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Isolation and Characterization of a Novel Thermophilic, Freshwater Methanogen

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A novel thermophilic, coccoid methanogen isolated from nonthermal freshwater sediments is described. Hydrogen plus carbon dioxide and formate were substrates for methanogenesis, and methane production was stimulated by yeast extract, Casamino Acids, and tryptose. Growth also occurred autotrophically. Elevated levels of sodium chloride were not required for maximum growth and were inhibitory above 2%. The minimum doubling time occurred at 57°C, and the upper and lower limits for methane production were 62 and 26°C, respectively. The optimum pH for growth was between 7.0 and 7.5. Inhibitory antibiotics included metronidazole, anisomycin, chloramphenicol, and lasalocid. Colonies were circular, dark yellow, shiny, and convex with entire edges. Cells were 1 to 2.5 μm in diameter, nonmotile, occurring singly or in pairs, and fimbriated. Cells were lysed by pronase or trypsin digestion, glass-distilled water, and 1% sodium dodecyl sulfate. Electron micrographs of thin sections showed a monolayered cell wall ca. 20 nm thick. The DNA base ratio was 49.2 mol% guanine plus cytosine. The whole cell protein pattern differed from that of other named coccoid methanogens.

The methanogenic bacteria inhabit diverse anaerobic niches and exhibit wide morphological and physiological variation (1). Species from the genera *Methanococcus*, *Methanogenium*, *Methanomicrobium*, *Methanococcoides*, *Methanoplanus*, and *Methanolobus* occur almost exclusively in marine environments and characteristically possess a protein or glycoprotein cell wall which is sensitive to detergents. Most show a requirement for elevated levels of sodium chloride for optimal growth; the exceptions are *Methanococcus vannielii* (11), an unnamed coccobacillus (6), and *Methanococcus maripaludis* (12); the last species does, however, require 20 to 70% (vol/vol) sea water.

To date, *Methanogenium olentangyi* is the only methanogenic species possessing an osmotically fragile cell wall to have been isolated from a freshwater environment (5). This species is an irregular coccus which utilizes hydrogen plus carbon dioxide as the substrate for methanogenesis and requires acetate. Although high levels of sodium chloride are not an essential requirement, optimum growth occurs in 1% sodium chloride at 37°C.

Thermophilic marine methanogens, *Methanococcus thermolithotrophicus* (9), *Methanogenium thermophilicum* (16), an unnamed coccobacillus (6), and a *Methanococcus* species (J. A. Leigh and W. J. Jones, Abstr. Annu. Meet. Am. Soc. Microbiol. 1983, I17, p. 142) have only been isolated from thermal habitats.

We describe here the isolation and characterization of a previously unreported autotrophic, moderately thermophilic methanogen from a nonthermal freshwater lake. This isolate possesses an osmotically fragile cell wall, utilizes hydrogen plus carbon dioxide or formate for methanogenesis, and exhibits no requirement for elevated levels of sodium chloride.

MATERIALS AND METHODS

Strains. *Methanococcus vannielii* DSM 1224, *Methanococcus voltae* DSM 1537, *Methanogenium cariaci* DSM 1497, *Methanogenium marisnigri* DSM 1498, *Methanogenium thermophilicum* DSM 2373, *Methanococcus thermolithotrophicus* DSM 2095, *Methanolobus tindarius* DSM 2278, and *Methanoplanus limicola* DSM 2279 were obtained from the Deutsche Sammlung von Mikroorganismen, Göttingen, Federal Republic of Germany.

Sample collection. Sediment was obtained by scuba divers from Fritton Lake, a freshwater lake with an average depth of 4 m in Norfolk, England. The samples were collected in screw-capped bottles previously gassed out with N_2-CO_2 (80:20) and were transported to the laboratory immediately.

Culture methods. All manipulations were performed in an anaerobic cabinet (model 1024; Forma Scientific, Inc., Mallinckrodt, Inc., Marietta, Ohio). Broth cultures were grown in screw-capped glass tubes fitted with butyl rubber septa (Bellco Glass, Inc., Vineland, N.J.). Solid medium was dispensed into plastic petri dishes and incubated in a stainless steel pressure vessel similar to that of Balch et al. (3).

Media. All media and supplements were prepared by the anaerobic techniques of Hungate (10). BM3 medium was a defined mineral salts medium containing (per liter): NaCl (0.9 g), $(NH_4)_2SO_4$ (0.9 g), KH_2PO_4 (0.45 g), $CaCl_2 \cdot 6H_2O$ (0.2 g), $MgSO_4 \cdot 7H_2O$ (0.2 g), $FeSO_4 \cdot 7H_2O$ (0.01 g), resazurin (1 mg), and 10 ml of trace mineral solution (2). The medium was mixed and purged with N_2-CO_2 (80:20) at 103 kPa for 5 min before boiling under the same gas mixture for 5 min. After cooling, anaerobic 8% (wt/vol) Na_2CO_3 was added to a final concentration of 0.2% (wt/vol), the pH was adjusted to 6.7, and 4.5-ml samples were dispensed under N_2-CO_2 (80:20). After autoclaving and before inoculation, the medium was reduced by the addition of 0.1 ml of a solution containing 1.7% (wt/vol) $Na_2S \cdot 9H_2O$ and 1.7% (wt/vol) cysteine hydrochloride. The final pH was 6.8 to 7.0. SM3 medium was prepared as BM3, but with the addition of (per litre) yeast extract (5 g), sodium formate (2 g), sodium acetate (2 g), K_2HPO_4 (0.9 g), tryptose (2 g; Difco Laboratories, Detroit, Mich.), Casamino Acids (2 g; Difco), and vitamin solution (10 ml; 3). Solid medium was prepared by the addition of 1% agar (Difco) and reduced with $Na_2S \cdot 9H_2O$ (0.5 g/liter) and cysteine hydrochloride (0.4 g/liter) before autoclaving.

Growth on H_2-CO_2 (80:20) was achieved by replacing the

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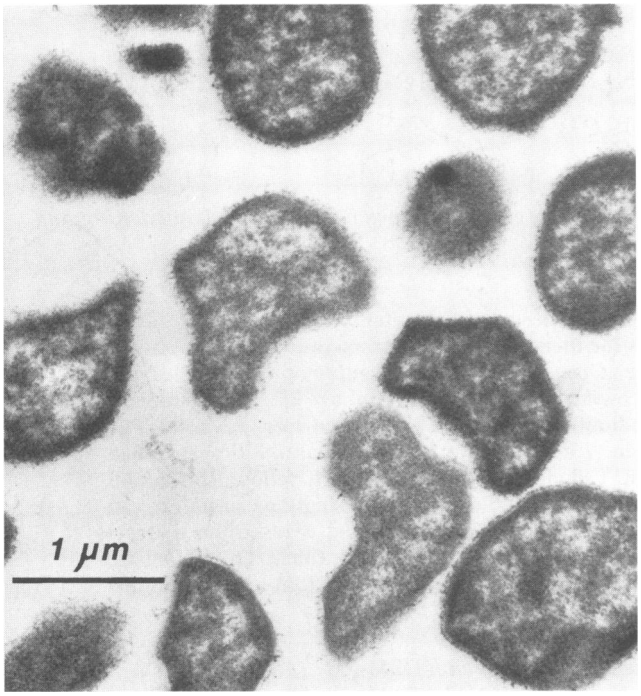

FIG. 1. Thin section of typical cells of FR-4.

gas atmosphere of broth cultures and pressure vessels with the gas mixture at 203 kPa. Substrates for methanogenesis were determined in both BM3 and SM3 media, the latter prepared without the addition of sodium formate and sodium acetate. The stimulatory effect of nutrients was examined in BM3 medium. Autotrophic growth was tested in cysteine-free BM3 medium reduced with 2.5% $Na_2S \cdot 9H_2O$ (0.1 ml). Both the optimum temperature for growth and the sensitivity to antibiotics were examined in SM3 medium. For determination of the optimum pH for growth, SM3 medium was adjusted by the addition of NaOH to cover the pH range 6 to 8.5. Sodium carbonate was replaced by TES buffer (*N*-tris[hydroxymethyl]methyl-2-aminoethanesulfonic acid) at 50 mM for the pH range 7 to 8.5. The concentration of NaCl for optimum growth was examined in appropriately modified SM3 medium prepared with TES buffer. All growth tests were performed in duplicate, and cultures were transferred sequentially at least twice in the test medium to confirm growth.

Other species of methanogens were cultured in medium 3 of Balch et al. (3).

Enrichment and isolation. Sediment samples (ca. 0.5 ml) were incubated in SM3 medium containing 100 μg of cefoxitin (Merck Sharp & Dohme, Hoddesdon, United Kingdom) per ml at 37°C and under H_2-CO_2 (80:20). Tubes producing methane and containing fluorescent bacteria (420 nm) were serially diluted and reincubated, and those producing methane were plated onto the same medium. Colonies of fluorescent bacteria were picked into broth. A pure culture was

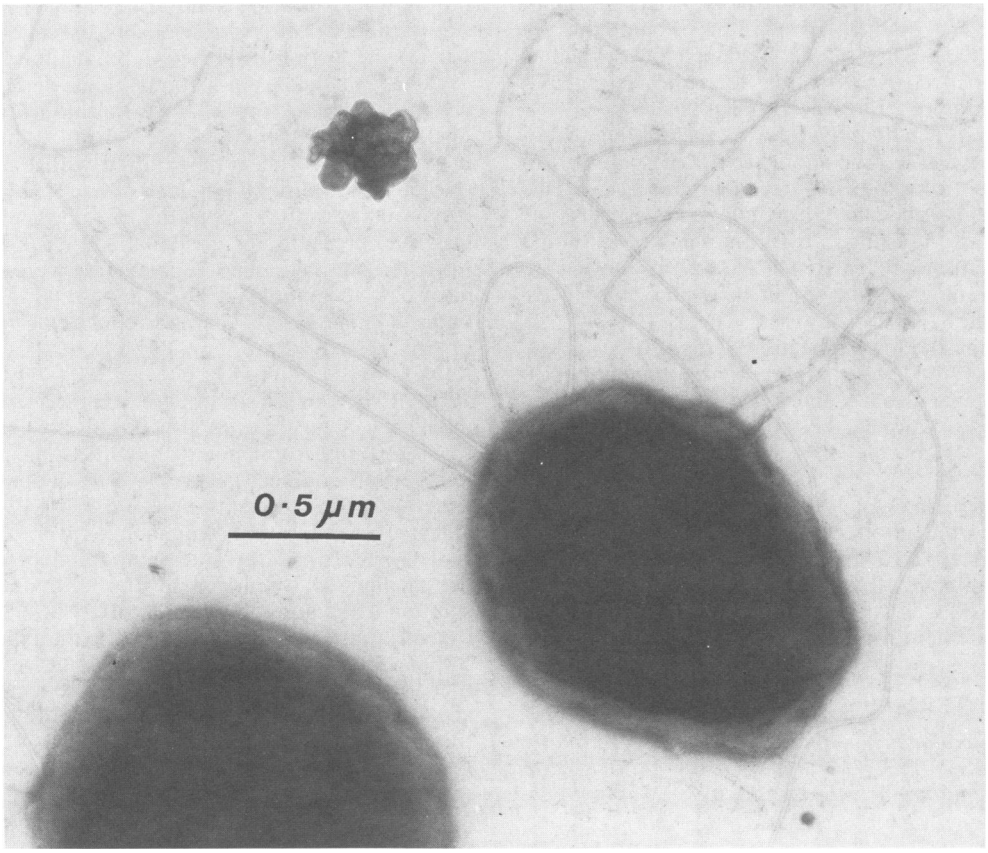

FIG. 2. Negative stain of whole cell of FR-4 showing fimbriae.

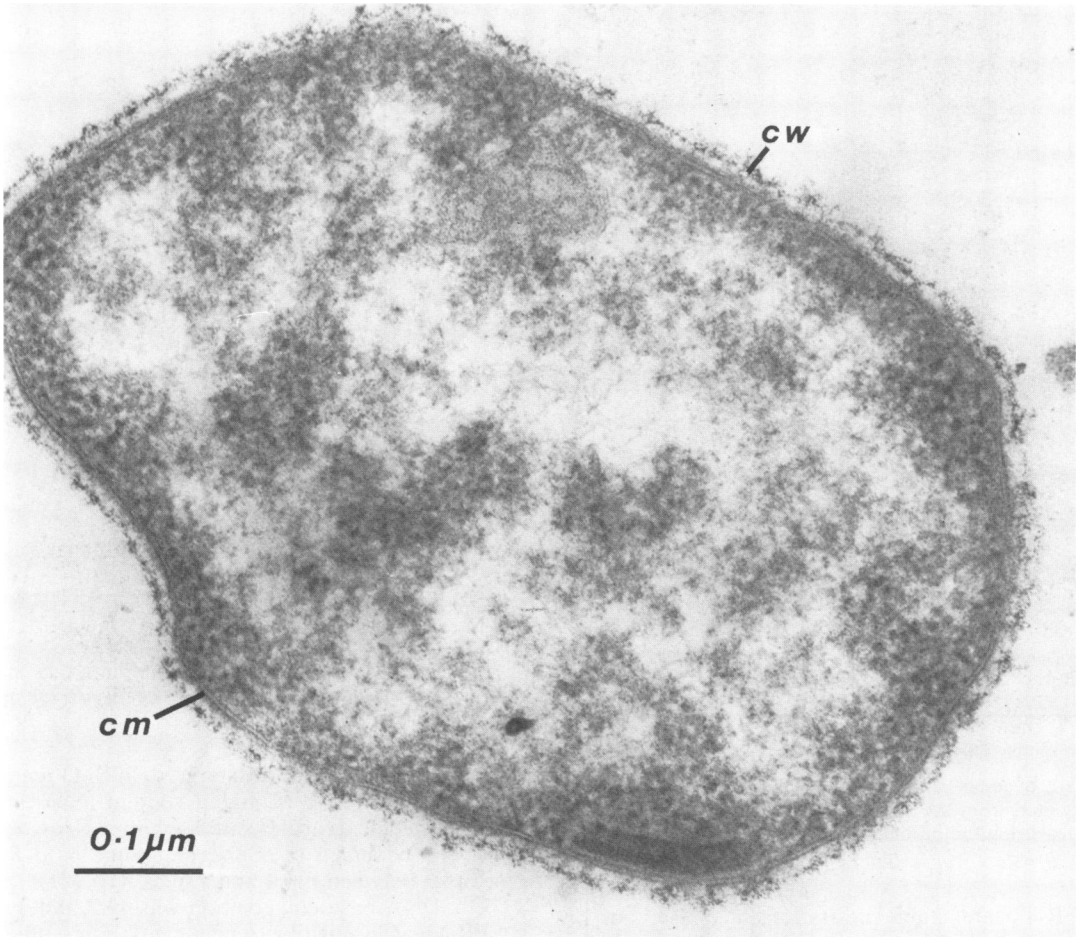

FIG. 3. Thin section of single cell of FR-4 showing the cell wall (cw) and cell membrane (cm).

obtained by repeating the procedure in the presence of vancomycin (50 μg/ml).

Microscopy. Phase contrast and fluorescence microscopy (2) were used to confirm the presence of methanogens and the purity of cultures. Log-phase cells were fixed for electron microscopy in 2% glutaraldehyde overnight before postfixation in 2% osmic acid, dehydration, and embedding (14). Thin sections were poststained with saturated aqueous uranyl acetate and 1.75% (wt/vol) lead acetate. Uranyl acetate was used for negative staining of whole cells. Electron micrographs were taken with an AEI 801 transmission microscope.

Measurement of growth. Production of methane was used as a measure of cell growth. Methane concentration was determined with a Pye 104 gas chromatograph equipped with flame ionization detector as previously described (2).

DNA isolation and analysis of base ratio. Cells were resuspended in Tris buffer (pH 8.0; 10 mM) and EDTA (100 mM) and lysed by the addition of 1% sodium dodecyl sulfate (SDS). DNA was purified from the lysate by the method of Chater et al. (4). Moles percent guanine plus cytosine (G + C) was determined by cesium chloride equilibrium density centrifugation with an MSE Centriscan 75 centrifuge (18) and *Escherichia coli* DNA as a reference.

SDS-polyacrylamide gel electrophoresis. Log-phase cells were solubilized, and samples were run on one-dimensional gels by the method of Laemmli and Favre (13).

RESULTS

There was no mud-water interface at the lake bed, and the samples were a fine suspension of sediment. The temperature and pH of the samples at the time of collection were 17.5°C and 6.5, respectively.

After repeated enrichment of sediment samples, fluorescent cocci became the predominant organisms. The purity of the culture was determined by microscopic examination and colony type on solid medium. The isolate was called FR-4. After 12 days of incubation at 37°C, colonies were 1 to 2 mm in diameter, dark yellow, shiny, and convex with entire edges. Cells were nonmotile, irregular cocci, occurring singly or in pairs, and measuring from 1 to 2.5 μm in diameter (Fig. 1). Gram staining was inconclusive due to the fragile nature of the cell wall. Negative staining revealed several fimbriae on each cell (Fig. 2).

TABLE 1. Stimulation of growth rate by nutrients

Added nutrient	Specific growth rate, μ (h^{-1})
None	0.024
Yeast extract	0.055
Tryptose	0.059
Casamino Acids	0.039
Vitamin solution	0.022

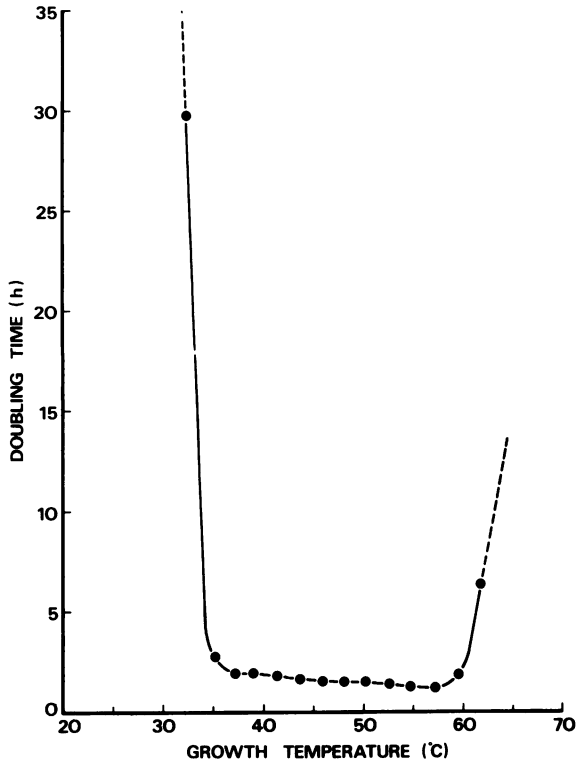

FIG. 4. Effect of temperature on the growth of FR-4. Doubling time was calculated from the slopes of exponential growth curves obtained by measuring the production of methane.

Cells of FR-4 were stable in stationary phase, but on exposure to oxygen they became spherical with some lysis. In anaerobic conditions immediate lysis occurred upon resuspension of cells in glass-distilled water or upon the addition of SDS to 1%. Incubation in trypsin or pronase also caused lysis of FR-4. Electron microscopy of thin sections showed the cell membrane to be surrounded by a cell wall ca. 20 nm thick (Fig. 3). Negatively stained preparations indicated a regular, crystalline structure in the cell envelope (C. Grief, personal communication).

Only H_2-CO_2 (80:20) and formate were substrates for methanogenesis. Methanol, acetate, monomethylamine, and trimethylamine were not metabolized in an N_2-CO_2 (80:20) gas atmosphere during incubation for 3 weeks. Sequential subculturing in BM3 medium with H_2-CO_2 as the substrate confirmed that FR-4 grows autotrophically and has no requirement for acetate. The rate of growth was stimulated markedly by yeast extract, tryptose, and Casamino Acids but not by the addition of vitamin solution (3) (Table 1).

The temperature for optimum growth on H_2-CO_2 was 57°C, although the doubling time was between 1 and 2 h over a broad range, from 37 to 60°C (Fig. 4). The upper and lower limits for growth, determined after incubation for 8 days, were 26 and 62°C, respectively.

Comparison of the influence of sodium chloride on growth of FR-4 with that on requiring (*Methanococcus voltae*) and nonrequiring (*Methanococcus vannielii*) marine species showed that salt is not essential for growth (Fig. 5). A marked optimal sodium chloride concentration was not observed, and methane production was similar over the range 0 to 1%. FR-4 was also less tolerant to elevated levels of salt than were both *Methanococcus voltae* and *Methanococcus vannielii*. The isolate grew in SM3 medium containing less than 0.001 M added sodium ions, although yeast extract may be a further source of ions. Growth and methanogenesis occurred over the pH range 6 to 8.25 and was maximal between pH 7 and 7.5.

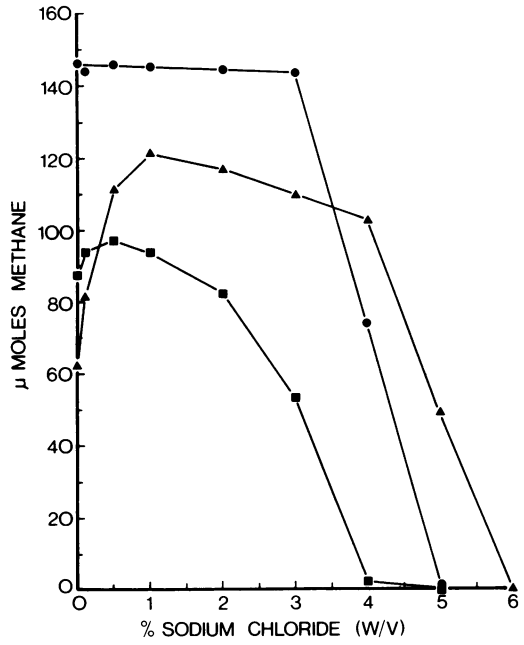

FIG. 5. Effect of sodium chloride concentration on the growth of FR-4. Symbols: ■, FR-4; ●, *Methanococcus vannielli*; ▲, *Methanococcus voltae*.

The DNA base composition was 49.2 mol% G + C.

FR-4 was insensitive to many antibiotics affecting cell wall biosynthesis and protein synthesis. Those found to be inhibitory are listed in Table 2. Metronidazole was the most effective antibiotic tested.

The pattern of total cell proteins of FR-4 revealed by SDS-polyacrylamide gel electrophoresis varies from that of each of the marine methanogens examined (Fig. 6). However, there is close resemblance to *Methanogenium thermophilicum*, with differences in only one major protein band.

DISCUSSION

The new isolate exhibits characteristics typical of archaebacteria (20), that is, insensitivity to many antibiotics affect-

TABLE 2. Sensitivity to antibiotics

Antibiotic[a]	MIC[b] (μg/ml)
D-Cycloserine	64
Tetracycline	16
Neomycin	8
Chloramphenicol	4
Anisomycin	1
Monensin	256
Lasalocid	2
Polymixin	16
Metronidazole	<1

[a] Antibiotics found not to be inhibitory at the maximum concentration tested (500 μg/ml) were cefoxitin, ampicillin, vancomycin, bacitracin, nisin, cycloheximide, erythromycin, lincomycin, streptomycin, and kanamycin.

[b] The MIC was defined as the lowest concentration of antibiotic inhibiting growth and was determined in SM3 broth by monitoring methane production over 8 days.

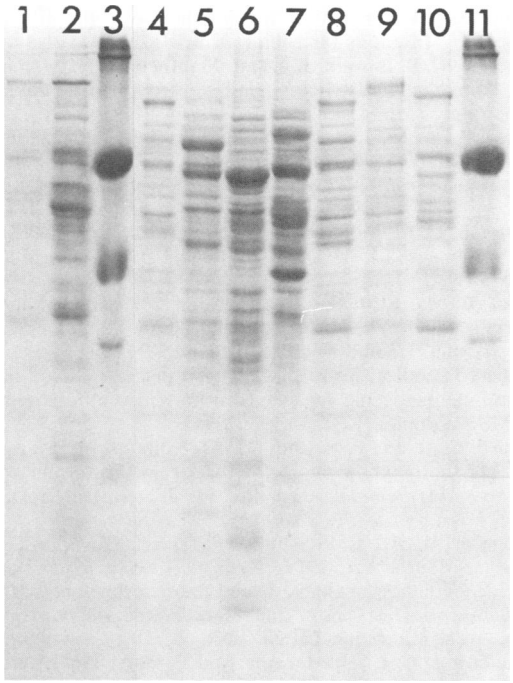

FIG. 6. SDS-polyacrylamide gel electrophoresis of whole cell proteins of marine methanogens and FR-4. Lanes: 1, *Methanoplanus limicola*; 2, *Methanolobus tindarius*; 4, FR-4; 5, *Methanococcus voltae*; 6, *Methanococcus vannielii*; 7, *Methanococcus thermolithotrophicus*; 8, *Methanogenium thermophilicum*; 9, *Methanogenium marisnigri*; 10, *Methanogenium cariaci*; 3 and 11, molecular weight standards: bovine serum albumin (68,000), aldolase (40,000), α-chymotrypsinogen (25,700), β-lactoglobulin (18,400), and cytochrome *c* (12,300).

ing peptidoglycan biosynthesis and protein synthesis in eubacteria and also the presence of ether-linked lipids (W. D. Grant, personal communication). From its ability to form methane from hydrogen plus carbon dioxide or formate and its fluorescence at 420 nm, the isolate is clearly a methanogen.

The sensitivity of FR-4 to antibiotics is similar to that of other methanogens tested (7, 8, 15; Pinn, unpublished data), especially inhibition by chloramphenicol, lasalocid, metronidazole, and anisomycin, although the MIC of anisomycin for *Methanococcus vannielii* was found to be unusually high at 500 μg/ml (15). In common with FR-4, other marine, coccoid methanogens were also inhibited by D-cycloserine, neomycin, tetracycline, and polymixin, but FR-4 differs by its insensitivity to bacitracin.

The isolate closely resembles other coccoid methanogens in morphology and in the characteristics of the cell envelope. The evidence suggests that the outer layer of the cell is composed of a periodic arrangement of protein subunits similar to the S-layers described for many marine methanogens (19), including the coccoid species *Methanococcus vannielii* (11), *Methanogenium cariaci* (17), and *Methanogenium marisnigri* (17).

The enrichment and isolation procedures used were not selective for thermophiles, but FR-4 showed optimal growth in the thermophilic range (49 to 60°C). Methane production was not detected at temperatures below 26°C in pure cultures of FR-4 even after incubation for 2 weeks. This suggests that the isolate may not grow in its natural habitat (17.5°C), although cells must remain viable for long periods or be introduced frequently from another source. Alternatively, interactions operating in the microbial community of the sediment may allow the growth of FR-4 in conditions in which growth of pure cultures was not observed, thus ensuring its continued survival.

The characteristics of this moderately thermophilic methanogen isolated from freshwater sediment suggest that it belongs to the family *Methanomicrobiaceae* (3). The isolate closely resembles members of the genera *Methanogenium* and *Methanococcus* in morphology, substrates utilized for methanogenesis, and characteristics of the cell envelope. *Methanogenium olentangyi* is the only species from either of these genera to have been isolated from a freshwater habitat and to show no requirement for, or tolerance to, high levels of sodium chloride (5). However, unlike *Methanogenium olentangyi*, FR-4 utilizes formate for methanogenesis, does not require acetate, is stimulated by yeast extract and tryptose, and has an optimum growth temperature of 57°C. *Methanogenium thermophilicum* shows optimal growth at 55°C, with a minimum doubling time of 2.5 h compared with 1.2 h for FR-4. This species also differs from FR-4 in requiring tryptose, trace vitamins, and 0.2 M NaCl (16). The DNA base ratios of FR-4 and *Methanogenium thermophilicum* are 49.2 and 59 mol% G + C, respectively.

The evidence suggests that FR-4 is not identical to any named species of *Methanococcus* or *Methanogenium*, and this is supported by comparison of whole cell proteins by SDS-gel electrophoresis. Since the DNA base ratio of FR-4 falls within the range 37 to 61.2 mol% G + C described for *Methanogenium* and is higher than that of the genus *Methanococcus* (30.7 to 40.5), the isolate may belong to this genus. Further phylogenetic evidence is needed to assign FR-4 unambiguously to genus level, but on the basis of the characteristics described here we suggest that the isolate is a new species and should be given the name *Methanogenium frittonii*.

The characteristics of this novel isolate are summarized below.

Morphology. The cells are nonmotile, fimbriated irregular cocci, 1 to 2.5 μm in diameter, occurring singly or in pairs. Cells are surrounded by a protein envelope (ca. 20 nm thick) and are disrupted by pronase and trypsin digestion, glass-distilled water, and 1% SDS.

Colony characteristics. Surface colonies are dark yellow, circular, convex, and shiny with entire edges.

Source. Anaerobic, watery sediment from Fritton Lake, a freshwater lake in Norfolk, England.

Physiology. Growth occurs over the temperature range 26 to 62°C, with the optimum at 57°C. The optimum pH for growth is 7 to 7.5, and the pH range is 6 to 8.25.

Nutrition. Strictly anaerobic and autotrophic. Hydrogen plus carbon dioxide and formate serve as substrates for methanogenesis. Yeast extract, tryptose, and Casamino Acids are all stimulatory to growth. Sodium chloride is not required and is inhibitory above 2% (wt/vol).

DNA base ratio. 49.2 mol% G + C.

This strain has been deposited in the Deutsche Sammlung von Mikroorganismen (DSM 2832).

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LITERATURE CITED

1. **Archer, D. B., and J. E. Harris.** 1985. Methanogenic bacteria and methane production in various habitats. *In* E. M. Barnes and G. C. Mead (ed.), Anaerobic bacteria in habitats other than man. Society for Applied Bacteriology Symposium Series 13. Blackwell Scientific Publications, Oxford.
2. **Archer, D. B., and N. R. King.** 1983. A novel ultrastructural feature of a gas-vacuolated *Methanosarcina*. FEMS Microbiol. Lett. **16**:217–223.
3. **Balch, W. E., G. E. Fox, L. J. Magrum, C. R. Woese, and R. S. Wolfe.** 1979. Methanogens: reevaluation of a unique biological group. Microbiol. Rev. **43**:260–296.
4. **Chater, K. F., D. A. Hopwood, T. Kieser, and C. J. Thompson.** 1982. Gene cloning in *Streptomycetes*. Curr. Top. Microbiol. Immunol. **96**:69–95.
5. **Corder, R. E., L. A. Hook, J. M. Larkin, and J. I. Frea.** 1983. Isolation and characterization of two new methane-producing cocci: *Methanogenium olentangyi*, sp. nov., and *Methanococcus deltae*, sp. nov. Arch. Microbiol. **134**:28–32.
6. **Ferguson, T. J., and R. A. Mah.** 1983. Isolation and characterization of an H_2-oxidizing thermophilic methanogen. Appl. Environ. Microbiol. **45**:265–274.
7. **Hilpert, R., J. Winter, W. Hammes, and O. Kandler.** 1981. The sensitivity of archaebacteria to antibiotics. Zentralbl. Bakteriol. Mikrobiol. Hyg. I Abt. Orig. C **2**:11–20.
8. **Hook, L. A., R. E. Corder, P. T. Hamilton, J. I. Frea, and J. N. Reeve.** 1983. Modification and use of an ultra-low oxygen chamber for the growth of methanogens. *In* W. R. Strohl and O. H. Tuovinen (ed.), Microbial chemoautotrophy. Ohio State University Press, Columbus.
9. **Huber, H., M. Thomm, H. Konig, G. Thies, and K. O. Stetter.** 1982. *Methanococcus thermolithotrophicus*, a novel thermophilic lithotrophic methanogen. Arch. Microbiol. **132**:47–50.
10. **Hungate, R. E.** 1969. A roll tube method for cultivation of strict anaerobes. Methods Microbiol. **3**:117–132.
11. **Jones, J. B., B. Bowers, and T. C. Stadtman.** 1977. *Methanococcus vannielii*: ultrastructure and sensitivity to detergents and antibiotics. J. Bacteriol. **130**:1357–1363.
12. **Jones, W. J., M. J. P. Paynter, and R. Gupta.** 1983. Characterization of *Methanococcus maripaludis* sp. nov., a new methanogen isolated from salt marsh sediment. Arch. Microbiol. **135**:91–97.
13. **Laemmli, U. K., and M. Favre.** 1973. Maturation of the head of bacteriophage T4. I. DNA packaging events. J. Mol. Biol. **80**:575–599.
14. **Lund, B. M., J. M. Gee, N. R. King, R. W. Horne, and J. M. Harnden.** 1978. The structure of the exosporium of a pigmented Clostridium. J. Gen. Microbiol. **105**:165–174.
15. **Pecher, T., and A. Bock.** 1981. *In vivo* susceptibility of halophilic and methanogenic organisms to protein synthesis inhibitors. FEMS Microbiol. Lett. **10**:295–297.
16. **Rivard, C. J., and P. H. Smith.** 1982. Isolation and characterization of a thermophilic marine methanogenic bacterium, *Methanogenium thermophilicum* sp. nov. Int. J. Sys. Bacteriol. **32**:430–436.
17. **Romesser, J. A., R. S. Wolfe, F. Mayer, E. Spiess, and A. Walther-Mauruschat.** 1979. *Methanogenium*, a new genus of marine methanogenic bacteria and characterization of *Methanogenium cariaci* sp. nov. and *Methanogenium marisnigri* sp. nov. Arch. Microbiol. **121**:147–153.
18. **Schildkraut, C. L., J. Marmur, and P. Doty.** 1962. Determination of the base composition of deoxyribonucleic acid from its buoyant density in CsCl. J. Mol. Biol. **4**:430–443.
19. **Sleytr, U. B., and P. Messner.** 1983. Crystalline surface layers on bacteria Annu. Rev. Microbiol. **37**:311–339.
20. **Woese, C. R., L. J. Magrum, and G. E. Fox.** 1978. Archaebacteria. J. Mol. Evol. **11**:245–252.