Methanogenic Archaea in the Human Digestive Tract Revisited

Project Report Submitted by

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Microbial Diversity
Marine Biological Laboratory
Woods Hole
Summer 1997
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Acknowledgment

Our gratitude is extended to the co-directors and faculty members of the Microbial Diversity course who created a conducive environment for learning and exchange of knowledge.

We are thankful to the MBL for offering us this unique opportunity, and also grateful for the people who provided the financial support to cover for the course tuition fees.

We are indebted to our colleagues who volunteered and participated in this project.
Introduction

Methanogenic archaea are obligate anaerobes that produce methane mainly from $\text{H}_2-\text{CO}_2$ and, in some cases, from formate, acetate, methanol or methylamine.

Methanogenesis is the terminal step in carbon flow in many anaerobic habitats, including marine and freshwater sediments, marshes and swamps, hot springs, sewage sludge and the intestinal tract of animals, including humans.

About one third of adult human population excretes methane, most of which is excreted from the body in flatus, and some methane is absorbed into the blood and excreted via the lungs. The methane-breath test allows a distinction between methane excretors and non-methane excretors. A negative breath test, however, does not prove a lack of infection with methanogenic archaea, because a low level of methane is always produced by the non-methane excretors.

So far, Methanobrevibacter smithii and Methanosphaera stadimanae were the only two methanogenic archaea species isolated from the human colon. The same species have been detected in dental plaques from patients with varying degree of periodontal disease.

This report describes our endeavor to re-examine the methanogenic archaea in the human digestive tract using culture-dependent methods as well as molecular biology techniques. We attempted to detect methanogenic archaea in dental plaques from healthy individuals, methane and non-methane excretors, as well as in fecal samples from non-methane excretors. Furthermore, we wanted to investigate the diversity of methanogenic archaea in the human digestive tract.
Materials and Methods

Volunteers
Six students of the Microbial Diversity course, 3 males and 3 females, volunteered to participate in this study. Four students were known as methane excretors and 2 were non-methane excretors as determined by the breath test.

Preparation of slurries
Freshly voided feces were collected from all volunteers, stored at 4 °C and processed within 2 - 4 hours. About 2.5 g ( wet weight) of a fecal specimen was transferred into a 20-ml serum bottle containing 5 ml of sterile anoxic standard medium and 5 glass beads (5 mm diameter). The bottles were sealed with butyl stoppers and were shaken vigorously to obtain homogenous slurries. The slurries were used for enrichment, enumeration, direct microscopy and nucleic acid extraction.

Enrichment and enumeration of fecal methanogens
Enrichment cultures of each fecal specimen were maintained in 60-ml serum bottles and incubated in the dark at three different temperatures: 30 °C, 37 °C and 40 °C. Before inoculation, the bottles contained 10 ml standard medium, gassed with H₂ - CO₂ (80 : 20) and pressurized to 0.5 atm. Each bottle was inoculated with 1 ml (0.5 g) of the slurry using a sterile syringe and needle.
Total fecal methanogens were enumerated by most probable number (MPN) estimation in triplicate using 60-ml serum bottles. The bottles contained 9 ml standard medium and gassed and pressurized as mentioned above. All MPN bottles were incubated at 37 °C in the dark. A positive growth was counted after five days if the methane level in the head space over the culture was higher than the mean of methane level in the head space of three sterile bottles.
Enrichment of dental plaques

Dental plaques collected from all volunteers were directly inoculated into 20-ml serum bottles containing 1 ml standard medium amended with penicillin G (2000 U/ml) and hemin. The bottles were immediately sealed, gassed and pressurized as indicated above, and incubated at 37 °C in the dark.

Methane analysis

Gas chromatography (GC) was used to determine methane levels in volunteers’ breath, and in the gas phase in the enrichment cultures and MPN bottles. The gas chromatograph (Shimadzu GC-14 A) was fitted with a porpak column and a flame ionization detector. The chromatographic conditions were: 50 °C running temperature and 80 °C detection temperature. A standard mixture of 10% of methane in air was used to calibrate the gas chromatograph.

Coenzyme F-420 fluorescence

A Zeiss microscope was used to observe coenzyme-420 fluorescence of bacteria wet mount preparation of slurries, enrichment cultures and MPN bottles. The microscope was arranged so that the same filed could be observed by epifluorescence and by phase contrast microscopy. Furthermore, the microscope was attached to a video camera, a monitor and a computer to enable image capture and processing.

Isolation of methanogenic archaea

Agar bottles (3 % agar) or agar plates (1.5 % agar) were used to isolate methanogenic archaea from positive enrichments. The agar bottles were inoculated aseptically under nitrogen and then were gassed and pressurized as described above. The agar plates were prepared and stored in a gassed and pressurized canister until were used. Transfers from enrichment cultures onto agar plates were done in the anaerobic chamber. The inoculated
agar plates were placed in the canister, gassed and pressurized. All subcultures were incubated at 37 °C in the dark.

**Fluorescent in situ hybridization (FISH)**
Rhodamine labeled archaea probe was used to detect methanogenic archaea in feces. Treatment of specimens and hybridization reaction protocols were carried out as described in the workshop manual provided by Sandra Nierzwicki-Bauer.

**Extraction of DNA**
The method provided during the course was used to extract DNA from fecal slurries and enrichment cultures. A slurry sample was suspended in 1 ml 0.01 mM Tris buffer, pH 7.6, sonicated for 1 min and briefly centrifuged at low speed (3000 x g for 1 min). An aliquot (0.1 ml) of the supernatant was washed in 1 ml Tris buffer and centrifuged at 13000 x g for 5 min. The resulting pellet was suspended in 0.1 ml Tris buffer. Similarly, 0.1 ml volume of the enrichment culture was mixed with 0.9 ml of Tris buffer, centrifuged at 13000 x g for 5 min and the pellet was suspended in 0.1 ml Tris buffer.

**PCR amplification of the 16S rDNA**
Universal archaea primers, 1Af forward (position 1) and 1100Ar reverse (position 1100) were used for PCR amplification of DNA extracted from slurries and enrichment cultures. The amplification was carried out in 50 µl reaction volume: 1 µl of template DNA with 49 µl of polymerase reaction mixture. Each reaction mixture received one bead containing the *Taq* polymerase. The PCR temperature profile was as follows: 94 °C for 5 min, 30 cycles of (94 °C for 5 sec, 62 °C for 45 sec, 72 °C for 90 sec), the amplification time of each cycle was extended for 5 sec. Amplified DNA was examined by horizontal electrophoresis in 1% agarose with 5 µl aliquots of the PCR product.
Cloning of 16S rDNA

Amplified 16S rDNA was made blunt ended and cloned using the ZeroBlunt PCR cloning kit (Invitrogen) according to the manufacturer instructions. To extract the cloned DNA four colonies of the resulting transformants were picked from every plate. Each colony was suspended in 0.1 ml sterile water, boiled for 5 min and centrifuged at 13000 x g for 1 min. From the supernatant 5 µl aliquot was taken and PCR amplified using Topo primers\(^1\) and the Vent (exo\(^{-}\)) polymerase in a 50 µl reaction volume. The PCR temperature profile was as follows: 94.5 °C for 5 min, 30 cycles of (94.5 °C for 45 sec, 53.5 °C for 45 sec, 72.5 °C for 100 sec). Amplified DNA was examined by horizontal electrophoresis as indicated in the previous section.

Restriction fragment length polymorphism (RFLP)

Aliquots of PCR products were mixed with the restriction endonucleases HinP1i or Msp I. Reaction mixtures of 10 µl containing: 5 µl PCR product, 1 µl restriction buffer NEBII and 0.5 µl restriction enzyme, were incubated overnight at 37 °C. Restricted DNA was analyzed by horizontal electrophoresis in 2% agarose.

Results and Discussion

Characterization of the volunteers based on breath test, MPN, and direct microscopy

According to the breath test Alex and Sabine can be considered as high methane excretors, Scott and David as low methane excretors and Yunko and Jeff as non methane excretors (Figure 1). Differences in the results of the two measurements of methane concentration in the breath might be due to a systematic error since two different persons carried out the measurement procedure.

Total number methanogens in the fecal of the volunteers was estimated by most probable

\(^1\) Topo forward primer (CCACTAGTAACGCCGCGCC) and reverse primer (CGGCCGCCAGTGATGATG)
number (MPN). Bottles with methane level above 0.01% after 6 days of inoculation were counted positive. The results of the MPN are not corresponded to the results of the breath test. The MPN only allows a distinction between methanogen harboring persons (9×10⁴ -2.5×10³ cells/g feces) and non-methanogen harboring persons (Figure 1). According to J.Dore et al. the threshold concentration of methanogens in the colon corresponding to the limit at which methane becomes detectable in the breath is 10⁷ - 10⁸ cells/g feces. They counted bottles positive when they contained methane levels above 200ppm after 14 days. The longer incubation time might be responsible for the higher cell number. Since the yielded cell number in Yunkos feces was within the error rate of the method (e.g.difficulties in setting up the dilution series because facial particle clogged the needle) she might be considered as non-methanogen harboring person.

Methanogens contain large concentration of F-420 coenzyme which make them detectable by fluorescent microscopy. Fluorescent cells were only visible in the feces of Alex and Sabine whereas no fluorescent cells were observed in the feces of David and Scott although their fecal MPN estimate were in the same order of magnitude as for Alex and Sabine.

**Primary enrichment**

Methane production was used as a indication of methanogenic activity. Methane production was detected in the enrichments from Alexs feces within 24h of inoculation. In case of Yunkos and Jeffs no methane production was measureable even after 188h. Since we were running out of compressed air it was only possible to determine the methane concentration after 138h - 189.5h in the fecal enrichments of the other volunteers (Figure 2). Within this incubation time the methane concentration in the headspace of the fecal enrichments of Alex, Sabine, David and Scott at the different incubation temperatures reached almost to the same amount of approximately 40% methane. Furthermore the addition of hemin and the enrichment in see water, respectively did not yield to a different methanogenic activity. (Figure 2)
A high variety in morphology of fluorescent cells was observed in the enrichment cultures of Alex, Sabine, David, and Scott. The population was dominated by short rods. Other observed forms were small cocci, long rods, cocci chains, and spindle shaped cells. The cells existed either as free cells, in clumps, or they staked to fecal particles. (Figure 9 - 11) These morphological types were seen in all enrichments. Methanogens were not observed in the enrichments of Yunko and Jeff by microscopy. Since no methanogenic activity and no fluorescence were observed for Yunko and Jeff no further enrichment experiments were carried out for them.

Secondary enrichment

1 ml of the primary enrichments of Alex, Sabine, David, and Scott was transferred to 9ml fresh medium to carry out a secondary enrichments at 30, 37, and 42°C. The aim of the experiment was to measure methane production with time, because this might show differences in the composition and amount of the methanogenic population in the feces of the volunteers.

An increase of the methane concentration in the headspace of the enrichment bottles was measured within 24h. (Figure 3)

At 30°C the methane production showed a linear development with time for all individuals. However the rise for David was lower than the rises of the other individuals which was the same for them. (Figure 5)

At 37°C the methane production of Alex and Sabine was exponential with time whereas Sabines enrichment showed a higher level of methane production. The methane production for David and Scott increased rapidly after 92,5h and 142h, respectively.

After 214h the methane concentration in the headspace of their bottles was as high as the methane concentration in Sabines enrichment. (Figure 5)

At 42°C the methane production increased with time for all individuals in different manner. However David and Scott showed the highest concentration of methane after 214h (Figure 5).
The highest methanogetic activity for Alex was observed at 30°C, whereas Sabines enrichments showed the highest level of methane production at 37°C. David and Scott methanogens were most highly active at 42°C. (Figure 3)

The observed discrepancies in methane production in the enrichments can be due to different cell numbers in the cultures or the cells might be in a different state of growth in the several enrichment cultures. The counting of fluorescent cells by microscopy after 142h was used to determine the number of active cells to calculate the methane production per active cell. The extraction of the coenzyme F-420 after 214h was used as an second estimate of the number of actively growing cells.

The cell count after 142h showed that the differences of methane concentrations at this time is mainly due to different numbers of active cells in the enrichments, since the methane production per active cell is in the same range for each enrichment culture (Figure 6). Appearance of the methanogens in clumps and as attachments to fecal particle caused difficulties in the cell count and might have led to errors in the determination of the cell number.

The OD 420 of the extracted material was considered as the concentration of the coenzyme F-420 and it was related to the methane concentration at 214h. This led to an estimation of methane produced by a certain amount of coenzyme F-420. (Figure 6) These ratios also showed that the methane production per “coenzyme” was the same for every enrichment. Thus the different determinations of methane production per active cell at two different time points led to the same conclusion.

A UV spectrogram of the extracted solution of each individual was carried out. (see figure 7) All of them except David showed the same absorption pattern (Figure 7). The extraction of Davids enrichment culture has two absorption peaks at 675nm and 400nm. The absorption at 675nm is characteristic for chlorophyll A. The absorptions at 400nm might be due to other chromophors in the fecal sample since the applied extraction method was not specific for the F-420 coenzyme.
Third enrichment

According to the cell count of the secondary enrichments, $10^7$ cells were inoculated in 9ml fresh medium at 30, 37, 42°. The third enrichments were carried out in duplicates. All enrichments started with the same amount of cells and hence different methane production rates can be due to different growth rates of the methanogenic population. The methane production rate was approximately the same for every enrichment culture except for Davids. (Figure 4) They produced methane at higher levels. As shown in the UV spectrogram there are differences in the composition of Davids enrichments which might account for the higher production rate (Figure 7).

The more often the methanogens where subcultivated the lower was the methane production in the enrichments. That might be due to a lack of fecal components or other species from the fecal sample in the enrichment media. A other reason could be that the cells were at an different growth states at the time they were subcultivated. That mean they were different in thier state of enzyme production and hence in their ability to adaptate to the new media.

Isolations

Enrichments at every state were streaked out on agar plates and agar bottles. Methane production was detectable in the agar bottles but no fluorescence were observed. Also, colonies grown on agar plates did not show fluorescent cells. Since no other detection pattern for methanogens was available it was not possible to determine that the isolated colonies are methanogens.

Enrichment of methanogens in dental plaque samples

No methane production and no fluorescent cells were detectable in the enrichment cultures of the dental plaque samples of each volunteer. Methanogens have been isolated from dental plaque by Belay et al., however all methanogen positive samples were from individuals with some degree of periodontal disease. Since non of the volunteers has such
a disease, we might have not been able to detect any methanogens in their dental plaque.

In this report we were able to confirm that methanogens are cultivateable from the feces of methane excretors whereas no methanogens were cultivateable from the feces of non-methane excretors. However the amount of excreted methane in the breath is not related to concentration of methanogens in the feces, since other factors - like e.g. the uptake of methane into the blood - also influence the excreted amount of methane.

The hydrogen produced by fermentive degradation of nutrients in the colon is utilized by three groups of anaerobic microorganisms methanogenic archaena (MA), sulfate-reducing bacteria (SRB) and acetogenic bacteria (AB). Since SRB are able to use other electron donors than hydrogen there are able to exist in the appearance of either MA or AB. Thus SRB might ferment lactate, transferring [H]-equivalents to dominant MA. AB and MA can only use hydrogen as a electron donor. Thus there is a competitive exclusion of them.

Thermodynamically methanogens always yield a more negative free energy than the acetogens. However, if the acetate concentration in the colon is in a nM range the AB gain a more negative free energy than the MA (Figure 8). Thus the synthetic acetate has to be immediately adsorbed by the host if the AB shall outcompete the MA. Hackstein and van Alen studied the appearance of methanogens in different species. They found that differences in the dietary habits were irrelevant for the strength of methane emission. They made the course of evolution responsible for the lost of methanogens due to the lost of a specific receptor for methanogens within the host.

**Fluorescent in situ hybridization (FISH)**

Fecal specimens from four methane excretors and one non-methane excretor, were examined for methanogenic archaea by FISH. Undiluted and diluted (1:100) specimens were hybridized with rhodamine labeled archaea probe and a combined mixture of three rhodamine labeled universal probes. Fluorescent cells were not seen in any of the examined specimens, undiluted or diluted, that were hybridized with either the universal or archaea probes. Since no signal was obtained with the universal probes it would be
possible that targeted molecules were not accessible for hybridization. In order to investigate this possible reason, the same probes and hybridization conditions were applied to samples from fecal enrichment cultures containing autofluorescent methanogens. Prior to hybridization, the samples were washed extensively (3 times) to eliminate any possible interfering substances that might be present in the growth medium. Following this treatment fluorescent cells were observed in samples hybridized with the universal but not with the archaea probes. Extensive washing could not have reduced the number of methanogens, because F-420 fluorescent cells were seen in the washed specimens. Many of these cells, however, were adhering to fecal particulate material which remained in the washed specimens. Probably the adherent cells were not easily permealized during fixation with methanol:formaldehyde, and hence cellular target molecules were inaccessible to the probe. Alternatively, the cells could have been accessible to the probe but the fluorescent signal was masked by the intense autofluorescence of the particulate matter which the cells were attached to. Autofluorescence could be overcome by using probes labeled with other fluorochromes. On the other hand, the used archaea probe might not have formed stable hybrids under the described hybridization conditions. To test this argument it would be useful to use different archaea probes individually or combined.

**Restriction fragment length polymorphism (RFLP)**

PCR-amplification of DNA extracted from fecal specimens and enrichment cultures (Table 1) resulted in barely visible bands in agarose gel. The amplified samples might contain low concentration of extracted DNA or inhibitors of the Taq polymerase activity. The former assumption is more convincing because original samples were tenfold diluted prior to extraction of DNA. The dilution step was carried out according to a previous experience during the course, where a tenfold diluted sample of enrichment for methanogens gave a stronger signal of 16S rDNA PCR-products than an undiluted specimen.
Table 1. Results of 16S rDNA PCR-amplification of DNA extracted from fecal specimens and enrichment cultures

<table>
<thead>
<tr>
<th>Subject</th>
<th>Sample for DNA extraction</th>
<th>Electrophoretic analysis of PCR product (16S rDNA)</th>
</tr>
</thead>
<tbody>
<tr>
<td>1 (methane excretor)</td>
<td>Feces</td>
<td>Faint band</td>
</tr>
<tr>
<td></td>
<td>Enrichment @ 37</td>
<td>Faint band</td>
</tr>
<tr>
<td></td>
<td>Enrichment @ 42</td>
<td>Faint band</td>
</tr>
<tr>
<td>2 (methane excretor)</td>
<td>Feces</td>
<td>Faint band</td>
</tr>
<tr>
<td></td>
<td>Enrichment @ 37</td>
<td>Faint band</td>
</tr>
<tr>
<td></td>
<td>Enrichment @ 42</td>
<td>Faint band</td>
</tr>
<tr>
<td>3 (non methane excretor)</td>
<td>Feces</td>
<td>Faint band</td>
</tr>
<tr>
<td></td>
<td>Enrichment @ 37</td>
<td>Faint band</td>
</tr>
<tr>
<td></td>
<td>Enrichment @ 42</td>
<td>Faint band</td>
</tr>
<tr>
<td>Total Samples</td>
<td></td>
<td>9</td>
</tr>
</tbody>
</table>

Cloning of the PCR-products, despite of their low DNA contents, resulted in variable number of transformant colonies (Table 2). It was possible to retrieve the inserts from 4 of the 16 clones (Table 2). The number of inserts could have been higher if more clones (> 4) were screened per sample.

Table 2. Results of cloning of PCR-amplified DNA from fecal specimens and enrichment cultures

<table>
<thead>
<tr>
<th>Subject</th>
<th>Original sample</th>
<th>No. of transformant colonies</th>
<th>No. of screened clones for insert</th>
<th>No. of retrieved inserts</th>
</tr>
</thead>
<tbody>
<tr>
<td>1 (methane excretor)</td>
<td>Feces</td>
<td>&gt;10</td>
<td>4</td>
<td>none</td>
</tr>
<tr>
<td></td>
<td>Enrichment @ 37</td>
<td>&gt;10</td>
<td>4</td>
<td>1</td>
</tr>
<tr>
<td></td>
<td>Enrichment @ 42</td>
<td>&gt;10</td>
<td>ND</td>
<td>ND</td>
</tr>
<tr>
<td>2 (methane excretor)</td>
<td>Feces</td>
<td>&gt;100</td>
<td>4</td>
<td>2</td>
</tr>
<tr>
<td></td>
<td>Enrichment @ 37</td>
<td>&lt;10</td>
<td>4</td>
<td>none</td>
</tr>
<tr>
<td></td>
<td>Enrichment @ 42</td>
<td>&gt;10</td>
<td>ND</td>
<td>none</td>
</tr>
<tr>
<td>3 (non methane excretor)</td>
<td>Feces</td>
<td>&gt;10</td>
<td>4</td>
<td>1</td>
</tr>
<tr>
<td></td>
<td>Enrichment @ 37</td>
<td>&gt;10</td>
<td>4</td>
<td>none</td>
</tr>
<tr>
<td></td>
<td>Enrichment @ 42</td>
<td>&gt;10</td>
<td>ND</td>
<td>ND</td>
</tr>
</tbody>
</table>
The retrieved inserts were PCR-amplified and analyzed with restriction endonucleases. Restriction with Hin P1I yielded one visible fragment of about 500 bp from each insert (Table 3). In one case (subject 1), an additional fragment of about 400 bp was also obtained. The Msp1 endonuclease produced visible but less resolved fragments ranging between 200 - 300 bp (Table 3).

<table>
<thead>
<tr>
<th>Subject</th>
<th>Original sample</th>
<th>No. of retrieved inserts</th>
<th>No. and approximate size of the restriction fragments</th>
</tr>
</thead>
<tbody>
<tr>
<td>1 (methane excretor)</td>
<td>Enrichment 37</td>
<td>1</td>
<td>Hin P1I</td>
</tr>
<tr>
<td>2 (methane excretor)</td>
<td>Feces</td>
<td>1</td>
<td>1</td>
</tr>
<tr>
<td></td>
<td>Feces</td>
<td>1</td>
<td>1</td>
</tr>
<tr>
<td>3 (non methane excretor)</td>
<td>Feces</td>
<td>1</td>
<td>1</td>
</tr>
</tbody>
</table>

* NR : poorly resolved bands.

*Hin* P1I restriction pattern indicates that the 3 inserts from subjects 2 and 3 may originate from the same 16S rDNA. In other words, these two subjects may harbor the same methanogenic archaea. Subject 1, however, may harbor a different species.

The PCR-amplified products from the retrieved inserts were submitted for sequencing. Inserts originating from DNA extracted from samples of subject 1 and subject 3 (Table 2) were not suitable for sequencing. Subject 1 was a methane-excretor and harbors methanogenic archaea in the colon as evidenced by MPN estimates and enrichment culture from which the DNA was extracted. On the other hand, subject 3 was a non-methane-excretor and methanogenic archaea could not be demonstrated in the fecal enrichment. Consequently, it is most likely that these inserts represent an artifact product of cloning.

The other two clones originated from DNA extracted from subject 2 who was methane-excretor, but fluorescent methanogenic archaea could not be demonstrated by direct examination of the fecal sample. Blast search analysis of sequences of these two inserts
showed that one clone\textsuperscript{2} was similar (95\%) to \textit{Methanobacterium thermoflexum}, whereas the other clone\textsuperscript{3} was similar (97\%) to \textit{Methanobacterium thermoautotrophicum}. In other words, the two clones might be closely related to the genus \textit{Methanobacterium}. It would have been more intriguing if these clones were produced from the enrichment cultures, particularly that grew at 42 °C. Furthermore, these findings could have been verified if pure cultures were obtained from the feces of this subject.

The only two methanogenic archaea described to occur in the human colon are \textit{Methanobrevibacter smithii} and \textit{Methanosphaera stadtmanaee} using culture-based techniques. The former is known to be the dominant species. Our findings, using the molecular approach, suggest that other methanogenic archaea may reside in the human colon, and pave the way for further investigation of the diversity of the methanogenic archaea in this habitat.

\textsuperscript{2} This clone was designated as Methanobacterium clone MBL-E7.

\textsuperscript{3} This clone was designated as Methanobacterium clone MBL-E8
Suggested References


Characterizations of volunteers based on breath test, MPN and direct microscopy

Breath test

Methane (%)

Alex  Sabine  David  Scott  Yunko  Jeff

breath test I  breath test II

MPN

Cells/g

Alex  Sabine  David  Scott  Yunko  Jeff

1.00E+05
1.00E+04
1.00E+03
1.00E+02
1.00E+01
1.00E+00

Direct microscopy - autofluorescence

Alex  Sabine  David  Scott  Yunko  Jeff

+  +  -  -  -  -

Figure: 1
Figure 3

Scott

David

Sable

Alex

Enrichment
How much methane is produced by one cell?
### System 1: variable temperature.

<table>
<thead>
<tr>
<th>Reaction No. (S.P)</th>
<th>Formula</th>
<th>State</th>
<th>Special remarks</th>
<th>Stoich. coeff.</th>
<th>Activity</th>
<th>Compound name</th>
<th>Not.</th>
</tr>
</thead>
<tbody>
<tr>
<td>4 s</td>
<td>H2</td>
<td>g</td>
<td></td>
<td>4</td>
<td>1,00E-04</td>
<td>Hydrogen</td>
<td></td>
</tr>
<tr>
<td>5 p</td>
<td>H2O</td>
<td>l</td>
<td></td>
<td>3</td>
<td>1,00E+00</td>
<td>Water</td>
<td></td>
</tr>
<tr>
<td>6 s</td>
<td>HCO3-</td>
<td>aq</td>
<td></td>
<td>1</td>
<td>1,00E-01</td>
<td>Bicarbonate</td>
<td></td>
</tr>
<tr>
<td>7 s</td>
<td>H+</td>
<td>aq</td>
<td></td>
<td>1</td>
<td>6,30E-08</td>
<td>Proton</td>
<td></td>
</tr>
<tr>
<td>8 s</td>
<td>CH4</td>
<td>g</td>
<td></td>
<td>1</td>
<td>7,30E-03</td>
<td>Methane</td>
<td></td>
</tr>
<tr>
<td>9 s</td>
<td>H2</td>
<td>g</td>
<td></td>
<td>4</td>
<td>1,00E-04</td>
<td>Hydrogen</td>
<td></td>
</tr>
<tr>
<td>10 p</td>
<td>H2O</td>
<td>l</td>
<td></td>
<td>4</td>
<td>1,00E+00</td>
<td>Water</td>
<td></td>
</tr>
<tr>
<td>11 s</td>
<td>HCO3-</td>
<td>aq</td>
<td></td>
<td>2</td>
<td>1,00E-01</td>
<td>Bicarbonate</td>
<td></td>
</tr>
<tr>
<td>12 s</td>
<td>H+</td>
<td>aq</td>
<td></td>
<td>1</td>
<td>6,30E-08</td>
<td>Proton</td>
<td></td>
</tr>
<tr>
<td>13 p</td>
<td>CH3COO</td>
<td>aq</td>
<td></td>
<td>1</td>
<td>v</td>
<td>Acetate</td>
<td></td>
</tr>
</tbody>
</table>

**Temperature (K):** 309

---

**Log plot:**

![Log plot](image)

**Reactions:**

- **Reaction 1:**
  - $dG(0)(T) = -173.15$  
  - $dG(T) = -42.62$

- **Reaction 2:**
  - $dG(0)(T) = -140.73$
  - $dG(T) = -50.808$

---

**Variables:**

- **Acetate:** 1,00E-10  1E-08  1E-06  0,0001  0,01

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**Figure:** 8