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Anaerobic Methane Oxidation with Fe^{3+}

Barbara Pomper

Max-Planck-Institut fuer terrestrische Mikrobiologie, Marburg, Germany

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Abstract

Several studies have confirmed that methane is consumed in anaerobic environments, including anoxic marine water, sediments of soda lakes, and freshwater sediments. Most of the methane oxidation occurs in the anaerobic bottom waters and sediments. Attempts to identify organisms responsible for environmental methane oxidation have yielded inconclusive results. Biochemical evidence indicates that the overall process involves a transfer of electrons from methane to sulfate and is probably mediated by more than one organism: a methanogen and a sulfate-reducer.

Using sulfate and nitrate as sole electron acceptors no single organism could be found consuming methane. By using Fe^{3+} as an electron acceptor, a decrease in methane and an increase in Fe^{2+} was measurable when using methane as sole carbon source. Isolated DNA from this sample was cloned and sequenced. Finally in the beginning of this study two different organismn has been identified, a gamma proteobacterium, *Acinetobacter calcoaceticus* and an alpha proteobacterium, *Porphyrobacter spec.*

Introduction

The atmospheric concentration of methane, a greenhouse gas, has more than doubled during the past 200 years. Therefor study of methane-consuming organisms, methanotrophs, has ecological importance. Methanotrophs, microaerophilic organisms widespread in aerobic soils and sediments, oxidize methane to derive energy and carbon for biomass. Because of their wide occurrence methanotrophs play an important role in regulating atmospheric methane content.

It is known that large amounts of methane is produced in marine sediments but is then consumed before contacting aerobic waters or the atmosphere. These findings suggest the presence of an anaerobic methane consumer but until now no organism has been isolated that can consume methane anaerobically. There is biogeochemical evidence that the overall process involves a transfer of electrons from methane to sulfate and is probably mediated by a consortium of methanogenic and sulfate-reducing bacteria.

Experiments to find a methane oxidizer using sulfate (SO_4^{2-}) and nitrate (NO_3^{2-}) as sole electron acceptor failed.

It seems to be the case that iron played an important role of early Earth history and this report summarizes first attempts to find anaerobic methane oxidizers using Fe^{3+} as electron acceptor.

Materials and Methods

Bacteria enrichment

For the enrichment of anaerobic methane oxidation bacteria was used an anaerobic mineral salt medium with a pH of 6.8.

Tab.1: Mineral salt medium components

K_2HPO_4	1.7 g
NaH_2PO_4	1.7 g
NH_4Cl	0.8 g
MgCl	0.2 g
Trace element solution	1 ml
dH_2O	ad 1 l

Tab. 2: trace element solution

EDTA	1.5 g
$ZnSO_4 \times 7\ H_2O$	4.4 g
$(NH4)Mo_7O_{21} \times 4\ H_2O$	0.22 g
$Ca\ Cl_2 \times 2\ H_2O$	1.47 g
$CuSO_4 \times 5\ H_2O$	0.3 g
$CoCl_2 \times 6\ H_2O$	0.3 g
$MnCl_2 \times 4\ H_2O$	1 g
$FeSO_4 \times 7\ H_2O$	1 g
dH_2O	ad 1l

30 ml of the medium was filled into 100 ml and 75 ml serum vials which are closed by stoppers.
Additional added by syringes to the vials was 10 mM $Fe(OH)_3$, 1 mM DTE and CH_4.
A rubber tire was inflated with methane as a reservior.
Methanol was also added to a few vials (0.15 ml per 30 ml medium).
The inoculum was a sample from School Street Marsh, Woods Hole, diluted 1:10.
Inoculated serum vials were shaken at 30°C in the dark.

Experimental structure

For finding anaerobic methane oxidizers two different carbon sources were chosen:
one attempt with methanol and methane and the other with methane alone.
Non inoculated vials were used as control for the determination of methane and iron.
In the beginning of the experiment a medium containing sulfate was incorrectly prepared and the first vials containing this medium have been inoculated.

Samples:
Only CH_4 + control
CH_4/methanol + control
CH_4/methanol/sulfate + control

Measurement of iron and methane

For the determination of iron compounds the chemical ferrozine was used (determination by Stookey et al.)
The methane concentration was measured at the Gas Chromatograph.

Cloning and sequencing

DNA isolation was followed by a given method from "Prepman"-ABI. For the PCR amplification of ribosomal DNA, a universal and eubacterial primer were used.
Eub8F: 5'-AGAGTTTGATCCTGGCTCAG-3'
Univ.1492R: 5'-GYTACCTTGTTACGACTT-3'

Tab. 3:PCR assay

Volume	**Component**
2.5 ul	10 x Reaction Buffer
2.0 ul	25 mM $MgCl_2$
2.0 ul	Nucleotide mix (1.25 mM each dNTP)
0.5 ul	forward primer
0.5 ul	reverse primer
0.2 ul	Taq DNA polymerase (5U/ul)
1.0 ul	Template DNA
21.6 ul	DH_2O
25 ul	**Total reaction volume**

PCR reaction without template.was used as a negative control.
For confirming PCR products, a 1% SeaKem agarose gel (made in 0.5x TBE buffer) was used.
PCR products were cloned with the Invitrogen TOPO TA cloning kit.
For RFLP analysis a small portion of a colony was added by a sterile pipette tip to a 25 ul total volume PCR reaction including TOPO primers to amplify 16 s rDNA insert.
The PCR product is separated in a 1.5% Metaphor agarose gel after a 3 hour digest by HINPI and MspI.
For sequence analysis 10 ul of PCR product was send to Bruce Pasteur's lab (analysed in a sequence gel).

Results

Iron and methane concentrations:

After several days a change of Fe^{2+} and methane concentration was observed in some vials.

Methane sample:
After five days an increase of Fe^{2+} ions was measurable (diagram 1) and also a decrease of methane. Under the microscope highly motile larger rods and less motile smaller rods could be found. After refilling methane the concentration dropped again from 100 % to 78% during one day.

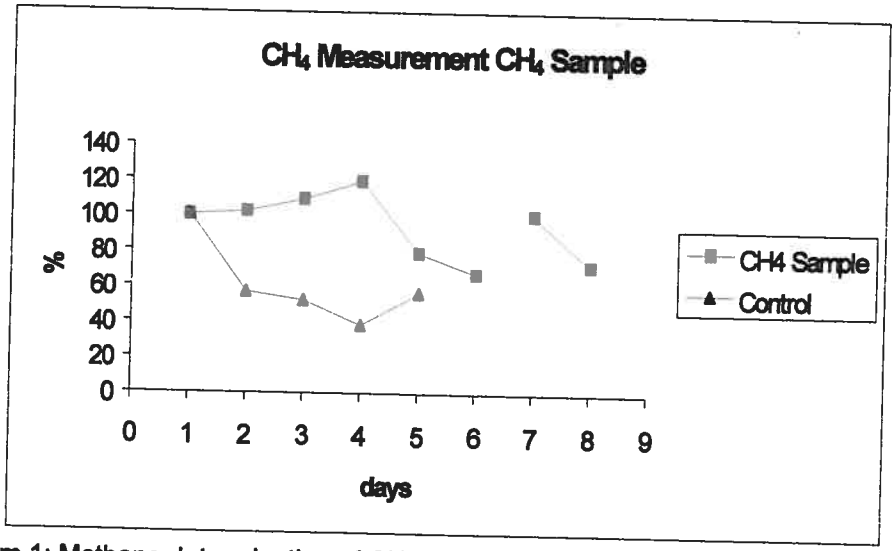

Diagram 1: Methane determination of CH_4 sample

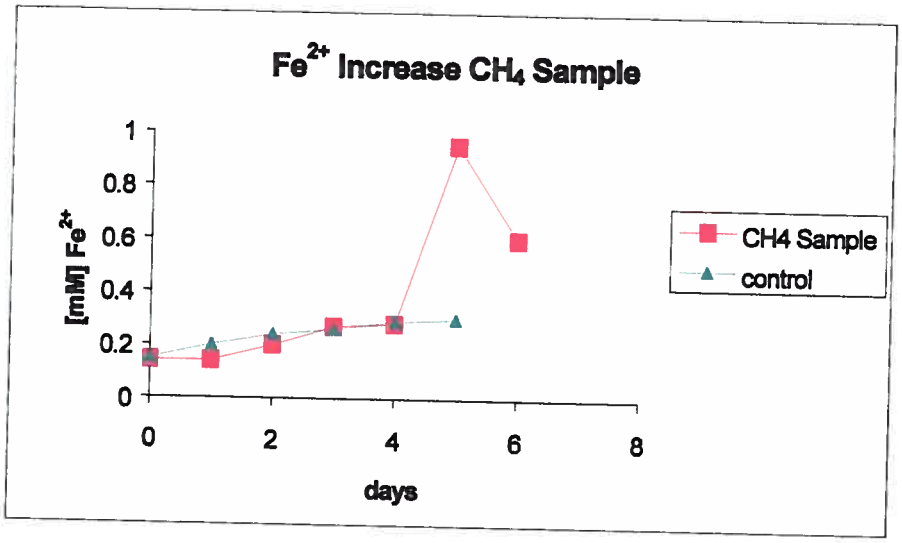

Diagram 2: Fe^{2+} determination of CH_4 sample

Methanol sample:

Compared to the control, no change of Fe^{2+} concentration could be found in the methanol sample but a decrease of methane (diagrams 3 and 4). Under the microscope only a few organisms were visible.

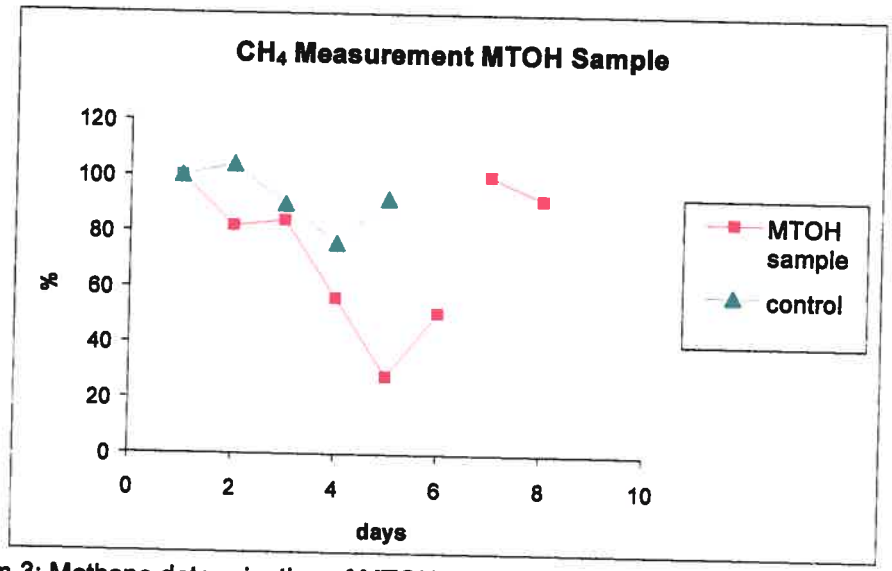

Diagram 3: Methane determination of MTOH sample

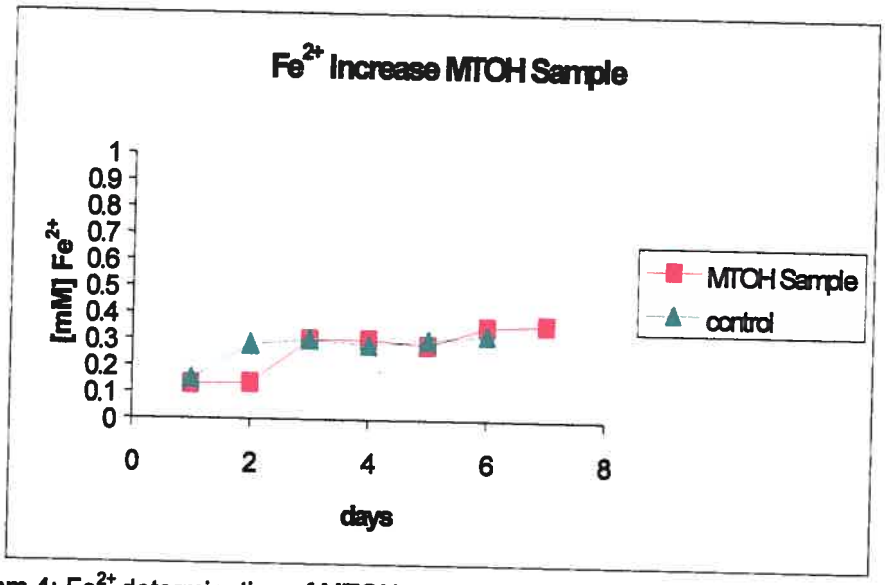

Diagram 4: Fe^{2+} determination of MTOH sample

Methanol/SO_4 containing sample:

After several days a change of the color of the medium from brown to gray was visible. Measurements of the iron concentration showed an increase of Fe^{2+} from 0.2 mM to 5 mM (diagram 6). The methane concentration dropped during the first five days from 100% to 30 %. After refilling the vial with methane again a decrease of the methane concentration could be found, but also later an increase (diagram 5).

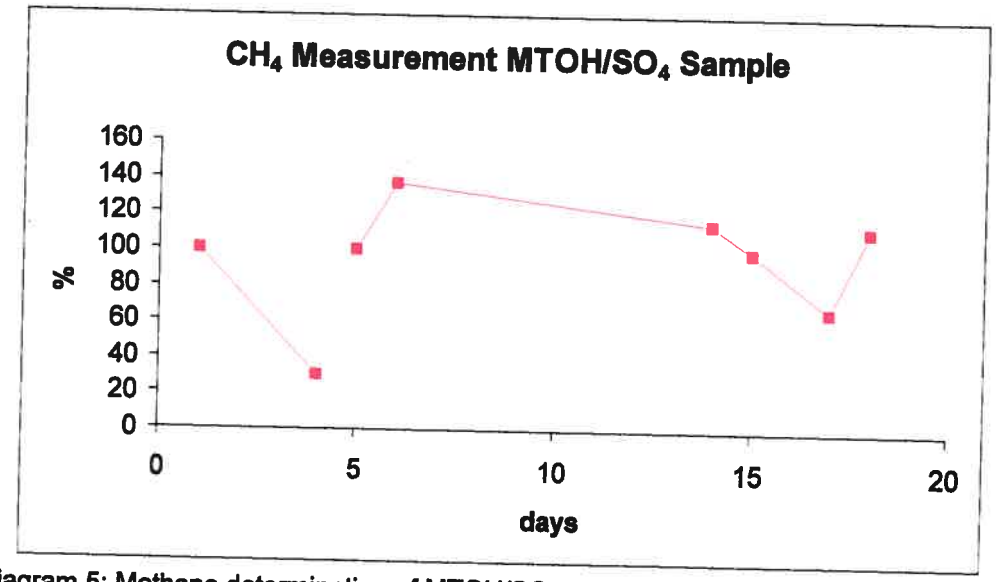

Diagram 5: Methane determination of MTOH/SO_4 sample

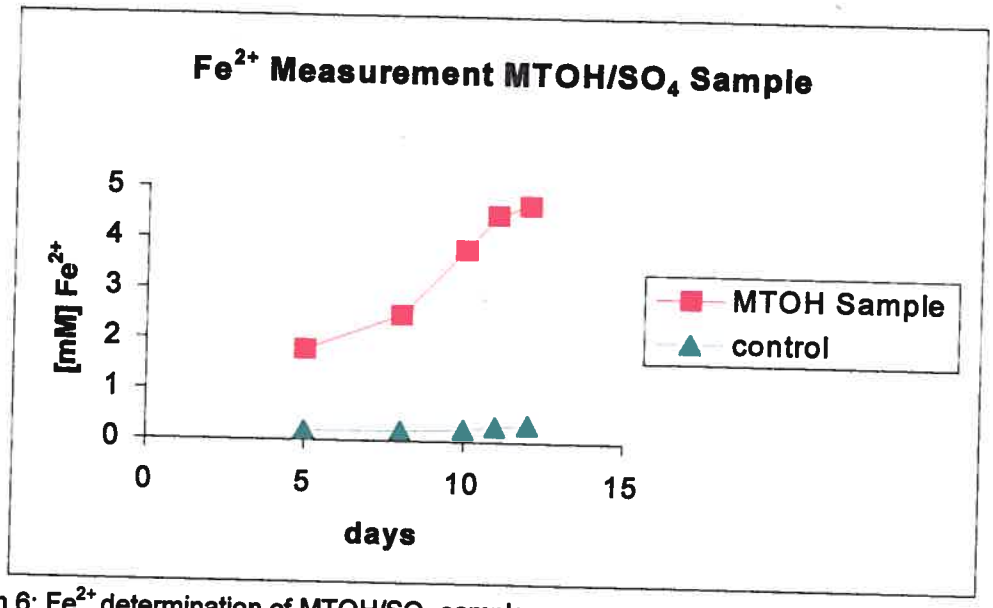

Diagram 6: Fe^{2+} determination of MTOH/SO_4 sample

On the other hand several control vials showed a decrease in methane which is a consequence of free diffusion though the stopper.

Cloning:

After successful DNA isolation and transformation the RFLP analysis of the CH_4 sample indicated two different patterns. The sequence analysis identified them as *Acinetobacter calcoaceticus* with an identity of 98% and *Porphyrobacter spec.* with 100% identity.

Discussion

To find an anaerobic methane oxidizer no oxygen should be present. Using serum vials containing anaerobic media and filled up with methan is not anoxic enough. The methane coming out of a tire was filled into the vial via a syringe, so it is likely that oxygen have been in the vial but at low concentrations.

The controls turned out how leaky the stoppers can be, so the decrease of methane could be the consequence of methane oxidation or simple diffusion.

Another critical point is the ferrozine iron determination. This method cauld high source of rror because of several dilution steps. this method has. For exact iron determination, constant measuring conditions are important to compare samples from different days and enrichment conditions.

The existence of *Acinetobacter calcoaceticus* in the enrichment indicates that the vial is not oxygen free. This bacterium is known as strict aerobic gram negative rod belonging to the gamma proteobacteria. The finding of this organism in the samples is very interesting because the conditions in the vial are more or less anoxic. This indicates that *Acinetobacter* perhaps can grow under less anoxic conditions as thought. Altough *Acinetobacter calcoaceticus* is generally nonmotile and in the enrichment were observed high motile rods but also it was not a pure culture.

For further studies the enrichment procedure has to be improved. One of the most important points is to create strict anoxic conditions and to improve the method of iron and methane measurement.

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