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Methanogenic Bacteria, Including an Acid-Tolerant Strain, from Peatlands

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Five pure cultures of methanogenic bacteria were isolated from Minnesota peatlands by enrichment culture techniques. One strain, identified as a member of the family *Methanobacteriaceae* by antigenic fingerprinting, was acid tolerant and able to produce methane at pH 3.1. Growth could not be demonstrated at pH less than 5.3.

Methanogenic bacteria are a distinct grouping of microorganisms, phylogenetically distant from typical bacteria (1, 8). They are part of a biological group of bacteria referred to as archaeobacteria. The biochemistry (e.g., 16S rRNA structure, DNA structure, intermediary metabolism, and lipid-cell wall composition) of archaeobacteria is considered to be as different from that of classic procaryotes as from that of eucaryotes. This has led to the suggestion that archaeobacteria be classified as a new primary kingdom (8).

Methanogens occupy a narrow ecological niche. They mediate the formation of methane from simple substrates (e.g., H_2-CO_2, formate, methanol, and acetate) in highly reducing and anaerobic environments. They have among the most stringent requirements of any known anaerobes for the absence of oxygen (less than 2 ppm), and for the growth they require a redox potential of less than −330 mV. Consequently, specialized techniques and equipment are required for their isolation and cultivation (2, 9).

In culture, known methanogenic bacteria metabolize best in the near-neutral pH range of 6.7 to 8.0 (9). Very low rates of methanogenesis, however, have been observed at slightly lower pH values (4). We are aware of only one report of growth of a methanogen at low pH. A Russian worker, Kuzneceorii (for a review, see reference 9), reported growth of a *Methanobacillus* organism at pH 4.0 in 1969. No subsequent work with this organism has been reported.

Peat bogs produce methane (7), and methanogenic bacteria have been enumerated in peatlands (6); however, we are aware of no reports detailing the isolation of methanogenic bacteria from such environments. The peat bogs we have studied in Minnesota varied between pH 3.5 and 4.5 (5). Consequently, they are excellent habitats in which to look for acid-tolerant or acidophilic methanogens.

During the present study, methanogenic bacteria were isolated from water-saturated peat samples collected from 10 to 30 cm below peatland surfaces (see references 5 to 7 for descriptions of peatland sites). An anaerobic chamber (Coy Manufacturing Co.) filled with 10% H_2–90% N_2 and a gassing manifold were used for all manipulations (1, 2). Positive enrichment cultures (CH_4 production) were obtained by inoculating complex (a mineral solution supplemented with acetate, formate, trypticase, yeast extract, and vitamins) or basal (a mineral solution supplemented with vitamins) media (6) at neutral pH with 0.1 ml of peat interstitial water. Methanogenic enrichments were maintained in our defined basal medium (6) under 2 atm (1 atm = 101.29 kPa) of 80% H_2–20% CO_2 to minimize the growth of nonmethanogenic bacteria. No consistent differences in methane production were observed between enrichments grown in complex versus basal media.

In attempts to enhance isolation and encourage better growth of peatland methanogens, two types of medium supplements were tested. The first additive used was autoclaved peat interstitial water. A second additive was prepared by sonicating an interstitial water-peat mixture (1:1 [vol/vol]). The resulting solution was then either filter sterilized or autoclaved. Neither of these additives significantly stimulated methane production when added over a range of concentrations (1 to 50% [vol/vol]) to the standard basal medium. Use of these additives as media supplements was discontinued before the establishment of enrichment cultures used to isolate the methanogens described in this paper.

Two strategies were employed to obtain pure cultures of methanogens from successful enrichments. The first involved the use of petri plate (purified agar in basal medium) streaking to obtain single colonies. These plates were incubated at 22°C in modified pressure cookers (2) filled with 1.5 atm of 80% H_2–20% CO_2. This approach failed. Even in cases in which methane was detected in the incubation chamber, no single colonies could be located. The growth of methanogens was always confluent with nonmethanogenic contaminants. A second approach, utilizing the addition of antibiotics, proved successful. Five methanogenic bacteria (strains M-1, M-2, M-3, O-1, and O-2) were obtained in pure culture after a series of transfers in basal medium containing streptomycin (1 mg/ml), gentamicin (0.1 mg/ml), and kanamycin (0.1 mg/ml). We believe these are the first authentic pure cultures of methanogens isolated from peatlands.

Microscopic examination and Gram stains of the pure cultures after growth in basal medium revealed the following: culture M-1, regular nonmotile cocci, gram negative; M-2, long nonmotile rods, often forming curved filaments with crooked cells, gram positive; M-3, generally short, single rods with occasional doublets but no chains, motile, gram positive; O-1, long straight rods with blunt ends, often in filaments, gram positive, nonmotile; O-2 blunt rods often in doublets but not chains, gram positive, nonmotile.

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TABLE 1. Methane production from H_2-CO_2 by cultures grown at various pHs

Culture no.	Initial pH	Methane produced[a] (nmol)	Final pH
O-1	7	12,440	6.6
	6	20,710	6.1
	5	10,148	5.4
	4	720	4.3
	3	410	3.1
O-2	7	1,802	6.6
	6	753	6.0
	5	140	5.3
	4	0	4.2
	3	0	3.1
M-1	7	20,700	6.4
	6	100	5.9
	5	56	5.4
	4	0	4.4
	3	0	3.1
M-2	7	12,400	6.4
	6	9,100	6.0
	5	12	5.4
	4	0	4.3
	3	0	3.1
M-3	7	28,700	6.4
	6	268	5.7
	5	12	5.3
	4	56	4.7
	3	0	3.1

[a] After 2.5 weeks of incubation at 22°C in basal medium under an atmosphere of 80% H_2–20% CO_2, uninoculated controls produced no methane, methane was quantitated by gas chromatography (7), cultures were grown in serum bottles containing 8 ml of medium, and headspace gases were collected by syringe.

TABLE 2. Methane production from H_2-CO_2 by culture O-1 in pH-adjusted complex and basal media

Medium	Initial pH	Methane produced[a] (nmol)	Final pH[b]
Complex	7	41,000	7.0
		39,100	—
	6	15,400	6.4
		7,200	—
	5	38,300	5.3
		19,000	—
	4	448	4.3
		121	—
	3	112	3.6
		78	—
Basal	7	30,600	6.7
		27,300	—
	6	38,300	6.4
		27,300	—
	5	21,600	5.3
		7,400	—
	4	267	4.1
		129	—
	3	69	3.6
		121	—

[a] After 2 weeks of incubation at 22°C (duplicate cultures) (for media compositions, see reference 6, uninoculated controls produced no methane, methane was quantitated by gas chromatography (7), cultures were grown in serum bottles containing 8 ml of medium, and headspace gases were collected by syringe.

[b] —, No pH value determined for the specified culture medium at the end of the 2-week incubation period.

Attempts to isolate acid-tolerant or acidophilic methanogens by enrichment in low pH media failed. However, the pH optima for methane production from H_2-CO_2 by the above cultures was investigated by using subsamples of the cultures to inoculate basal medium adjusted to various pHs (Table 1). The methane production of culture O-1 observed at pH 3 and 4 was particularly interesting. To confirm these results, the pH range experiment with culture O-1 was repeated with duplicate cultures and both basal and complex media (Table 2). They confirm the ability of the culture to produce methane at pH 3 to 4. Growth, however, could only be detected at pH 5.3 and above. Approximately 5×10^4 methanogens per ml, as determined by acridine orange direct count (6), were present in the basal and complex media after inoculation. After 2 weeks of incubation, all tubes with an initial pH ranging from 5 to 7 contained between 8.3×10^5 and 6.8×10^6 methanogens per ml. The numbers present in tubes with an initial pH of 3 or 4, however, did not contain significantly more methanogens than initially added by inoculation. These observations confirm the existence of acid-tolerant methanogens, though a true acidophilic methanogen was not isolated.

Isolate O-1 was subjected to antigenic fingerprinting by the indirect immunofluorescence technique (3). The indirect immunofluorescence reaction was 2+ with anti-*Methanobacterium bryantii* MoHG, 3+ with anti-*Methanobrevibacter arboriphilus* DH, and negative with 15 other S probes. Therefore, isolate O-1 is immunologically more related to the *Methanobacteriaceae* family than to either the *Methanococcaceae* or *Methanomicrobiaceae* family.

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