Biogenesis of methane in primate dental plaque

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Dental plaque samples collected from monkeys (*Macaca mulatta*) were found to contain a large amount of dissolved methane gas (0.6 nmol CH_4/mg wet wt plaque). Enrichment cultures inoculated with dental plaque obtained from *Macaca fascicularis* produced methane when the medium contained ethanol, methanol, lactate, acetate or a hydrogen + CO_2 atmosphere. Methane formation in the enrichments was inhibited by oxidation of the culture medium, autoclaving or the addition of 2-bromoethane sulfonic acid (BES). The methane producing enrichments were observed to contain fluorescent cocci occurring singly and in short chains. It was concluded that methane formation in the monkey dental plaque was the result of the presence of methanogenic bacteria.

Dental plaque methanogenesis *Methanogenesis* *Dental plaque* *Enrichment culture* *Methane* *Methane formation*

1. INTRODUCTION

Methane represents the most reduced form of carbon. The biogenesis of methane from hydrogen and carbon dioxide or other compounds such as acetate, formate and methylamines is due to methanogenic bacteria and has been well documented [1–3]. To date, the sources of methanogenic activity reported have been mainly sediments, sewage and the intestinal tract of man and ruminant animals [1,4–7]. In this report, we describe the presence of methane in samples of dental plaque obtained from monkeys and the methanogenic activity of enrichment cultures made from monkey plaque. Methanogenesis in dental plaque may be significant because methanogenic organisms represent the bottom of a carbon food chain and function to remove excess hydrogen from the environment. This process is an important factor in the regulation of carbon flow from substrates to reduced products [2,8,9].

2. MATERIALS AND METHODS

2.1. *Enrichment culture medium*

Enrichment cultures of dental plaque were maintained in serum stoppered 18 × 150 mm tubes (Bellco Glass, Vineland NJ). The tubes contained 5 ml standard medium 1 (from [1]), modified by the addition of 1% of the recommended yeast extract and trypticase and the omission of sodium acetate and formate.

2.2. *Methane analysis*

The quantity of methane present in the gas phase above samples of dental plaque or enrichment cultures was determined by gas–liquid chromatography. The analysis was performed using a Hewlett-Packard 5840A gas chromatograph (Hewlett-Packard, Palo Alto CA) fitted with a 90 × 0.2 cm Porapak-QS column and a flame ionization detector. The chromatographic conditions were: oven, 100°C; injector, 250°C; detector, 300°C; carrier gas, nitrogen 10 ml/min. A standard mixture of 100 ppm CH_4 in nitrogen

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(Supelco, Bellefonte PA) was used to calibrate the chromatograph.

3. RESULTS

Samples of whole mouth dental plaque were obtained from monkeys (*Macaca mulatta*). The plaque was removed with a dental scaler, placed in glass vials, transported to the laboratory and processed within 2 h of collection. The presence of methane in dental plaque was shown by assaying the gas phase above the samples before and during incubation at 60°C (table 1). This temperature was considered sufficiently high to release gas from the aqueous phase of the samples as a result of decreased solubility without causing a physicochemical disruption of the material. We found that the methane present in these samples was fully released after 60 min incubation. The methane concentration in the plaque sample was calculated on the assumption that the majority of the methane released had been dissolved in the fluid surrounding the cells. Assuming that plaque fluid comprised 35% of the wet weight of dental plaque [10] we found 2 μmol methane/ml which is close to the saturation point of methane in water.

The biogenesis of methane in enrichment cultures made from monkey plaque samples obtained from *Macaca fascicularis*, was inhibited by the presence of 2-bromoethane sulphonic acid (BES) in the enrichment medium (table 2). This compound specifically inhibits microbial methanogenesis through competitive inhibition with the methanogen-specific compound coenzyme M [11]. Methane formation was also inhibited by the exposure of the enrichment culture to sterilizing conditions or oxygen, or by the absence of hydrogen.

Table 1

Concentration of methane in the gas phase above *Macaca mulatta* dental plaque incubated at 60°C

Sample no.	Wet wt (mg)	nmol CH_4/mg wet wt	
		0 min	60 min
1	208	0.12	0.60
2	81	0.04	0.19

Sample 1 represents full mouth plaque samples pooled from 3 monkeys. Sample 2 is a full mouth sample from an individual monkey of the same species. The plaque samples were placed in 1 ml conical vials fitted with serum caps. Methane, in 50 μl aliquots of the gas phase above the sample, was determined by gas–liquid chromatography

Table 2

Methanogenesis during incubation of tubes inoculated with an enrichment culture of *Macaca fascicularis* dental plaque

Incubation conditions	CH_4 (nmol/ml gas phase)			
	0 days	7 days	14 days	21 days
N_2	0	30	33	n.d.
N_2 + BES	0	0	0	0
H_2	0	130	1221	7348
H_2 + BES	0	0	5	5
H_2 (autoclaved)	0	0	0	0

Serum stoppered 18 × 150 mm tubes with 5 ml medium and 23 ml gas phase were given a 2% (v/v) inoculum in the absence or presence of 1.4×10^{-2} M BES. The atmosphere in each tube was adjusted to 2 atm. pressure with either 80% H_2 + 20% CO_2 or 80% N_2 + 20% CO_2 and incubated at 37°C. One set of hydrogen-containing tubes was sterilized in an autoclave (121°C for 20 min) after inoculation. The values presented represent the mean of 2 tubes; n.d., not determined

Further evidence for the microbial origin of methane in plaque was obtained by direct microscopic examination of plaque smears and aliquots of the enrichment cultures. The methanogen-specific chromophore, factor 420 [1] exhibits fluorescence upon excitation with longwave UV light. Fluorescent cocci, occurring singly and in short chains were observed in both types of samples with the aid of a UV microscope.

The methanogenic potential of the plaque enrichments with respect to different substrates was determined by incubation of the enrichments in a N_2 + CO_2 atmosphere with a variety of substrates (fig.1). The greatest conversion of substrate other than hydrogen to methane was observed with ethanol. Acetate, methanol and lactate also supported methane formation but to a lesser extent than hydrogen or ethanol. The production of methane from acetate has been documented [1]. The formation of methane from methanol and lactate was delayed with respect to ethanol and acetate and may indicate that

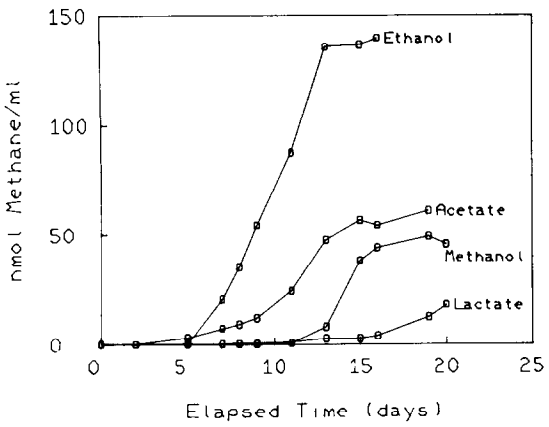

Fig.1. Methane production from various substrates by enrichment cultures derived from *Macaca fascicularis* dental plaque. The data presented are the [CH_4] (nmol/ml gas phase). Each tube contained substrate (30 mM) and an atmosphere of 80% N_2 + 20% CO_2 pressurized to 2 atm. The tubes were inoculated to a 2% (v/v) final conc. with an enrichment culture growing in an atmosphere of 80% H_2 + 20% CO_2.

methanol- and lactate-utilizing methanogens were present in low numbers in the sample inoculum or that methane was formed from a breakdown product of these substrates by other members of the enrichment culture. An example of the latter may be found in the *Veillonella* species present in dental plaque, which actively metabolize lactate to H_2, CO_2, acidic acid and propionic acid. The resulting H_2 could then be used by the methane bacteria for the reduction of CO_2 to CH_4. In addition to the substrates discussed above methanogenesis from N_2 + CO_2 and the mono-, di- and trimethylamines was tested and found to be negative after 20 days' incubation. The methylamines are metabolized by members of the *Methanosarcinaceae* [1] and the absence of methanogenesis from these compounds suggests that these methanogenic bacteria were not present in the plaque enrichment culture.

4. DISCUSSION

Here we show that the presence of methane in samples and enrichment cultures of dental plaque obtained from monkeys was due to the presence of methanogenic bacteria. The significance of these organisms in the microbial ecology of dental plaque may be by functioning as a mechanism to relieve the environment of excess hydrogen. The production of hydrogen by members of a microbiota ultimately inhibits the ability of other members to metabolize their primary substrates [2]. The continual removal of hydrogen from the environment by methanogenic bacteria could eliminate its inhibitory effect and thereby allow a more efficient utilization of substrates by the microbiota. The production of hydrogen by the anaerobic Gram-negative rods and Gram-positive cocci in dental plaque could thus be regulated by oral methanogens. In this respect the presence or absence of methanogenic bacteria in dental plaque may regulate the flow of carbon and perhaps determine which bacterial species will predominate in that microbiota. The isolation and characterization of the oral methanogens is necessary to fully understand the role of these bacteria in oral ecology.

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