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Methanogenic Bacteria from Human Dental Plaque

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Samples of human dental plaque were examined for the presence of methanogenic bacteria. Of 54 samples from 36 patients, 20 yielded H_2/CO_2-using methanogenic enrichment cultures. All methanogen-positive samples were from patients with some degree of periodontal disease. The predominant populations in the enrichments had morphologies characteristic of *Methanobrevibacter* spp. In six enrichments derived from three patients, the common methanogen was antigenically similar to *Methanobrevibacter smithii*. The same was true for the three methanogenic isolates obtained in axenic culture from a fourth patient. The six enrichments and two of the three isolates were antigenically closer to strain ALI than to PS. Two of the enrichments also had subpopulations with weak antigenic similarity to *Methanosphaera stadtmanae*. The data indicate that methanogens in the oral cavity of humans are antigenically close to those found in the intestinal tract.

Methanogenic bacteria belong to the group of organisms known as the archaebacteria (2, 9). They are strict anaerobes characterized by the ability to produce methane from H_2/CO_2 and, in some cases, from formate, acetate, methanol, or methylamine. Methanogenic bacteria have been isolated from sewage sludge, lake sediments, hot springs, and the intestinal tracts of animals, including humans. About one-third of the adult human population in the United States is reported to carry methanogens in the large intestine, with *Methanobrevibacter smithii* being the predominant methanogen present (5, 6, 18). However, the presence of methanogens in the human oral cavity has not been examined. Recently, the presence of methanogens in dental-plaque samples from monkeys was reported (13). We describe in this report our examination for methanogens in subgingival dental-plaque samples from patients at the University of Iowa College of Dentistry Clinics.

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With H_2/CO_2 (80:20 [vol/vol]) as the main source of carbon and energy, cells were grown in serum tubes (2048-00150; Bellco Glass, Inc., Vineland, N.J.) containing 5 ml of medium or in serum bottles (223746; Wheaton Industries, Millville, N.J.) containing 10 ml of medium by using the techniques described by Balch and Wolfe (3) and Daniels et al. (8). The medium consisted of the following components in distilled and deionized water: K_2HPO_4 (4.7 mM), $MgCl_2 \cdot 6H_2O$ (0.3 mM), NH_4Cl (7.5 mM), $CaCl_2 \cdot 2H_2O$ (0.3 mM), sodium acetate (20 mM), $Na_2S \cdot 9H_2O$ (2.0 mM), and resazurin (0.003 mM). A total of 10 ml of vitamin mix (21) and 10 ml of trace minerals elixir (as used for *Methanococcus thermolithotrophicus* [8]) was added per liter of medium. A total of 1.0 to 2.0 g/liter each of yeast extract (Difco Laboratories, Detroit, Mich.) and Trypticase peptone (BBL Microbiology Systems, Cockeysville, Md.) and 56 μg/ml each of filter-sterilized streptomycin and vancomycin were also added. The pH was adjusted to 6.5 by adding Na_2CO_3 while bubbling the medium with N_2/CO_2 (80:20 [vol/vol]). For growth experiments testing substrates other than H_2/CO_2, the medium was prepared under N_2/CO_2 and supplemented with formate (130 mM), acetate (117 mM), or methanol (116 mM).

Subgingival plaque was removed from the tooth with a sterile curette (Gracey 5/6, Hu-Friedy-Immunity) and placed into a tube or bottle containing medium. The medium was maintained under a stream of H_2/CO_2 (80:20 [vol/vol]) during transfer of the plaque sample by using a portable gas cylinder equipped with a regulator and hoses tipped with sterile needles. The samples were transported to the laboratory and further processed within 1 to 2 h. Each sample container was flushed thoroughly (for 15 min) with H_2/CO_2 (80:20 [vol/vol]) and pressurized to 140 kPa. To replace sulfide lost during gassing, a sterile sodium sulfide solution was added to a concentration of 1.4 mM. Each sample container was shaken vigorously to disperse the plaque. Incubation was carried out at 37°C, with the tubes or bottles standing upright. Each sample was shaken by hand one or two times daily, and methane production was monitored periodically for up to 21 days. Methane was measured by gas chromatography as described previously (4). Enrichments were plated on agar (2%) medium under a H_2/CO_2 gas phase by using the techniques described previously (3, 12). Isolated colonies were streaked twice, and final colonies were grown in liquid medium. Growth of the isolates was determined by measurement of methanogenesis and by culture turbidity measured at 600 nm in a Spectronic 20 spectrophotometer (Bausch & Lomb, Inc., Rochester, N.Y.).

Isolates and enrichment cultures were examined by using a phase-contrast microscope and a JEOL 100B transmission electron microscope operating at 60 kV. Cells for electron microscopy were negatively stained with 2% phosphotungstic acid with bacitracin to aid spreading.

Immunologic identification of methanogens in the enrichment and axenic cultures was done by comparing antigenic fingerprints according to published procedures (14, 15). Antigenic fingerprinting was done by using indirect immunofluorescence, quantitative immunoenzymatic assay (7), and a panel of polyclonal antibody probes for reference methanogens. The S probe (i.e., the last dilution in the

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plateau of the antiserum titration curve) was used throughout. The degree of antigenic similarity of each isolate in axenic culture with the relevant reference methanogens was measured by quantitative immunoenzymatic assay.

The results of our survey for methanogenic activity in human dental-plaque samples are shown in Table 1. Of 54 samples from 36 patients, 20 samples were positive for the presence of methanogenic bacteria. The periodontal disease status of each tooth from which a sample was taken was determined according to criteria of the American Dental Association (1). Of the 14 samples from no-periodontitis and early-periodontitis cases, 7% were positive for the presence of methanogens, whereas 26% of 23 moderate-periodontitis cases and 76% of 17 advanced-periodontitis cases yielded methanogen-positive samples. All methanogen-positive enrichments were examined with a phase-contrast microscope, which showed that the predominant population of cells in each case was morphologically similar to *Methanobrevibacter* species.

The methanogen-positive sample enrichments were maintained by continued transfer into the H_2/CO_2 medium containing yeast extract, Trypticase, and antibiotics. Some of the cultures were transferred into medium lacking yeast extract and Trypticase as well, with the aim of reducing heterotrophic populations and facilitating the isolation of methanogenic species. Isolates were obtained from three of the enrichment samples (30A, 30B, and 30C, as shown in Table 1) that grew well in the absence of yeast extract and Trypticase. The isolates have been designated Hdm-30A, Hdm-30B, and Hdm-30C. Examination with a phase-contrast microscope showed homogenous cultures with morphologies resembling the morphology of *Methanobrevibacter* species in all cases. Figure 1 shows a transmission electron micrograph of one of the isolates, Hdm-30C.

Each isolate used either H_2/CO_2 or formate as the substrate for growth and methane production. None of the isolates grew on acetate or methanol. Doubling times during growth on H_2/CO_2 (in the presence of 0.1% yeast extract)

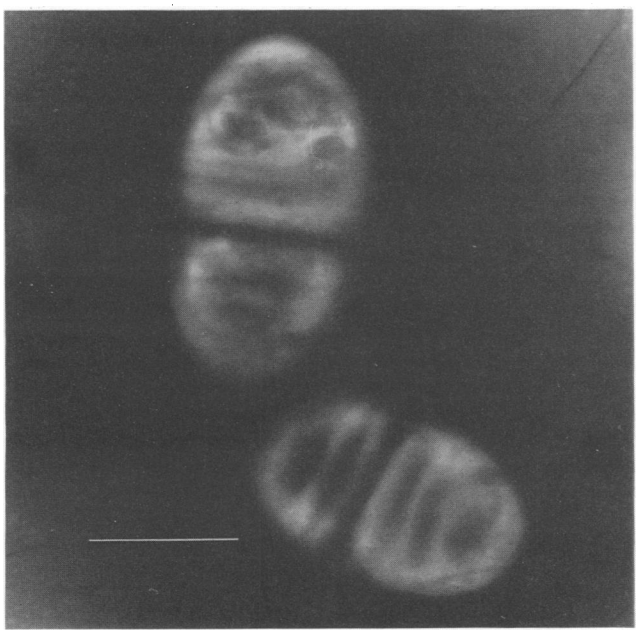

FIG. 1. Transmission electron micrograph of Hdm-30C, a methanogen isolated from human dental plaque. Bar, 0.5 μm.

TABLE 1. Periodontal condition by tooth and methanogenesis in human dental-plaque samples

Sample no.[a]	ADA periodontal classification[b]	Tooth no.[c]	μmol of CH_4/gas phase[d] (days of incubation)
1A	IV	3	37 (7)
1B	IV	5	402 (5)
2	II	18	0 (21)
3A	IV	15	0 (21)
3B	II	18	0 (21)
4	I	15	0 (21)
5A	III	23	84 (21)
5B	III	24	51 (21)
6	I	14	0 (21)
7A	III	19	0 (21)
7B	III	28	0 (21)
8	III	31	0 (21)
9A	III	25	182 (9)
9B	III	19	119 (9)
10A	III	13	0 (21)
10B	III	30	0 (21)
11	II	19	0 (21)
12	I	30	0 (21)
13A	II	19	0 (21)
13B	II	30	0 (21)
14A	II	14	0 (21)
14B	III	3	0 (21)
15A	I	3	0 (21)
15B	I	30	0 (21)
16	III	21	0 (21)
17	II	19	0 (21)
18A	IV	30	114 (11)
18B	IV	22	106 (11)
19	III	30	0 (21)
20A	IV	20	101 (11)
20B	IV	28	111 (11)
21	III	27	0 (21)
22	III	25	92 (11)
23	IV	14	0 (21)
24	IV	26	0 (21)
25	III	27	0 (21)
26	III	3	0 (21)
27	III	3	0 (21)
28	III	19	0 (21)
29	II	19	123 (6)
30A	IV	22	115 (6)
30B	IV	27	122 (6)
30C	IV	7	113 (6)
31	III	29	106 (6)
32	II	14	0 (19)
33A	IV	20	87 (6)
33B	IV	22	97 (6)
33C	IV	8	76 (6)
33D	IV	12	0 (19)
33E	IV	7	95 (6)
34	III	30	0 (19)
35	III	3	0 (19)
36A	III	29	0 (19)
36B	III	21	0 (19)

[a] Numbers indicate different patients, and letters indicate different samples from the same patient.

[b] Types: I, gingivitis but no bone loss; II, early periodontitis; III, moderate periodontitis; IV, advanced periodontitis. Types II to IV all show bone loss. ADA, American Dental Association.

[c] Tooth numbering is by the universal numbering method, starting with the upper rear right molar and proceeding through the upper rear left and lower rear left molars to the final lower rear right molar.

[d] Samples 1A and 1B were cultured in 70-ml serum bottles in 10 ml of medium, and all other samples were cultured in 25.7-ml serum tubes in 5 ml of medium.

were 17, 18, and 22 h for isolates Hdm-30A, Hdm-30B, and Hdm-30C, respectively. Streptomycin or vancomycin, at a concentration of 56 μg/ml, had no effect on growth of the isolates or on any of the enrichment cultures. However, all isolates and most of the enrichment cultures were inhibited (complete growth inhibition) by kanamycin at a concentration of 26 to 53 μg/ml.

Immunologic analysis of six of the enrichment cultures showed a minority cell subpopulation antigenically related to the reference methanogen *Methanobrevibacter smithii* ALI (Table 2). Two of these six cultures also showed a predominant cell subpopulation with a weak antigenic similarity to the reference methanogen *Methanosphaera stadtmanae* MCB3.

Partial antigenic fingerprinting of the three isolates by indirect immunofluorescence and quantitative immunoenzymatic assay showed a close antigenic relationship with the reference species *Methanobrevibacter smithii* but not with *Methanobrevibacter ruminantium* M1 or *Methanobrevibacter arboriphilus* strains AZ, DC, and DH1. A quantitative comparison of the antigenic similarities of the three isolates with each other and with the two *Methanobrevibacter smithii* PS and ALI reference strains showed that isolates Hdm-30B and Hdm-30C were different from PS (Fig. 2A), since the rates of reaction indicated by the change in A_{450} were distinct for PS. On the other hand, the three isolates were similar to ALI (Fig. 2B).

This study is the first demonstration of the presence of methanogenic bacteria in human dental plaque or in any human oral location. The immunologic data show that a common methanogen in the human dental-plaque samples (both enrichments and axenic cultures) is closely related to *Methanobrevibacter smithii*. This finding is of interest since previous microbiological and immunologic data showed that a predominant methanogen in human feces is also *Methanobrevibacter smithii*; isolation of several antigenically distinct strains has also been reported (18, 19). In addition, in two enrichment cultures we found a population of bacteria with a weak antigenic similarity to *Methanosphaera stadtmanae*, which is an organism originally isolated from human feces (16, 17). It should be noted that the three isolates that showed a close immunologic relationship with *Methanobrevibacter smithii* ALI and *Methanobrevibacter smithii* PS are from a patient different from those from which the enrichment cultures shown in Table 2 were derived, in which the predominant methanogen was antigenically related with *Methanobrevibacter smithii* ALI only. Data in Table 2, a quantitative comparison of antigenic fingerprints, and the measurements shown in Fig. 2, taken together, indicate that the predominant methanogen in human dental plaque is more closely related to *Methanobrevibacter smithii* ALI than to any other methanogen.

TABLE 2. Antigenic characteristics of bacterial subpopulations in methanogenic enrichment cultures of human dental plaque[a]

Enrichment culture	IIF reaction intensity[b] with S probe for:	
	Methanobrevibacter smithii ALI	*Methanosphaera stadtmanae* MCB3
1A	2 (15)	0
1B	3 (20)	0
5A	3 (20)	0
5B	2 (5)	0
9A	3 (30)	1 (55)
9B	3 (5)	1 (95)

[a] These enrichment cultures were obtained by transferring a 10% inoculum from the initial plaque enrichment tubes and allowing about 1 week for growth.

[b] The indirect immunofluorescence (IIF) reaction intensity was quantified from 0 (no reaction) to 4 (maximum reaction). The reaction intensities were 0 with the S probe for other reference methanogens, which included *Methanobacterium formicicum* MF, *Methanobacterium bryantii* MoH, *Methanobacterium bryantii* MoHG, *Methanobacterium thermoautotrophicum* ΔH, *Methanobacterium thermoautotrophicum* GC1, *Methanobrevibacter ruminantium* M1, *Methanobrevibacter arboriphilus* DC, *Methanobrevibacter arboriphilus* DH1, *Methanobrevibacter arboriphilus* AZ, *Methanococcus vannielii* SB, and *Methanococcus voltae* PSv. The percentages of all cells reacting with the probe are given in parentheses.

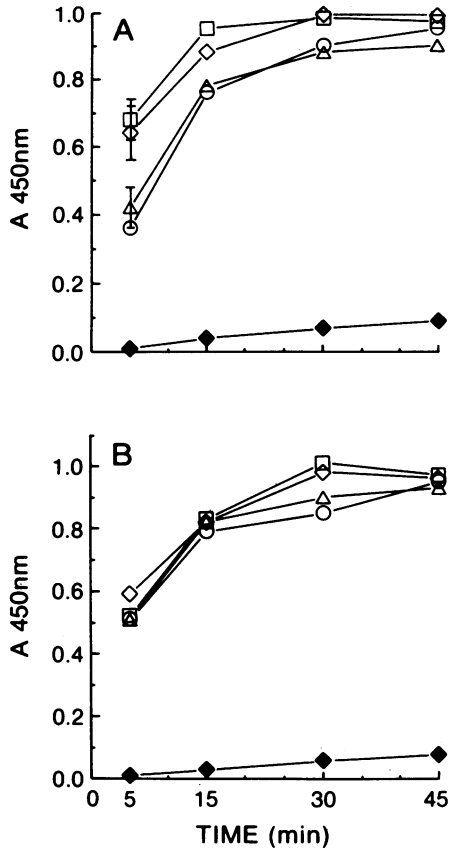

FIG. 2. Degree of antigenic similarity of each methanogenic isolate from human dental plaque with the reference strains *Methanobrevibacter smithii* PS (□, panel A) and *Methanobrevibacter smithii* ALI (□, panel B), determined by quantitative immunoenzymatic assay. The isolates were Hdm-30A (◇), Hdm-30B (○), and Hdm-30C (△). ◆, Negative control containing bacteria but normal serum. Results represent the average of three determinations. An illustrative example of range is shown only in panel A (5 min) for clarity.

In the United States and the United Kingdom, about 35% of adults have detectable methane levels in their breaths (10), which has been interpreted as a reflection of the presence of methanogens in their bowels. However, Hoshi et al. (11) have recently reported that in Japan, fewer than 10% are breath methane producers, while about 40% have methanogens in their feces. The differences between the U.S.-British and Japanese populations may possibly reflect differences in the abundances of dental-plaque methanogens. Further studies would be warranted to define the correlation between breath methane and those methanogens from dental plaque and the large intestine.

Of the 36 patients examined (54 samples), 9 (20 samples) yielded methanogen-positive samples. A significantly greater

likelihood for the presence of methanogenic bacteria was observed in patients with moderate- or advanced-periodontitis compared with those with no periodontitis and early periodontitis. Periodontal disease is characterized by growth in the subgingival area of a wide variety of anaerobic heterotrophic bacteria, including *Actinomyces*, *Bacteroides*, *Fusobacterium*, *Eikenella*, and *Selenomonas* species, which can lead to severe tissue damage and destruction (20). It is likely that the methanogen is taking advantage of the local microbial activity that ultimately produces the end products (e.g., formate or H_2/CO_2) that it needs for growth. The removal of these products by methanogenic activity in turn could increase total microbial activity and contribute to local tissue damage, in analogy with the role of methanogenesis in anaerobic degradation in waste conversion to methane and carbon dioxide.

The numerical significance of methanogens in relation to the total viable anaerobic microbial population needs to be determined to learn the possible ecological role of these bacteria in periodontal disease. It will be interesting to pursue such ecological study, as well as to examine the biochemical properties of the isolated methanogens in greater detail.

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