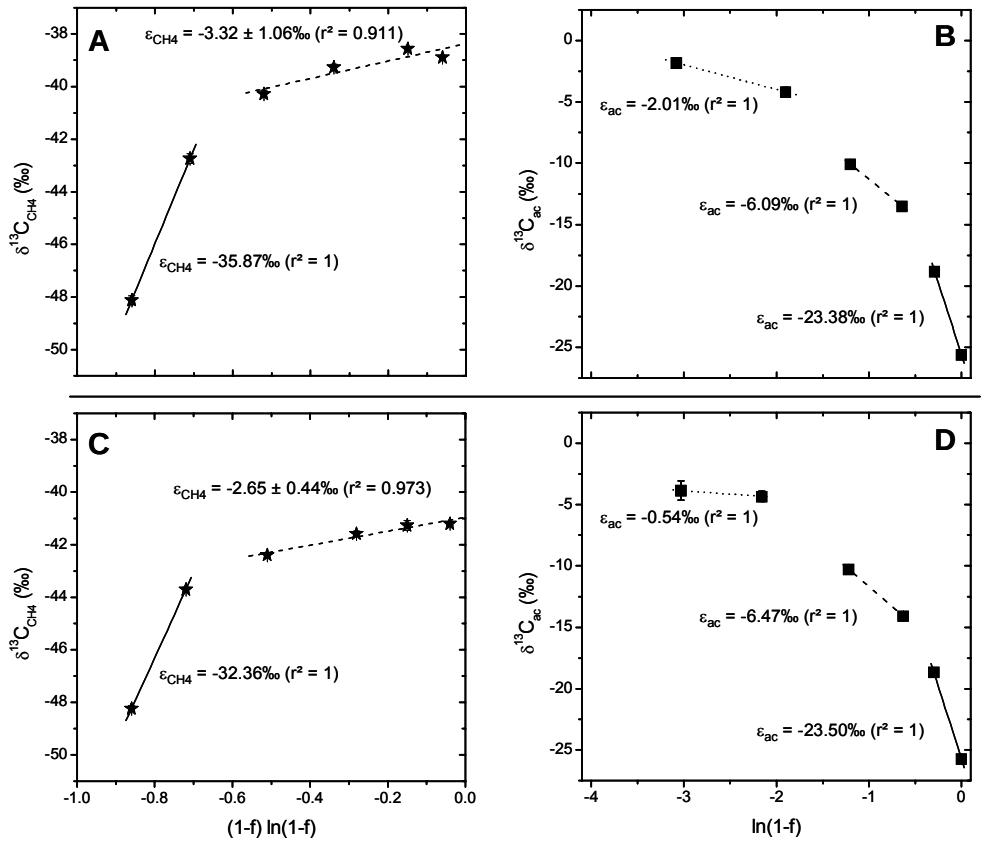

Figure 4: Isotope enrichment during consumption of acetate in rice field soil. The plots are based on equations derived by Mariotti et al. (1981) to determine isotope fractionation factors of CH_4 (★) and acetate (■). (A) and (B) Soil incubations amended with acetate, (C) and (D) Soil incubations amended with acetate and sulfate. The values are means ± standard errors (*n* = 3).

Diversity of archaea in paddy soil incubations

Soil incubations with acetate amendment were used to determine the archaeal diversity of 16S rRNA genes by performing T-RFLP analysis. The relative amplicon frequencies of T-RFs (assigned as in Chin et al., 2004) were determined for the incubation days 0, 6, and 10 to see whether the diversity changed during the incubation. However, T-RF patterns of the different sampling days were highly similar (Fig. 5). The most abundant T-RF was found at 185 bp, representing *Methanosarcinaceae*, accounting for more than 60% of the total archaeal community. The other archaeal family capable of utilizing acetate, *Methanosaetaceae*, was only detected at the beginning of the incubation with a low frequency (284 bp T-RF). Other abundant groups were *Methanomicrobiaceae*/Rice cluster IV (RC-IV) at 83 bp, *Methanobacteriaceae* at 91 bp, and RC-I at 392 bp.

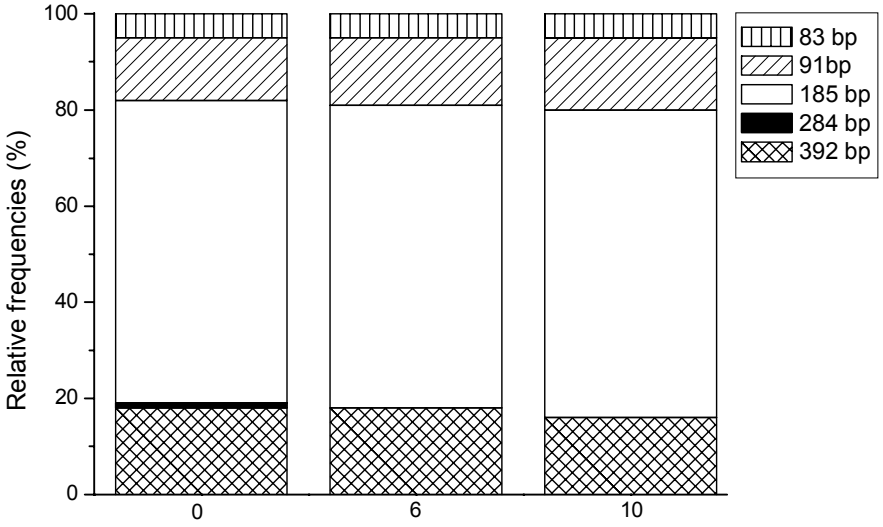

Figure 5: T-RFLP analysis for determination of archaeal diversity of 16S rRNA genes. Relative abundances for incubation days 0, 6, and 10 are shown. 83 bp = *Methanomicrobiaceae*/Rice cluster IV (RC-IV), 91 bp = *Methanobacteriaceae*, 185 bp = *Methanosarcinaceae*, 284 bp = *Methanosaetaceae*, 392 bp = RC-I. Average values of triplicate incubations.

Discussion

The results of the experiment with the competing co-cultures *D. postgatei* and *M. acetivorans* showed a clear dominance of the sulfate-reducing bacterium that outcompeted the methanogenic competitor for acetate. Only for a short time both processes were running simultaneously before sulfate reduction became dominant and inhibited acetoclastic methanogenesis. Thus, *D. postgatei* clearly asserted its isotopic signature against *M. acetivorans* finally resulting in a similar fractionation factor as observed previously in pure cultures of *D. postgatei* and *D. hydrogenophilus* (Goevert and Conrad, 2008). Also the fractionation factor associated with acetoclastic methanogenesis was comparable to the corresponding ε_{ac} determined for pure cure cultures of *M. acetivorans* (see Results III.1). This shows that the deviation from the optimal growth conditions (temperature, growth medium), e.g., incubating at 30°C instead of 37°C, caused no or only minor changes in the isotopic fractionation. However, future work should try to establish the competition for acetate under continuous conditions in a chemostat to investigate how the isotopic fractionation is affected when the two processes are running simultaneously.

In anoxic rice field soil, the competition for acetate between methanogens and sulfate reducers does not seem to play an important role. Indeed, enumeration of SRB showed that H_2-utilizing sulfate reducers were more abundant in paddy soil than those using fatty acids such as acetate (Wind and Conrad, 1995). During our experiments the competition for acetate had only little impact on carbon isotope fractionation. Either, acetotrophic sulfate reducers were poorly active in the soil and their isotopic signature was suppressed by the signature of

acetoclastic methanogens or, active sulfate reducers expressed a similar isotopic fractionation as the methanogens.

The most abundant archaeal family in the paddy soil, detected by T-RFLP analysis, was *Methanosarcinaceae*. The other acetoclastic family, *Methanosaetaceae*, was detected only at the beginning of incubation and only to a minor extent. We assume that *Methanosarcinaceae* dominated, since, at relatively high acetate concentrations, they can grow faster than *Methanosaetaceae*. As *Methanosarcina* spp. have a higher threshold and K_m value for acetate compared to *Methanosaeta* spp. (Jetten et al., 1992) it is reasonable to find *Methanosaeta* spp. mainly in habitats with low acetate concentrations. Indeed, it has been shown previously that *Methanosarcina* spp. are the dominant acetoclastic methanogens on rice roots (Chin et al., 2004) and on decomposing rice straw (Weber et al., 2001), which also show high concentrations of acetate. Interestingly, the carbon isotope fractionation which we determined in rice field soil was in the same range than the observed fractionation in pure cultures of *Methanosarcina* spp. (see Results III.1). However, minor differences occurred which may result from the low isotopic data set that we obtained from the rice field incubations. Here, ε-values for the different phases of acetate consumption relied on only two data points each and thus variations could have appeared easily. Nevertheless, it seems that *Methanosarcina* spp. show a similar fractionation in pure cultures and in rice field soil. Hence, isotope ratios associated with acetoclastic methanogenesis obtained from pure cultures may also be used for soils and sediments.

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IV. General discussion

In the previous chapter various isotope fractionation factors associated with different processes during the anaerobic degradation of acetate were presented. These data help to constrain anaerobic degradation pathways of acetate which lead to the production of CH_4 and CO_2. The question whether biochemical pathways may be detected by only measuring carbon isotope signatures will be the subject of the following discussion.

IV.1 Acetoclastic methanogenesis

Methane is the end product of the anaerobic degradation of organic matter. In most terrestrial systems, CH_4 is almost exclusively produced acetoclastically or hydrogenotrophically (Conrad and Claus, 2005). Theoretically, more than 67% of the total CH_4 production result from acetate consumption and thus acetate is the most important precursor for the microbial production of the greenhouse gas CH_4. Here, the carbon isotope fractionation during this process in methanogenic cultures and in an environmental model system will be discussed.

Only two archaeal families are capable to produce CH_4 from acetate, *Methanosaetaceae* and *Methanosarcinaceae*. It has been found that the carbon isotope fractionation between those families differs significantly. The fractionation factor (α) during acetoclastic methanogenesis in *Methanosarcina* spp. typically ranges from 1.021 to 1.027 (Krzycki et al., 1987; Zyakun et al., 1988; Gelwicks et al., 1994; this study), whereas isotope fractionation in *Methanosaeta* spp. is apparently weaker, ranging between 1.007 for *Methanosaeta thermophila* (Valentine et al., 2004) and 1.010 for *Methanosaeta concilii* (Penning et al., 2006a). So far it was believed that the fractionation factors determined for pure cultures of *Methanosarcina* spp. could also be used for environmental systems. This study now showed for the first time that not only isotopic signatures differ within the genus *Methanosarcina* but also that differences occur in the isotopic distribution compared to environmental samples where *Methanosarcina* is the most abundant methanogen.

Table IV-1 shows fractionation factors during growth of *M. barkeri* and *M. acetivorans* and during incubation of rice field soil amended with acetate. The calculated values for isotope enrichment in the methyl group of acetate ($\varepsilon_{ac\text{-}methyl}$) agreed between *M. barkeri* and *M. acetivorans*. However, enrichment factors of total acetate (ε_{ac}) and CH_4 (ε_{CH4}) differed by approx. 6 and 4‰, respectively. Nevertheless, these data showed that the differences between the two acetoclastic genera *Methanosarcina* and *Methanosaeta* are even bigger than observed before. Thus, this strengthens the assumption that under methanogenic conditions ε_{ac}-values of ≥ –20‰ indicate a predominant abundance of *Methanosarcina* spp.

Table IV-1: Isotope fractionation factors associated with acetoclastic methanogenesis in pure cultures and rice field soil.

Studied system	ε_{ac} [‰]	$\varepsilon_{ac\text{-}methyl}$ [‰]	ε_{CH4} [‰]
Methanosarcina barkeri	–30.5	–25.6	–27.4
Methanosarcina acetivorans	–36.9	–25.2	–23.8
Rice field soil[a]	–23.4	n.d.	–35.9

[a] amended with 5 mM acetate
n.d. = not determined

Interestingly, incubations of rice field soil which were amended with acetate yielded fractionation factors that differed to some extent to those obtained by pure culture studies. In environmental samples T-RFLP analysis showed a dominant abundance of *Methanosarcinaceae* (185 bp T-RF) compared to *Methanosaetaceae* (284 bp T-RF) which were in most of the samples not even detected (Fig. IV-1).

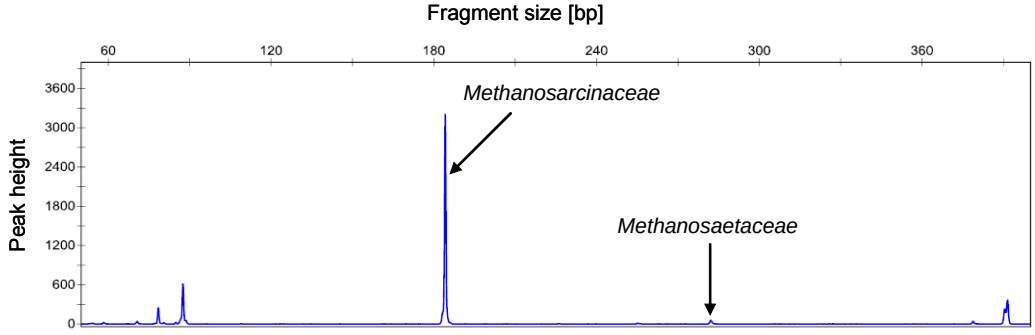

Figure IV-1: Exemplary T-RFLP pattern obtained by T-RFLP analysis of archaeal 16S rRNA genes from rice field soil. 83 bp = *Methanomicrobiaceae*/Rice cluster IV (RC-IV), 91 bp = *Methanobacteriaceae*, 185 bp = *Methanosarcinaceae*, 284 bp = *Methanosaetaceae*, 392 bp = RC-I.

Other detected peaks were related to hydrogenotrophic methanogens. Hence, the determined carbon isotope fractionation of ε_{ac} = –23.4‰ (and presumably also of ε_{CH4} = –35.9‰, due to the high abundance of *Methanosarcinaceae*) was exclusively expressed by *Methanosarcina* spp. Compared with the fractionation factors for pure cultures of *Methanosarcinaceae*, ε-values obtained from soil incubations were lower in acetate (by ~ 7 – 13‰) and higher in CH_4 (by ~ 8 – 12‰). These differences were presumably caused by the few $\delta^{13}C$-data that were available to differentiate isotopic distribution for different phases of acetate consumption in rice field soil. Since mostly only two $\delta^{13}C$-values served for the calculation of ε_{ac} and ε_{CH4} for each phase, fractionation factors might have been

determined inaccurately. Nevertheless, these isotopic data indicate that *Methanosarcinaceae* fractionate similarly in rice field soil and in pure cultures. This finding is in agreement with a previous study on isotope fractionation in rice roots (Penning et al., 2006b), which represents a model system where *Methanosarcinaceae* is the dominant acetoclastic methanogenic group (Chin et al., 2004). For this system the calculated ε-values were also in proximity to pure culture determinations. Hence, it seems that for *Methanosarcinaceae* no strong differences in carbon isotope fractionation at environmental and pure culture conditions exist. Consequently, isotope ratios associated with acetoclastic methanogenesis which were determined in pure cultures may also be used for environmental systems if fractionation factors are calculated precisely for different phases of acetate consumption.

IV.2 Dissimilatory sulfate reduction

Sulfate-reducing bacteria also play an important role during the production of CH_4. They can successfully compete with methanogens for H_2 and acetate (Fig. IV-2) and thus inhibit microbial methanogenesis. Although much research has been done in the last decades to understand the processes during this competition, carbon isotope fractionation of sulfate reducers is poorly studied and isotopic data are rare.

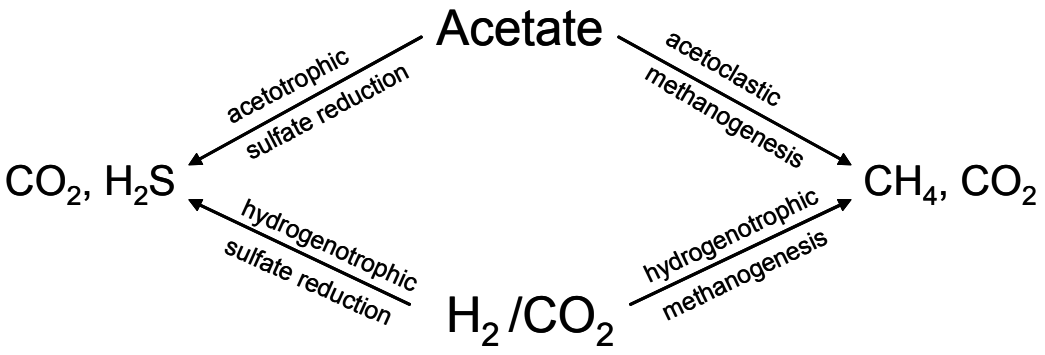

Figure IV-2: Competition for acetate and H_2 between sulfate-reducing bacteria and methanogenic archaea.

By studying the fractionation of stable carbon in acetotrophic sulfate reducers, significant differences were observed. Sulfate reducers using the acetyl-CoA pathway, like *Desulfobacca acetoxidans*, showed a 'normal' fractionation meaning that the relative heavy ^{13}C isotope was discriminated. By contrast, *Desulfobacter postgatei* and *Desulfobacter hydrogenophilus* which are oxidizing acetate via the TCA cycle, did not discriminate against ^{13}C and expressed an 'inverse' fractionation where the heavier isotope is preferably consumed. Generally, this abnormal fractionation occurs during branched pathways when

e.g., the substrate is utilized by two different reactions. In this case, one reaction might express the normal fractionation and will preferably consume the lighter isotope and consequently, the other reaction has to utilize the remaining heavy isotope and will express inverse fractionation. Since during acetate degradation no branched pathway occurs, the inverse or positive fractionation was explained with an equilibrium isotope effect (EIE) during protonation of acetate and the uptake of acetic acid. As drawn in Figure IV-3, for EIE the highest abundance of the heavy isotope is usually found in the dense phase and in the compound having the largest molecular mass. Consequently, we would expect to find relatively more ^{13}C in form of undissociated acetic acid rather than acetate.

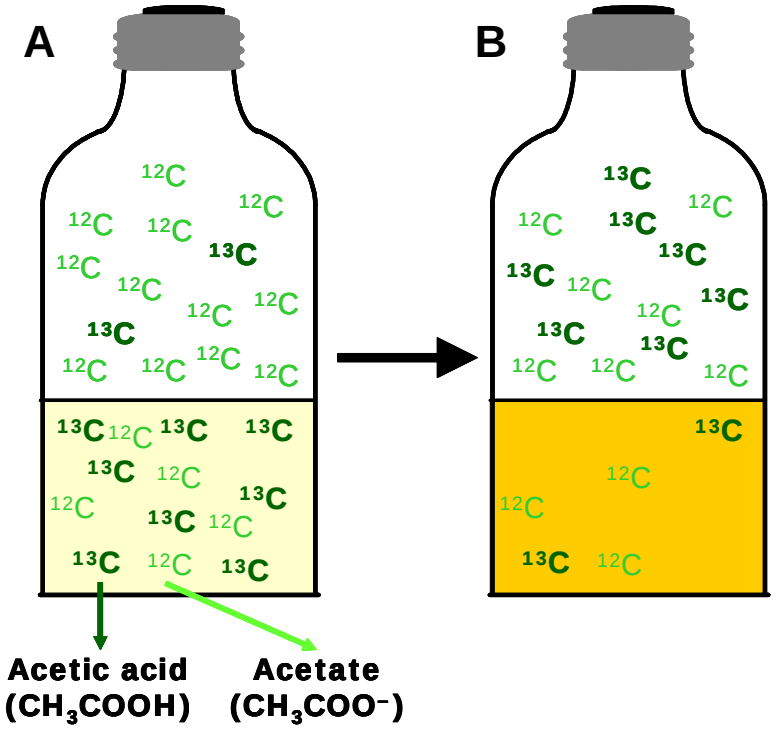

Figure IV-3: Equilibrium isotope effect during the uptake of acetate. In this approach, relatively more ^{13}C can be expected in acetic acid compared to acetate and in the liquid phase rather than in the headspace (A). If acetic acid is consumed, the remaining acetate would be depleted in ^{13}C (B).

If we assume that acetate is taken up by the cells as acetic acid, ^{13}C would be preferentially consumed according to the EIE between acetate and acetic acid. This would result in a depletion of ^{13}C in the remaining acetate, as observed, and hence explain the inverse fractionation of acetate in sulfate reducers.

However, it might be useful to determine carbon isotope fractionation in further SRB. E.g., *Desulfotomaculum acetoxidans* and *Desulfobacterium autotrophicum* are also known to

oxidize acetate via the acetyl-CoA pathway (Schauder et al., 1987; Spormann and Thauer, 1988). The determination of isotope fractionation expressed by these organisms might help to proof whether the fractionation of stable carbon in sulfate reducers depends on the metabolic pathway for the oxidation of acetate. So far we can only suggest this.

IV.3 Acetotrophic reduction of sulfur

A further group which is able to oxidize acetate under anoxic conditions are sulfur reducers. All acetotrophic sulfur-reducing bacteria that were isolated so far use the TCA cycle for the oxidation of acetate (Fig. IV-4).

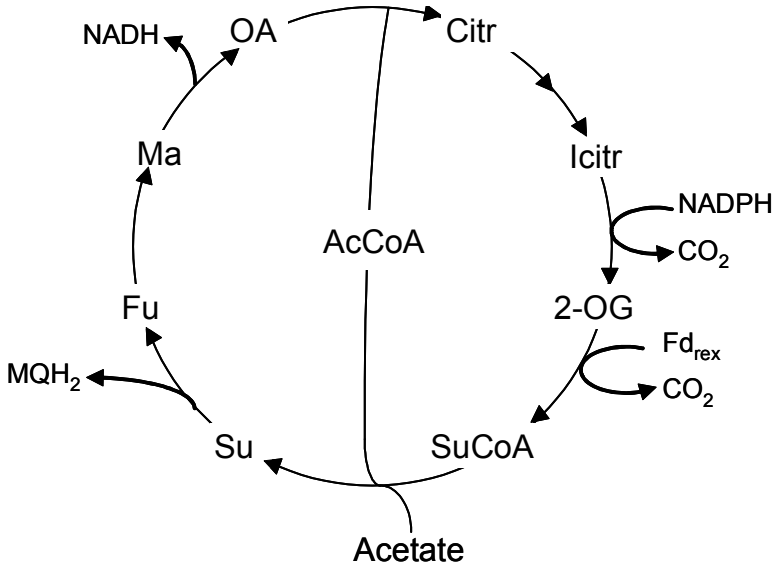

Figure IV-4: TCA cycle operative in *Desulfuromonas acetoxidans* growing on acetate plus elemental sulfur. *AcCoA,* Acetyl-CoA; *Citr,* citrate; *Icitr,* isocitrate; *2-OG, 2*-oxoglutarate; *SuCoA,* succinyl-CoA; *Su,* succinate; *Fu,* fumarate; *Ma,* malate; *OA,* oxaloacetate; *MQ,* menaquinone; *Fd,* ferredoxin (Thauer et al., 1989; modified).

By studying isotope ratios of stable carbon in sulfur-reducing bacteria differences in isotope fractionation between *Desulfuromonas acetoxidans* and *Desulfurella acetivorans* were observed. Here, this difference in the overall fractionation can not be linked to the pathway for acetate oxidation since both organisms use the TCA cycle. It was therefore speculated that the stronger fractionation in *Desulfuromonas* is caused by the initial activation of acetate. In this reaction the two acetotrophic sulfur reducers differ biochemically. In *Desulfuromonas acetoxidans* the activation of acetate proceeds via a succinyl-CoA:acetate-CoA-transferase. Interestingly, *Desulfurella acetivorans* uses different enzymes to activate acetate for the same

pathway (Fig. IV-5). These are acetate kinase and phosphate acetyltransferase which are usually found in the first step of acetate oxidation during the acetyl-CoA pathway. Or in other words, while *Desulfurella* activates acetate in two steps at the expense of one high-energy phosphate bond, *Desulfuromonas* activates acetate directly. It might be assumed that the activation by *Desulfuromonas* spp., which is performed in only one step, has a greater reversibility. Thus, this would also explain the stronger isotope effect of the later C–C bond cleavage of acetyl-CoA.

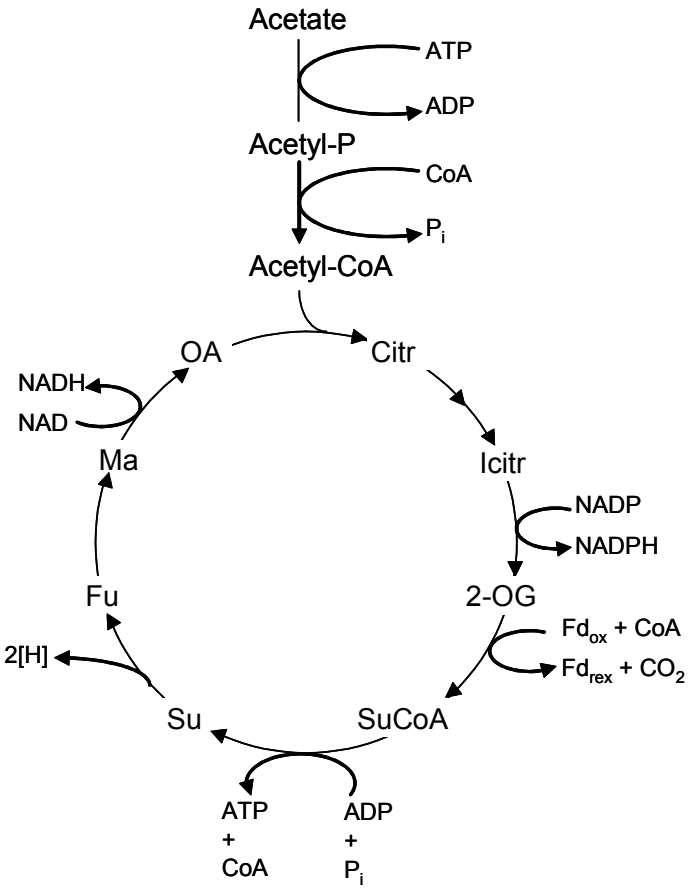

Figure IV-5: Pathway of acetate oxidation to CO_2 in *Desulfurella acetivorans*. *OA*, oxaloacetate; *Citr*, citrate; *Icitr*, isocitrate; *2-OG*, 2-oxoglutarate; *SuCoA*, succinyl-CoA; *Su*, succinate; *Fu*, fumarate; *Ma*, malate; *Acetyl-P*, acetyl phosphate; *Pyr*, pyruvate; P_i, inorganic phosphate; Fd_{red}, reduced ferredoxin; Fd_{ox}, oxidized ferredoxin (Schmitz et al., 1990; modified).

Since isotopic signatures of stable carbon were so far only determined in the two mentioned sulfur reducers we can only speculate that carbon isotope fractionation differs among sulfur reducing bacteria due to different mechanisms for the activation of acetate. Further organisms should be analyzed to verify this hypothesis. Unfortunately, the mechanisms of acetate activation in acetotrophic sulfur reducers are poorly studied. Nevertheless, for *Geobacter sulfurreducens*, which also utilizes acetate, enzymes for both mechanisms were found (Galushko and Schink, 2000). By measuring isotopic signatures during the consumption of acetate one might be able to predict which initial reaction for the oxidation of acetate is used in this bacterium.

Furthermore, if in future a sulfur reducing bacterium will be isolated that uses the acetyl-CoA pathway it would be very interesting to study the carbon isotope fractionation in this organism. Thus, the question could be addressed whether fractionation in sulfur reducers may also depend on the metabolic pathway and not only on the initial step of acetate oxidation.

IV.4 Conclusions and outlook

Carbon isotope fractionation during the anaerobic degradation of acetate among acetoclastic methanogenic archaea as well as acetotrophic sulfate and sulfur reducing bacteria was studied in this work (summarized in Table IV-II). All tested organisms using the acetyl-CoA pathway showed a comparatively strong fractionation in total acetate and ac-methyl. $\delta^{13}C_{ac\text{-}methyl}$ was not determined in sulfate and sulfur reducing bacteria since no differences between ε_{ac} and $\varepsilon_{ac\text{-}methyl}$ are expected in these microorganisms because ac-methyl and ac-carboxyl are not split during oxidation as it is the case during acetoclastic methanogenesis. Indeed, random analysis of $\delta^{13}C_{ac\text{-}methyl}$ and the resulting determination of $\varepsilon_{ac\text{-}methyl}$ yielded no significant differences compared to ε_{ac} (data not shown). Thus, it may be argued that ε_{ac}-values (and $\varepsilon_{ac\text{-}methyl}$) of ≥ –20‰ (i.e., –20‰ or more negative values) indicate a predominance of organisms using the acetyl-CoA pathway.

The results of experiments with sulfate and sulfur reducers using the TCA cycle yielded strongly differing ε_{ac}-values ranging between –10‰ and +2‰. These data indicate that no general isotope fractionation factor associated with the TCA cycle can be given. The reason for that might be due to the different rate-limiting steps that were suggested to be responsible for the overall fractionation in SRB and sulfur-reducing bacteria. Carbon isotope fractionation in the latter seems to be expressed during the initial activation of acetate as discussed before. Thus, fractionation factors differ when different mechanisms for this reaction are used. In SRB it was assumed that an EIE controls isotope fractionation during the uptake of acetate. This EIE may overlay a KIE e.g., during activation of acetate. It could also be possible that a small EIE is effective in sulfur reducers as well but is suppressed by a stronger KIE.

However, sulfate-reducing bacteria using the TCA cycle seem to express positive fractionation which is quite unique. Consequently, isotopic signatures can be used to identify these organisms in microbial communities. As discussed above, for sulfur reducers it is suggested that the mechanism for acetate activation is the rate-limiting step causing isotope fractionation. Since isotopic data for these microbial groups are very rare, more research has to be done to completely understand which factors control carbon isotope fractionation.

Table IV-2: Isotope fractionation factors determined during anaerobic degradation of acetate by methanogens, sulfate reducers, and sulfur reducers

Microorganism	ε_{ac} [‰]	$\varepsilon_{ac\text{-}methyl}$ [‰]	ε_{CH4} [‰]	Pathway of acetate oxidation	Mechanism for acetate activation
Methanosarcina barkeri	–30.5	–25.6	–27.4	acetyl-CoA	Reaction 1[a]
Methanosarcina acetivorans	–36.9	–25.2	–23.8	acetyl-CoA	Reaction 1
Desulfobacca acetoxidans	–19.1	n.d.	–	acetyl-CoA	Reaction 1
Desulfobacter postgatei	1.8	n.d.	–	TCA cycle	Reaction 2[b]
Desulfobacter hydrogenophilus	1.5	n.d.	–	TCA cycle	Reaction 2
Desulfuromonas acetoxidans	–10.6	n.d.	–	TCA cycle	Reaction 2
Desulfurella acetivorans	–4.7/–6.2[c]	n.d.	–	TCA cycle	Reaction 1

[a] Reaction 1: acetate + ATP → acetyl-phosphate + ADP using acetate kinase plus phosphate acetyltransferase
[b] Reaction 2: acetate + succinyl-CoA → acetyl-CoA + succinate via succinyl-CoA:acetate-CoA-transferase
[c] Depending on the ln(1 – *f*) range that was used for the calculation of ε
n.d. = not determined

In addition, differences in fractionation between pure cultures and environmental systems were studied. For that, the model system rice field soil was used. Interestingly, the calculated values for ε_{ac} and ε_{CH4} were in the same range in pure cultures of *Methanosarcina* spp. (average values of ε_{ac} = –33.7‰ and ε_{CH4} = –25.6‰) and in paddy soil in which *Methanosarcinaceae* were the dominant acetoclastic methanogens (ε_{ac} = –23.4‰ and ε_{CH4} = –35.9‰). Hence, it was concluded that isotope fractionation determined in pure cultures can be applied to environmental systems if fractionation factors are determined precisely for different phases of acetate consumption. Future work should focus on further environments such as lake sediments where *Methanosarcina* might be the dominant acetoclastic species e.g., in Priest Pot, a small eutrophic lake in the UK (Earl et al., 2003).

Furthermore, the effect of the competition of acetate between methanogens and sulfate reducers on isotope fractionation was studied in co-cultures and in rice field soil. Cultures of the sulfate-reducing bacterium *Desulfobacter postgatei* outcompeted the methanogenic archaeon *Methanosarcina acetivorans* for acetate. Since methanogenesis was inhibited completely, ε_{CH4} could not be determined. Nevertheless, it was observed for the first time that during the competition for acetate *D. postgatei* expressed the same isotopic signature as when grown in pure culture ($\varepsilon_{ac} \approx +2‰$). Also the isotope fractionation in *M. acetivorans* was similar in the co-culture at the beginning of the competition ($\varepsilon_{ac} = –30.4 \pm 3.3‰$) compared to the pure culture ($\varepsilon_{ac} = –36.9 \pm 1.2‰$). But as soon as sulfate reduction became the dominant process during the competition, carbon isotope fractionation showed inverse behavior which seems to be prevalent in *Desulfobacter* spp. By contrast, during incubation of rice field soil which was amended with acetate and sulfate, the competition for acetate had only little effect on the carbon isotope fractionation of acetoclastic methanogens. By applying molecular methods such as T-RFLP, the sulfate-reducing community in the soil should be analyzed to verify whether acetotrophic sulfate reducers were abundant. If this was the case, then the present sulfate reducers might express a similar fractionation as the abundant methanogens.

In future, the competition for acetate should be established in a continuous co-culture to have both processes, sulfate reduction and methanogenesis, running simultaneously. Furthermore, co-culture studies were so far performed only under marine salt concentrations. Future experiments should also proceed under freshwater concentrations e.g., by growing a competing co-culture of *Methanosarcina barkeri* and *Desulfobacca acetoxidans* to follow how this microbial combination influences the isotope fractionation of stable carbon.

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VII. Appendix

Index of figures

Figure I-1: Pathway of anaerobic degradation of organic matter by different groups of microorganisms 10
Figure I-2: Competition for acetate between acetoclastic methanogens (1) and acetotrophic sulfate reducers (2) 11
Figure I-3: Schematic representation of kinetic isotope fractionation in a closed system as a reaction proceeds to completion 14

Part III.1
Figure 1: Catabolism of acetate in a pure culture of *Methanosarcina barkeri* 37
Figure 2: Carbon isotope fractionation during the conversion of acetate to CH_4 by *Methanosarcina barkeri* 38
Figure 3: Batch culture of *Methanosarcina acetivorans* growing on 20 mM acetate as sole substrate 39
Figure 4: Isotope enrichment during acetoclastic methanogenesis by *Methanosarcina acetivorans* 40

Part III.2
Figure 1: Acetate oxidation by *Desulfobacca acetoxidans* using the acetyl-CoA/CODH pathway .. 52
Figure 2: Acetate oxidation by sulfate-reducing bacteria using the tricarboxylic acid cycle 53

Part III.3
Figure 1: Acetate oxidation by *Desulfuromonas acetoxidans* activating acetate via succinyl-CoA:acetate-CoA-transferase 60
Figure 2: Acetate oxidation by *Desulfurella acetivorans* activating acetate using acetate kinase and phosphate acetyltransferase 61

Part III.4
Figure 1: Competition between *Desulfobacter postgatei* and *Methanosarcina acetivorans* during the degradation of acetate 70
Figure 2: Substance conversions during anaerobic incubation of rice field soil 71
Figure 3: Carbon isotope ratios during anaerobic incubation of rice field soil with different amendments 72
Figure 4: Isotope enrichment during consumption of acetate in rice field soil 73
Figure 5: T-RFLP analysis for determination of archaeal diversity of 16S rRNA genes 74

Figure IV-1: Exemplary T-RFLP pattern obtained by T-RFLP analysis of archaeal 16S rRNA genes from rice field soil 79
Figure IV-2: Competition for acetate and H_2 between sulfate-reducing bacteria and methanogenic archaea 80
Figure IV-3: Equilibrium isotope effect during the uptake of acetate 81
Figure IV-4: TCA cycle operative in *Desulfuromonas acetoxidans* growing on acetate plus elemental sulfur 82
Figure IV-5: Pathway of acetate oxidation to CO_2 in *Desulfurella acetivorans* 83

Index of tables

Table II-1: Growth conditions for microorganisms used in this study 17
Table II-2: Different salt concentrations for culture media (in g/l) 17
Table II-3: Stock solutions for media 18/19
Table II-4: Compositions of low and high salt medium (in ml/l) 20
Table II-5: Composition of medium for growth of *Desulfobacca acetoxidans* (in ml/l) 20
Table II-6: Composition of HS medium for growth of *Methanosarcina* spp. (in ml/l) 21
Table II-7: Compositions of media for growth of *Desulfurella* and *Desulfuromonas* (in ml/l) 22
Table II-8: Operating data for gas chromatograph 23
Table II-9: Operating data for ion chromatograph 24
Table II-10: Operating data for GC-C–IRMS 25
Table II-11: Operating data for HPLC-IRMS 25
Table II-12: Operating data for EA-IRMS 26
Table II-13: Oligonucleotide primers used for T-RFLP analysis of partial archaeal 16S rRNA genes . 30
Table II-14: PCR reaction to amplify 16S rRNA genes from rice field soil 31
Table II-15: PCR conditions for analysis of the microbial diversity of 16S rRNA genes 31

Part III.1

Table 1: Isotope enrichment factors for acetate, ac-methyl, ac-carboxyl, and CH_4 during acetoclastic methanogenesis by *M. barkeri* and *M. acetivorans* 40

Table IV-1: Isotope fractionation factors associated with acetoclastic methanogenesis in pure cultures and rice field soil 79
Table IV-2: Isotope fractionation factors determined during anaerobic degradation of acetate by methanogens, sulfate reducers, and sulfur reducers 85

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Erklärung

Ich versichere, dass ich meine Dissertation

„Carbon isotope fractionation during the anaerobic degradation of acetate“

selbständig und ohne unerlaubte Hilfe angefertigt habe und mich keiner anderen als der von mir ausdrücklich bezeichneten Quellen und Hilfen bedient habe.

Diese Dissertation wurde in der jetzigen oder einer ähnlichen Form noch bei keiner anderen Hochschule eingereicht und hat noch keinen sonstigen Prüfungszwecken gedient.

Marburg, im März 2008