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Isolation of an Antigenically Unique Methanogen from Human Feces

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A methanogenic bacterium with the morphological and physiological properties of the genus *Methanobrevibacter* was isolated from the feces of a Japanese man who excreted methane in his breath. Indirect immunofluorescence staining revealed that the isolate had an antigenicity unrelated to that of any known members of the genus *Methanobrevibacter*.

The human population can be divided into two groups, methane producers and methane-nonproducers, on the basis of methane excretion in breath (2). In both the United States and Great Britain, about 35% of the adult human population are methane producers (2, 7), while in Japan <10% are producers (8). The reason for such a remarkable difference between the Japanese and resident of other countries remains to be understood. Although several methanogens were isolated from the feces of American methane producers, they were all identified as *Methanobrevibacter smithii* (12, 14). From these findings, Miller et al. (12) have suggested that *M. smithii* is the dominant methanogen in the human large intestine. In this communication, we report the isolation from a Japanese methane producer of a methanogen with an antigenicity unrelated to *M. smithii*.

Fresh feces were obtained from a healthy 35-year-old man who excreted methane in his breath. The feces were suspended in 3 volumes of phosphate-buffered saline, pH 7.2, and homogenized gently with a spatula and then vigorously with an anaerobic homogenizer (Towa Chemicals Ltd., Tokyo, Japan). The resulting homogenate was 10-fold serially diluted in liquid medium (15) containing 30% bovine rumen fluid. Each of the dilutions was inoculated on agar plates prepared with medium containing 2.5% agar (Difco Laboratories) and 30% rumen fluid, and the plates were incubated at 37°C in an anaerobic chamber (model 1024; Forma Scientific Co., Marietta, Ohio) filled with a gas mixture of H_2–CO_2–N_2 (10:10:80). On day 28 after inoculation, to identify the colonies of methanogens by epifluorescence (5, 6, 13), the cultures were exposed to UV light of 365 nm from a distance of 15 cm for 3 min, using a UV lamp (type UVSL-25; UV Products, Inc., San Gabriel, Calif.). The fluorescent colonies were picked up from the plate where only a few colonies were grown and then subcultured on new agar plates. Even after repeated subcultures, it was not possible to obtain a pure culture of methane-producing bacteria. The fluorescent colonies were always surrounded by a number of nonfluorescent tiny colonies consisting of gram-positive spore-bearing bacteria. It was previously shown that none of the methanogenic bacteria so far isolated contained a peptidoglycan structure (9, 16), so that the inhibitors of peptidoglycan synthesis, e.g., fosfomycin, D-cycloserine, vancomycin, penicillin G, and cephalosporin, selectively blocked the growth of the contaminants without inhibiting the growth of methanogens (14, 16). Therefore, we streaked the fluorescent colonies on agar plates, on which pulp disks containing 50 μg of carbenicillin were placed. After an incubation period of 28 days, fluorescent colonies were seen within the zone where growth of nonfluorescent bacteria was inhibited. There was no difference in the morphology of the fluorescent colonies formed in the presence and absence of carbenicillin. These colonies could be transferred to the agar plate lacking carbenicillin without further contamination by nonfluorescent colonies. As a result, we have obtained a pure culture of methanogenic bacteria free of any other contaminants. The purity of the culture was further supported by uniformity in cell morphology, methane production, autofluorescence of the cells under UV excitation, and lack of growth under either aerobic or anaerobic conditions in media deficient in the nutrients required for the growth of methanogens (data not shown). All cells of the isolate were gram-positive short rods, arranged mostly in pairs or sometimes in short chains.

The gram-positive methanogens are divided into two families, *Methanobacteriaceae* and *Methanosarcinaceae* (1). The rod-shaped methanogens are grouped into the former family. Balch et al. (1) described two genera in the family *Methanobacteriaceae*: i.e., *Methanobrevibacter*, short rods; and *Methanobacterium*, long rods. Balch et al. (1) also described three species in the genus *Methanobrevibacter*: *M. smithii*, *M. ruminantium*, and *M. arboriphilus*. Although these three species are morphologically similar, they are distinguishable from one another by physiological and serological properties as well as by 16S rRNA codon catalogs (1, 4). Recently, Conway de Macario et al. (4) compared the antigenicity of 17 methanogenic bacteria covering the entire range of available species by indirect immunofluorescence and found that different species of the same genus showed virtually no cross-reaction, whereas strains of the same species exhibited a high level of cross-reaction. To identify the species of our isolate, we attempted to compare its antigenicity with those of three standard methanogens from the genus *Methanobrevibacter* by indirect immunofluorescence. Members of the genus *Methanobacterium* were also analyzed for comparison. These standard methanogens were all purchased from the Deutsche Sammlung von Mikroorganismen (Göttingen, Federal Republic of Germany). For this experiment, the isolate and the standard methanogens were grown in liquid media prepared according to the protocol of Balch et al. (1), using Hungate-type culture tubes with rubber stoppers (Bellco Glass, Inc., Vineland,

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TABLE 1. Antigenic analysis of isolate by indirect immunofluorescence staining

Antigen	Antibody titer[a] of antiserum to:						
	Isolate	M1	PS	DH1	MF	MoH	ΔH
Isolate	3,200	—[b]	—	—	—	—	—
Methanobrevibacter							
M. ruminantium M1	—	6,400	—	—	—	—	—
M. smithii PS	—	—	6,400	—	—	—	—
M. arboriphilus DH1	—	—	—	3,200	—	—	—
Methanobacterium							
M. formicicum MF	—	—	—	—	12,800	—	—
M. bryantii MoH	—	—	—	—	—	6,400	—
M. thermoautotrophicum ΔH	—	—	—	—	—	—	3,200

[a] Antibody titer is expressed as the highest antiserum dilution showing positive immunofluorescence.
[b] —, <100.

N.J.) (10, 11). Immediately after inoculation with methanogenic bacteria, using a needle extensively flushed with sterile O_2-free N_2, the gas phase in each tube was exchanged by a gas mixture of H_2–CO_2 (80:20) and pressurized to 2 atm. The cultures were incubated at 37°C for 14 days and then used for the preparation of either antiserum or a bacterial smear. The rabbit antiserum against each of the methanogens was prepared as follows: bacteria fixed with Formalin according to the method of Conway de Macario et al. (3) were suspended in phosphate-buffered saline to give an optical density of about 0.5 at 660 nm and emulsified in an equal volume of Freund complete adjuvant, and 2-ml portions of the emulsion were used for each of the primary and secondary immunizations. A primary dose was injected into the bilateral rear footpads of a rabbit, and a secondary dose was given 28 days later by subcutaneous injections into different spots on the neck. The animal was then bled from the ear vein 14 days after the booster. The heat-fixed bacterial smears made on glass slides were covered with various dilutions of the rabbit antiserum prepared as above. After incubation at 37°C for 45 min, the antiserum was removed and the smear was again covered with 1:40-diluted, fluorescein isothiocyanate-labeled goat immunoglobulin to rabbit gamma globulin (Behringwerke AG, Marburg, Federal Republic of Germany). After further incubation at 37°C for 45 min, the bacterial smear was extensively washed with phosphate-buffered saline, dried with warm air, and then observed with an epifluorescence microscope (Leitz Ortholux II; Ernst Leitz Wetzlar GMBH, Wetzlar, Federal Republic of Germany). The results of these experiments are summarized in Table 1. When the homologous antisera, even highly diluted, were used, all six standard methanogens were stained brightly. On the other hand, fluorescent cells were not seen when the bacteria were reacted with any of the heterologous antisera. Thus, no cross-reaction was observed among the six standard methanogens, confirming the previous findings of Conway de Macario et al. (4). The results also indicated that our isolate reacted strongly with the homologous antiserum but not at all with antiserum against any of the standard methanogens. It was further found that antiserum against our isolate was able to stain only the isolate itself. Based on these observations, we conclude that the present isolate is antigenically different from any of the six species of methanogens tested. Therefore, this isolate may be a new member of the genus *Methanobrevibacter*. To confirm this view, we are now analyzing the structure of 16S rRNA of the isolate by an oligonucleotide fingerprinting technique.

Isolation of methanogenic bacteria from the feces of some Americans was reported by Nottingham and Hungate (14) and Miller et al. (12), and all of these isolates were shown to be identical to *M. smithii*. Our data suggest, however, that an organism other than *M. smithii* may be involved in the production of methane gas in the human large intestine. It appears of importance to determine whether the dominant methanogen of Japanese people is the same organism as the present isolate, or *M. smithii*, or others. Previous work from our laboratory (8) has demonstrated that methanogenic bacteria can be detected in the feces of not only methane producers but also about 40% of methane nonproducers and that in either case the concentrations of methanogen in the feces range from 10^1 to 10^5 cells per g, wet weight. It may be of interest to see whether the methanogens detected in the feces differ between producers and nonproducers of methane gas.

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