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Methanogenic Bacteria in Human Vaginal Samples

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Twelve vaginal samples were collected from separate patients, processed anaerobically, and added to methanogenic enrichment medium. Methanogenic activity was detected in two samples, both of which were from patients with bacterial vaginosis. None of the samples from healthy patients yielded positive methanogen cultures. One sample from a patient with bacterial vaginosis did not show any detectable methanogenic activity. Two methanogen isolates were obtained from one of the methanogen-positive samples, and both were identified as *Methanobrevibacter smithii* on the basis of morphological, cultural, and immunological features.

Methanogenic bacteria are strict anaerobes and derive their energy from conversion of simple substrates such as H_2-CO_2, formate, acetate, and methanol to methane. They are members of the recently established group of organisms known as the archaeobacteria (7). Methanogens have been found in a variety of ecological niches, including sewage sludge, marine and lake sediments, geothermal springs, deep-sea hydrothermal vents, and the intestinal tracts of animals (5, 7, 8). Moreover, about one-third of the adult human population in the United States harbors methanogenic bacteria in the large intestine (13), and the presence of methanogens in human dental plaque was recently documented (3, 4). *Methanobrevibacter smithii*, which uses either H_2-CO_2 or formate for growth, is the most prominent species of methanogen in both the human large intestine and dental plaque (3, 13). All individuals with methanogenic activity in their dental plaque had some degree of periodontal disease, although this was not the case with all diseased individuals (3). In the present study, we have tested for the presence of methanogenic bacteria in human vaginal samples.

The anaerobic techniques and procedures described by Balch and Wolfe (1) and Daniels et al. (6) were used throughout the study, and the medium consisted of the following components in distilled and deionized water (millimolar concentration): KH_2PO_4 (3.09), K_2HPO_4 (1.26), NH_4Cl (7.48), sodium acetate (20), NaCl (5.13), $NaHCO_3$ (23.8), $MgCl_2 \cdot 6H_2O$ (0.32), $CaCl_2 \cdot 2H_2O$ (0.33), sodium selenate (0.0006), sodium tungstate (0.0006), and 2-mercaptoethanesulfonate (0.0005); 10 ml each of vitamin mix (14) and trace minerals elixir (as used for *Methanococcus thermolithotrophicus* [6]) per liter was added, and 1 g each of yeast extract (Difco Laboratories, Detroit, Mich.) and tryptone (BBL Microbiology Systems, Cockeysville, Md.) per liter was also added. Adjustment of the pH was done by the addition of Na_2CO_3 while the medium was bubbled with N_2-CO_2 (80:20 [vol/vol]); the pH was adjusted to 6.6 for enrichments, to 7 for pure-culture studies, and to various levels for pH experiments.

Vaginal fluid samples were collected anaerobically from consenting patients at the University of Iowa Hospitals and Clinics Gynecology Clinic. All samples were obtained with 3.5 ml of an anaerobically reduced salts solution (0.85% NaCl, 0.01% dithiothreitol, 0.001% resazurin) which was aspirated from a rubber-stoppered tube with a sterile needle and syringe. After a sterile speculum was inserted, the solution was sprayed into the vagina and mixed with vaginal discharge by vigorous swabbing of the vaginal walls, fornices, and ectocervix with a cotton-tipped applicator. At least 3 ml of the mixture was aspirated with a sterile syringe and plastic catheter and placed into the stoppered tube. All specimens were transported to the laboratory within 30 min of collection and placed in an anaerobic environment (anaerobic glove box; Coy Manufacturing, Ann Arbor, Mich.).

To enrich for methanogens, we transferred the entire volume of a sample (approximately 3 ml) into a sterile anaerobic serum tube (2048-00150; Bellco Glass, Inc., Vineland, N.J.) containing 3 ml of double-strength medium. The tube was flushed thoroughly (15 min) with H_2-CO_2 (80:20 [vol/vol]) and pressurized to 140 kPa. Cysteine hydrochloride and $Na_2S \cdot 9H_2O$ were added to a final concentration of 1.2 mM each, and methanol was added to a final concentration of 0.25% from sterile anaerobic stock solutions. Streptomycin and vancomycin were also added to a final concentration of 50 μg/ml each from filter-sterilized anaerobic stock solutions. The enrichments were incubated at 37°C under static conditions, with the tubes standing upright; the tubes were shaken by hand once or twice daily. Methane production was monitored by gas chromatography as previously described (2). The isolation of methanogenic bacteria was carried out by plating enrichment cultures on medium with 1% Gelrite (Kelco, San Diego, Calif.) by previously described techniques (1, 9). Colonies were restreaked, and the colony picks were grown in liquid medium with 50 μg each of streptomycin and vancomycin per ml. After one more transfer into medium containing 50 μg each of oleandomycin and kanamycin per ml, the pure cultures were grown in medium without antibiotics and further studies on the isolates were carried out. Unless indicated otherwise, the isolates were incubated in a gyratory shaker at 150 rpm with the tubes in a horizontal position. Growth of cultures was determined by measurement of turbidity in a Spectronic 20 spectrophotometer (Bausch & Lomb, Inc., Rochester, N.Y.) and by monitoring methanogenesis. Data from all studies on the isolates are average values for duplicate tubes. Immunological identification of isolated methanogens was done by comparing antigenic fingerprints as described previously (3, 10, 11).

Data on methanogenic activity observed in enrichments of

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TABLE 1. Methanogenic activity in enrichments from human vaginal samples

Sample	Vaginal condition	μmol of CH_4/tube[a]	Days of incubation
1	Healthy[b]	0	14
2	Bacterial vaginosis	115	8
3	Healthy	0	19
4	Healthy	0	12
5	Bacterial vaginosis	87	10
6	Healthy	0	18
7	Healthy	0	12
8	Healthy	0	12
9	Healthy	0	12
10	Healthy	0	17
11	Erosive lichen planus	0	17
12	Bacterial vaginosis	0	12

[a] Total volume, 26.7 ml; culture volume, 6.2 ml.
[b] Healthy patients reporting for routine physical examinations.

vaginal fluid samples are summarized in Table 1. Examination of the positive enrichments by phase-contrast microscopy showed that the predominant population of cells in both samples was morphologically similar to *Methanobrevibacter* species.

From the methanogen-positive enrichment of sample 2, two lines of transfer were made. Isolate HVMm was maintained in the presence of both H_2-CO_2 and methanol, while isolate HVMh was propagated on H_2-CO_2 as the sole growth substrate in medium lacking methanol. Methanol was included in the original enrichments to promote methanogens that use methanol plus H_2-CO_2 in addition to those that grow on H_2-CO_2, because a methanogen isolated from human feces, *Methanosphaera stadtmanae*, is known to require both methanol and H_2-CO_2 (12). The isolation of methanogens in pure cultures was done from both lines of enrichment. Examination of isolates HVMm and HVMh by phase-contrast microscopy revealed both to be cells with a *Methanobrevibacter*-type morphology (Fig. 1).

The two isolates were examined for their nutritional and growth characteristics. Deletion of methanol from the HVMm culture had no effect on the growth of this isolate on H_2-CO_2. In addition to H_2-CO_2, the isolates were tested for growth on other substrates. Cultures were transferred into medium under a gas phase of N_2-CO_2 (80:20 [vol/vol]) and supplemented with 130 mM each methanol, formate, or acetate. Both isolates were able to grow on H_2-CO_2 or formate but not on the other substrates. Figure 2 shows growth and methanogenesis by isolate HVMh with H_2-CO_2 as a substrate. The optimum temperature and pH for the growth of both isolates were 37 to 40°C and 6.8 to 7.0, respectively. Thus, the isolates were identified as *Methanobrevibacter* species on the basis of morphological, nutritional, and growth characteristics.

Partial antigenic fingerprinting by indirect immunofluorescence and the quantitative slide immunoenzymatic assay showed a close antigenic relationship with the reference organisms *M. smithii* PS and ALI. This result is similar to previous observations showing *M. smithii* strains to be the predominant strains isolated from the large intestine of humans (13) and from human dental plaque (3).

This study is the first demonstration of the presence of methanogenic bacteria among the microbial flora of the human vagina. Although methanogens were not detected in all samples that were obtained from diseased individuals, the methanogen-positive samples were from females with bacterial vaginosis. One methanogen-negative subject had erosive lichen planus, and another had bacterial vaginosis. Interestingly, a similar study of methanogenic activity in human dental plaque involving patients with a variety of

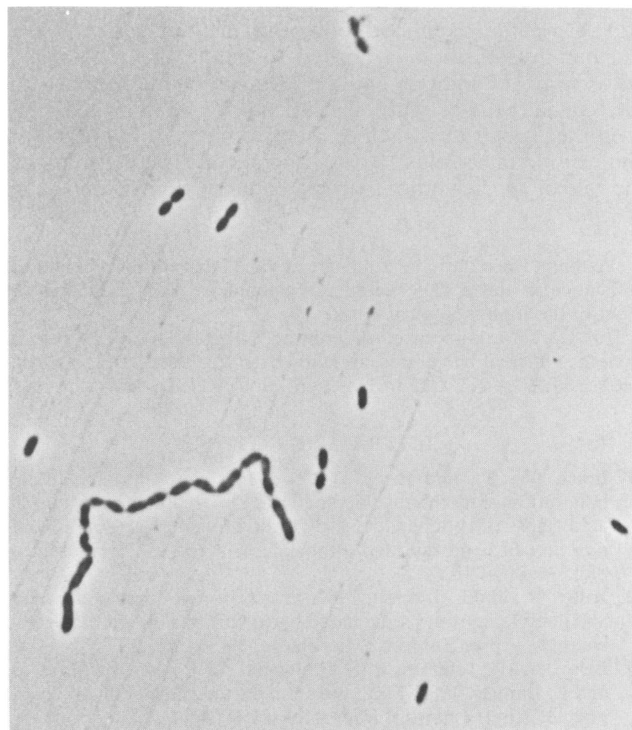

FIG. 1. Phase-contrast micrograph of *M. smithii* HVMm, isolated from a human vaginal sample.

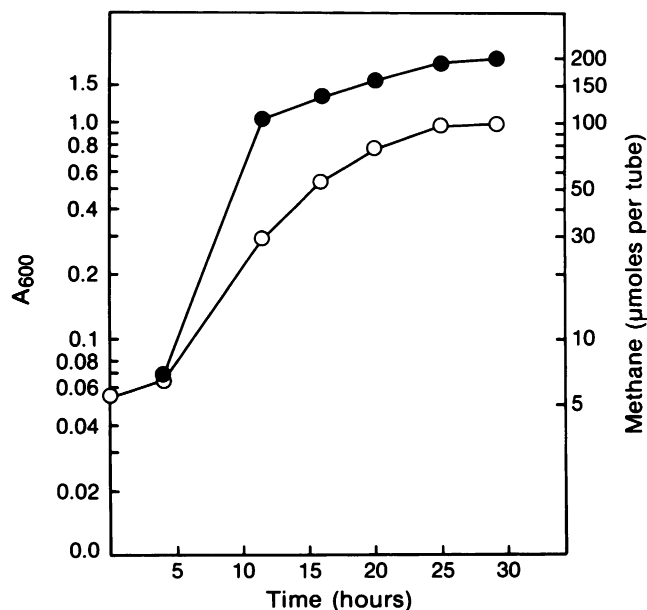

FIG. 2. Growth and methanogenesis of *M. smithii* HVMh, a methanogen isolated from a human vaginal sample. Symbols: ○, growth (culture turbidity at A_{600}); ●, methane formation. The cultures were incubated without shaking with the tubes in an upright position for the initial 4 h.

periodontitis case histories (3) showed that diseased patients had a greater likelihood of being methanogen positive. Further investigation is needed to define the relationship between host condition and the presence of methanogens in the vagina. Studies with enrichments for methanogens that use other substrates, such as methylamines, formate, methanol, or methanol plus H_2-CO_2 and acetate, may also reveal the role of species other than the common human isolate *M. smithii*.

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