Report on Individual Project during MBL Summer Course Microbial Diversity 1995

On the Use of Molecular Techniques for the Characterization of Methanogenic and Sulfate-Reducing Enrichments

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Abstract

Sediment from Cedar swamp was diluted with anaerobic mineral medium and incubated at 15°C and 35°C. Cellobiose was added to some of the cultures and methane formation and organic acid production was followed in time. In cultures incubated at 35°C where cellobiose was added, the pH dropped and a lot of organic acids such as propionate and butyrate accumulated. This process was slower at 15°C and in addition also acetate could be observed. Methane was formed in all cultures but only in small amounts. DNA was extracted from these cultures after 8 days of incubation. Even after purification and dilution of this DNA, it was not possible to obtain a PCR product of the 16S rRNA. It was therefore also not possible to study a possible shift in the methanogenic population by making bacterial community fingerprinting of amplified 16S rRNA gene sequences and restriction endonuclease analysis. The sediment sample from Buzzards Bay that was used to enumerate the sulfate-reducing bacteria contained $2 \cdot 10^4$ SRBs per g dry sediment as estimated by a MPN count. It was impossible to enumerate the sulfate-reducing bacteria by *in situ* hybridization. Only a small percentage of the bacteria showed up after *in situ* hybridization with a universal probe compared with the amount of DAPI stained cells. The bacteria did apparently not contain enough ribosomal RNA. Although about 20-30% of the cells showed a signal with the universal probe, none could be detected with the SRB probe.

Introduction

The use of gene probes for groups of bacteria makes it possible to observe specific bacteria directly in environmental samples. The probes are mainly designed with 16S rRNA as a target and there exist already a fair amount of specific probes and the number is increasing with every month. This new methodology perfectly complements the methods used so far in microbial ecology. Groups of bacteria were normally either

enumerated by plate counts or most probable number (MPN) counts. The activity was assessed by measuring metabolic products or by measuring the turn-over of labelled substrates. All methods on its own have their drawbacks. Either one found metabolic products but did not know what kind of bacteria were responsible for the formation or one found a group of bacteria present in an environmental sample but did not know whether it was metabolically active or not. With the gene probes technique one can visualize bacteria specifically in an environmental sample and quantify them. However, one can only visualize them if they are metabolically active since only cells which contain high enough amounts of ribosomal RNA can be detected. A combination of more traditional methods with molecular probing techniques makes it certainly possible to obtain a better insight in the microbial world in the environment.

The aim of this project is to apply molecular techniques to observe shifts in bacterial populations or to enumerate them. In methanogenic enrichments at mesophilic temperatures, methane formation is a result of hydrogenotrophic and acetoclastic methanogenesis, whereas at psychrophilic temperatures acetoclastic methanogenesis is the major methane forming process. The latter seems to be the result of a preferential hydrogen consumption by acetogens at low temperatures. The objective of this part of the project is therefore to investigate whether a difference of the methanogenic community can be observed if enrichments are incubated at 15°C and 35°C. The differences in the population will be evaluated by making bacterial community fingerprinting of amplified 16S rRNA gene sequences and restriction endonuclease analysis. In the second part of the project, the sulfate-reducing populations of a sediment sample of Buzzards Bay will be enumerated by either the MPN-method or by in situ hybridization and microscopically counting. The results of the two enumeration methods will then be compared.

Materials and Methods

Source of inoculum

For the methanogenic enrichments, sediment was collected from the Volta flame pond. It was stored at 15°C for four days before it was used. Buzzards Bay sediemnt for the exoeriment with sulfate reducers was collected during the cruise on July 1 and 2, 1995. It was stored at 15°C under aerobic conditions for 9 days prior to use.

Methanogenic enrichments

The medium utilized contained (g/l, if not otherwise indicated): KH_2PO_4, 0.2; NH_4Cl, 0.25; KCl, 0.5; $CaCl_2x2H_2O$, 0.15; NaCl, 1.0; $MgCl_2x6H_2O$, 0.62; $NaHCO_3$, 2.5; Na_2S, 0.24; trace element solution SL-10, 1 ml; vitamin solution, 1 ml; vitamin B_{12}, 1 ml;

resazurin solution, 1 ml. The first six salts together with the resazurin solution were dissolved in 950 ml of water. The solution was heated until boiling and cooled under flushing with N_2/CO_2 (4/1; v/v). An aliquot of 38 ml was dispensed in 150 ml serum bottles. The bottles were sealed with butyl rubber stoppers and the gas phase was changed with N_2/CO_2 (4/1; v/v). After autoclaving, 1 ml of a filter-sterilized stock solution containing the vitamins and the trace elements and 1.2 ml of a stock solution containing $NaHCO_3$ and Na_2S was added aseptically by syringe. To four bottles cellobiose was added from a stock solution to a final concentration of 25 mM. The enrichments were prepared in the anaerobic glovebox. Approximately 200 ml of the Volta flame pond sediment that contained a lot of decomposing leaves was washed with 100 ml medium and sieved through a mosquito net with mesh size of approximately 1 mm. This resulted in 180 ml sediment slurry. An aliquot of 18 ml was added to nine bottles with 40 ml medium. Outside the glovebox the gas phase was changed to N_2/CO_2 (4/1; v/v) again. Two bottles without substrate and two bottles with substrate (cellubiose) were incubated at 15°C and 35°C, respectively. Methane was measured after 4 h of incubation and once every second day. A sample for the acetate measurement at time 0 was taken from the ninth bottle. From this bottle also three samples of 1.5 ml were collected and microcentrifuged for 5 minutes. The pellet was frozen until DNA extraction.

MPN of sulfate reducers

The medium utilized contained (g/l, if not otherwise indicated): Citric acid, 0.12; Na_2SO_4, 4.0; $FeSO_4x7H_2O$, 0.1; KH_2PO_4, 0.2; NH_4Cl, 0.25; KCl, 0.5; $CaCl_2x2H_2O$, 0.15; NaCl, 20.0; $MgCl_2x6H_2O$, 3.0; $NaHCO_3$, 2.5; Na_2S, 0.02; $Na_2S_2O_4$, 0.02; trace element solution SL-10, 1 ml; vitamin solution, 1 ml; vitamin B_{12}, 1 ml; resazurin solution, 1 ml; Na-acetate, 0.8; Na-lactate, 1.1; Na-propionate, 0.6. The first nine salts together with the resazurin solution and the substrates acetate, lactate, and propionate were dissolved in 950 ml of water. The solution was heated until boiling and cooled under flushing with N_2/CO_2 (4/1; v/v). An aliquot of 9.5 ml was dispensed in Hungate tubes. The tubes were sealed with butyl rubber stoppers and the gas phase was changed with N_2/CO_2 (4/1; v/v). After autoclaving, 0.25 ml of a filter-sterilized stock solution containing the vitamins and the trace elements and 0.3 ml of a stock solution containing $NaHCO_3$, $Na_2S_2O_4$,and Na_2S was added aseptically by syringe. The medium was still pink. One gram of sediment was added to one tube. Aliquotes of 1 ml of this sediment slurry were transferred to three tubes by syringe. The sediemnt contained only very fine material and a 22 gauge did not get clogged. These tubes were considered to be the 100x dilutions of the MPN count. The dilutions were continued down to 10^{-9}. The tubes were incubated at 30°C and FeS precipitation was checked to occur after 15 days of incubation.

Extraction of cells followed by fixation versus direct fixation of cells

Bacteria were extracted from Buzzards Bay sediment either by sonication or treatment with pyrophosphate±Nonidet P-40. For the extraction by sonication, 1 g of sediment was suspended in 10 ml PBS (130 mM NaCl, 7 mM Na_2HPO_4, 3 mM NaH_2PO_4, pH 7.2), sonicated at low power three times for 10 s on ice. Subsequently, the slurry was vigorously vortexed for 1 min. The tubes were set on ice for 20 min, centrifuged in the table centrifuge for 2 min, and the supernatant was transferred to a new tube. The supernatant was centrifuged for 5 min at 5000 rpm in a centrifuge of the Physiology Course. The pellet was resuspended in 1.5 ml PBS and transferred to 2 ml tubes. The pellet was washed three times with PBS. Finally, the pellet was resuspended in 1.5 ml fixation buffer (40 g/l paraformaldehyde in 1xPBS, pH 7.2-7.4) and incubated at 4°C overnight.

For the extraction with pyrophosphate, 1 g of sediment was suspended in 10 ml of a 0.1% sodium pyrophosphate solution (pH 7.2). To one tube a drop of Nonidet P-40 was added in addition. These samples were subsequently treated as described for the sonication extraction after sonication.

For direct fixation, 1 g of sediment was suspended in 5 ml fixation buffer. After 2 min centrifugation in the table centrifuge, the supernatant was transferred to a new tube and the pellet was re-extracted with 2 ml fixation buffer. After subsequent centrifugation in the table centrifuge (2 min) the supernatants were combined and incubated at 4°C overnight.

The next morning, the samples were microcentrifuged, washed twice with PBS, and finally resuspended in 1 ml PBS/ethanol (1/1; v/v). They were stored at -20°C.

DAPI staining

Aliquots of 10 µl of the samples or of dilutions of it were applied on gelatine coated slides. After air-drying at 45°C, the sample was dehydrated in 50, 80, and 96% ethanol for 3 min and air-tried in a vertical position. On each well, 5 µl PBS and 5µl DAPI solution (10 µg/ml) were applied. After 5 minutes incubation in the dark, the slides were rinsed with destilled water and air-dried. After dehydration and before DAPI staining, also *in situ* hybridizations were carried out.

In situ hybridization

The *in situ* hybridization was carried out as described in the general procedure (step 3 and further) of the handouts of Sandra Nierzwicki-Bauer with some modifications. The hybridization mix was warmed up to 45°C prior to application on the slides and the slides were rinsed with 1xSET after hybridization.

PH

DNA extraction, PCR amplification of ribosomal DNA fragments, and restriction with endonucleases

The DNA was extracted from environmental samples after a protocol handed out during the course and developed by A. Sghir and J. Doré. The DNA was afterwards purified with the QIAEX II Gel Extraction Kit from QIAGEN or the XTreme Spin Column Kit from Pierce. The PCR was carried out following a protocol for Micro-PCR also provided by J. Doré.

Analyses

Methane was measured by GC/FID. Organic acids were analyzed by HPLC connected to a differential refractometer.

Microscopy

Slides with DAPI stained cells and cells after *in situ* hybridization were examined with a Zeiss Axioplan microscope equipped with the corresponding filter sets.

Results and Discussion

Shift in the methanogenic population by making bacterial community fingerprinting of amplified 16S rRNA gene sequences and restriction endonuclease analysis

Sediment from Cedar swamp was diluted with anaerobic mineral medium and incubated at 15°C and 35°C. Cellobiose was added to two cultures and two cultures did not obtain any additional substrate. Methane formation and organic acid productio n was followed in time. In cultures where cellobiose was added and which were incubated at 35°C, the pH dropped to pH 4.5 and a lot of organic acids such as propionate and butyrate accumulated (Fig.1). This process was slower at 15°C and in addition also acetate could be observed (Fig.3). The lack of acetate accumulation at 35°C is surprising since the low methanogenic activity in all cultures could not explain this phenomenon. The pH of the cultures was adjusted to neutral pH by adding sodium carbonate every second day. Methane was formed in all cultures but only in small amounts (Fig.1-4). The formate observed in all cultures at day 6 and 8 is probably a systematic error rather than actual formation in the cultures.

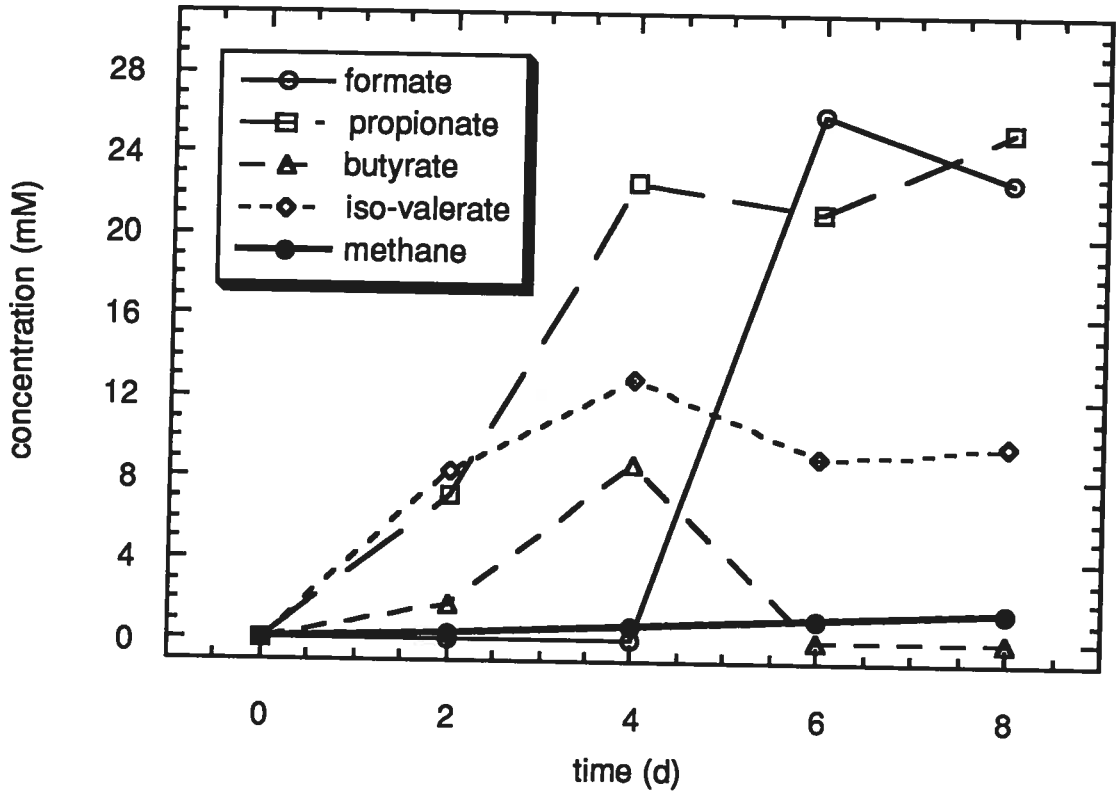

Fig.1. Formation of methane and organic acids in a methanogenic enrichment at 35°C amended with cellobiose

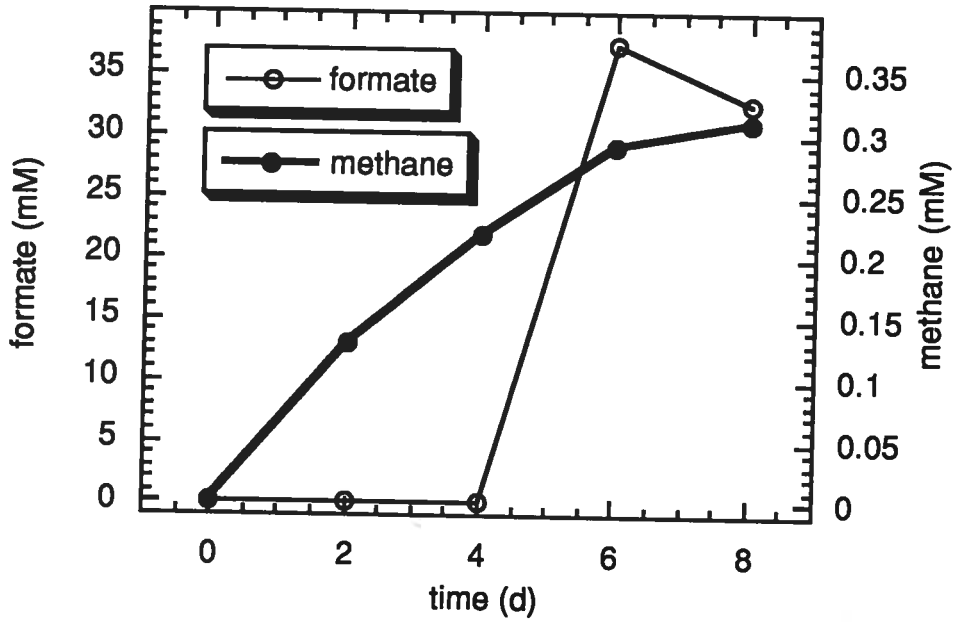

Fig.2. Formation of methane and formate in a methanogenic enrichment at 35°C.

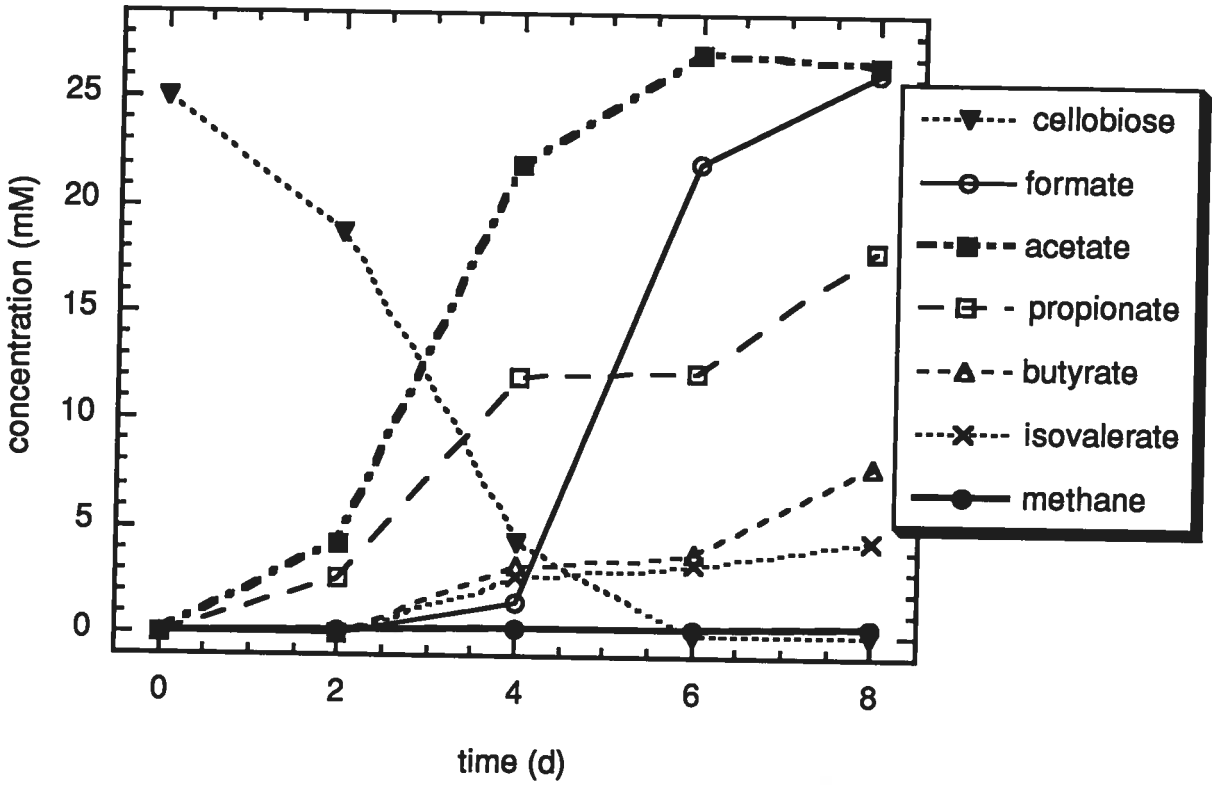

Fig.3. Formation of methane and organic acids in a methanogenic enrichment at 15°C amended with cellobiose

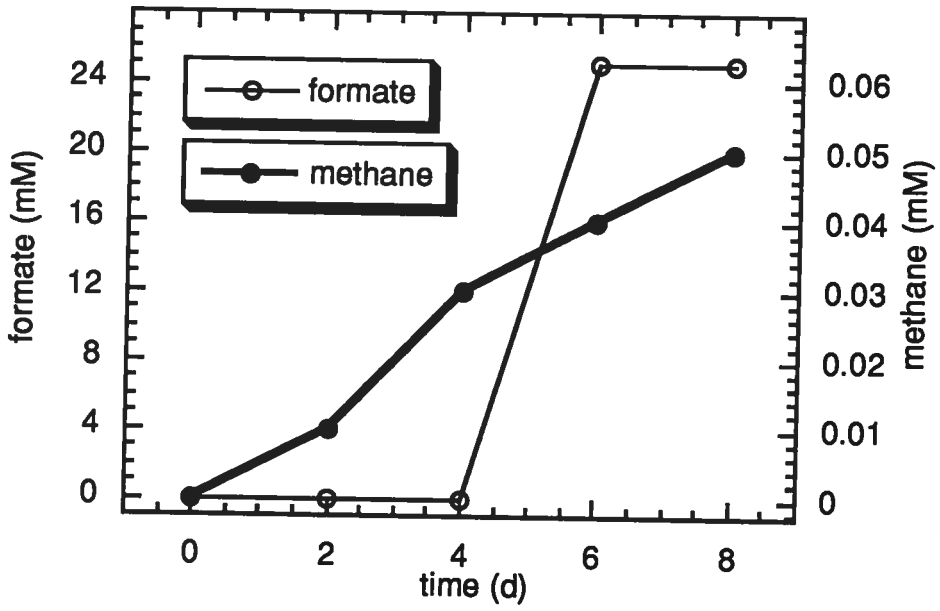

Fig.4. Formation of methane and formate in a methanogenic enrichment at 15°C.

DNA was extracted from these cultures after 8 days of incubation. Even after purification and dilution of this DNA down to 10^{-4}, it was not possible to obtain a PCR product of the 16S rRNA either with universal or archaea primers. The DNA was still brownish after the purification although much less compared with the color before the purification. The positive controls with DNA from *Bacteroides* and *Methanobrevibacter* showed that not one of reagents of the PCR kit was responsible for the lack of a PCR product but probably an inhibitory component still present in the DNA from the environmental sample, possibly humic substances. It was therefore not possible to test whether different fingerprints could be observed upon restriction of these PCR products with endonucleases and to obtain first indications of a shift in the methanogenic population after incubation at two different temperatures.

Enumeration of the sulfate reducing bacteria in a sediment sample by the MPN method and by in situ *hybridization*

The sediment sample from Buzzards Bay that was used to enumerate the sulfate-reducing bacteria either by the MPN method or *in situ* hybridization contained $2 \cdot 10^4$ SRBs per g dry sediment as estimated by a MPN count. Tests with different extraction procedures to recover the cells from the sediment material compared with direct fixation of the cells showed that direct fixation was the most effective method. The sediment sample contained a lot of very fine particles that did not sedimentate very fast after extraction. Therefore the samples still contained high amounts of particles. For mounting the samples on the slides, they had to be diluted 100 times to obtained best resolution under the microscope. In addition, part of the material was washed off the slide when the 10 times dilution was applied. It was impossible to exactly enumerate the cells because they were not evenly distributed over the slide. DAPI stained cells were only observed in fields where also a lot particles were present. It was also impossible to enumerate the sulfate-reducing bacteria by *in situ* hybridization. Only a small percentage of the bacteria showed up after *in situ* hybridization with a universal probe compared with the amount of DAPI stained cells. Furthermore, the bacteria were only visible with the universal probe if the signal was integrated during 30 seconds. The bacteria did apparently not contain enough ribosomal RNA to allow detection after hybridization with 16S rRNA gene probes. Although about 20-30% of the cells showed a signal with the universal probe, none could be detected with the SRB probe. This is not too surprising provided that the amount of SRBs present in the sediment as determined by the MPN count was a good estimate of the exact amount of SRBs. The total amount of bacteria was not determined. However, if one would assume that approximately 10^7 bacteria per g dry sediment were present, the chance to see $2 \cdot 10^4$ SRBs under the microscop is quite small.