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Methanospirillum, a New Genus of Methanogenic Bacteria, and Characterization of *Methanospirillum hungatii* sp.nov.

JAMES G. FERRY,[1] PAUL H. SMITH, and R. S. WOLFE

Department of Microbiology, University of Illinois, Urbana, Illinois 61801, and Department of Microbiology, University of Florida, Gainesville, Florida 32601

A new genus of methanogenic bacteria is described. The colonies produced by these bacteria are yellow, circular, and convex with lobate margins; an optical pattern of regular, light and dark striations throughout the colonies is a most unique and distinguishing characteristic. These striations are two cell lengths apart. Cells are gram negative and occur in filaments up to 100 μm in length. Tufts of polar flagella and a striated cell surface are revealed in electron micrographs; cell ends are blunt, not rounded. The deoxyribonucleic acid base composition of the type species is 45 mol% guanine plus cytosine. Formate or hydrogen and carbon dioxide serves as a substrate for methane formation and growth; acetate, pyruvate, methanol, ethanol, and benzoate do not. The name *Methanospirillum* is proposed for this new genus of spiral-shaped methanogenic bacteria. The type species, *Methanospirillum hungatii* sp. nov., is named in honor of R. E. Hungate. The type strain of *M. hungatii* is JF1 (ATCC 27890).

In 1966, P. H. Smith reported the isolation of a new spiral-shaped methanogenic bacterium from sewage sludge (8). In the present paper we define the characteristics of and propose a name for this new organism. A report on some of these findings has appeared previously (J. G. Ferry and R. S. Wolfe, Abstr. Annu. Meet. Amer. Soc. Microbiol. 1973, G121, p. 46).

MATERIALS AND METHODS

Bacterial strains. Strain BM was received from Barry C. McBride (University of British Columbia, Vancouver), strain 3-PS was received from Paul H. Smith (University of Florida, Gainesville), and strain JF1 was isolated in the present study. The inoculum from which each strain was isolated was sewage sludge (8, 9).

Media. Sterile growth media were prepared, and the organisms were cultivated under a strictly anaerobic (80% H_2-20% CO_2) atmosphere by a modification of the Hungate technique (4) as described by Bryant and Robinson (3). The maintenance medium contained the following constituents in distilled water at the indicated final percentage compositions (wt/vol): K_2HPO_4, 0.023; KH_2PO_4, 0.023; $(NH_4)_2SO_4$, 0.023; NaCl, 0.046; $MgSO_4 \cdot 7H_2O$, 0.009; $CaCl_2 \cdot 2H_2O$, 0.006; sodium formate, 0.2; yeast extract (Difco), 0.2; Trypticase (BBL), 0.2; resazurin, 0.0001; Na_2CO_3, 0.4; cysteine hydrochloride, 0.025; $Na_2S \cdot 9H_2O$, 0.025. In addition, 1% (vol/vol) each of vitamin solution and trace mineral solution were added to the medium (10). Before sterilization, the medium was adjusted to pH 7.2 with 1 M HCl. The final pH after sterilization and equilibration with a gas mixture of 80% H_2-20% CO_2 was 7.0. Solid medium for roll-tube cultures and slants was prepared by including Noble agar (Difco) at a final concentration of 2% (wt/vol). Cultures were incubated at 37 C. The liquid benzoate-enrichment medium contained the following constituents in distilled water at the indicated final concentration (wt/vol) in percent: sodium benzoate, 0.2; NH_4Cl, 0.075; K_2HPO_4, 0.04; $MgCl_2$, 0.01; resazurin, 0.0001; Na_2CO_3, 0.15; $Na_2S \cdot 9H_2O$, 0.025. Before sterilization, the medium was adjusted to pH 7.2 with HCl. The final pH after sterilization and equilibration with a gas mixture of 95% N_2-5% CO_2 was 7.0. This medium was inoculated with sludge from an anaerobic sewage-sludge digester, and the crude benzoate-degrading enrichment was maintained under strict anaerobic conditions by the Hungate technique (3, 4), about 20% of the medium being replaced with sterile medium once a week. A 95% N_2-5% CO_2 atmosphere was used.

Methods. Photomicrographs were made with a Carl Zeiss universal photomicroscope. Wet mounts of cultures were prepared on glass slides or slides which had been coated with 2% washed Noble agar (Difco) and dried. Colonies were photographed as they appeared on the agar surface of the growth medium. A Jeolco model JEM-T6S electron microscope was used to obtain shadowed micrographs. Aqueous solutions of washed whole cells were dried on support grids and shadowed at an angle of 5 to 10° with gold and platinum alloy (1:1). An RCA EMU-3C electron microscope was used for observing whole cells which had been negatively stained with a 0.02% (wt/vol) aqueous solution of phosphotungstic acid adjusted to pH 7.0 with potassium hydroxide.

Cells were lysed with sodium dodecyl sulfate, and deoxyribonucleic acid (DNA) was purified from the

[1] Present address: Department of Biochemistry, University of Georgia, Athens, Ga. 30601.

cell lysate by the method of Marmur (6); the buoyant density of the DNA was determined by cesium chloride density gradient centrifugation in a Beckman model E ultracentrifuge. The base ratio was calculated by the method of Schildkraudt et al. (7). DNA from *Micrococcus lysodeikticus* (a gift from C. L. Hershberger) was used as a standard.

Methane was quantitated with a Packard 7800 series gas chromatograph equipped with a Porapak QS column connected to a hydrogen flame detector. The column was operated at 50 C. The flow rate of helium carrier gas was 60 ml/min.

RESULTS

Direct isolation of the new microorganism was accomplished by making serial dilutions of the benzoate enrichment in agar maintenance-medium roll tubes containing an 80% H_2-20% CO_2 atmosphere. Methanogenic colonies appeared after other colonies had reached a maximal size, and their appearance correlated with the formation of methane. To obtain pure cultures, we picked an isolated colony with a bent, sterile transfer pipette and dispersed the cells in liquid maintenance medium; serial dilutions of this suspension were carried out in agar maintenance medium. All strains were maintained by weekly transfer on agar slants of maintenance medium.

Colonies of strain JF 1 in deep agar were diffuse, round, light yellow in color, and always less than 3 mm in diameter. Surface colonies on agar were light yellow, circular, and convex with lobate margins. Microscopic examination of colonies revealed an optical pattern of regular light and dark striations approximately two cell lengths (16 μm) apart (Fig. 1).

Single cells (Fig. 2c) were curved rods measuring 7.4 by 0.5 μm and most often existing in filaments (Fig. 2a). In phase-contrast micrographs of older cultures, filaments exhibited areas of lysed cells in which an envelope remained, suggesting a sheathlike structure encasing the individual cells (Fig. 2b). No spores or sporelike structures were observed.

All strains exhibited weak motility with progressive movement. Electron micrographs of gold-shadowed whole cells showed tufts of polar flagella and a striated surface structure (Fig. 3a). Zwillenberg (12) has shown that surfaces of this type are characteristic of certain gram-negative bacteria. The striated surface was made more visible by negatively staining the whole cells with phosphotungstic acid. The most conspicuous feature of negatively stained cells was the generally disturbed dark patches of phosphotungstic acid that appeared to be located just beneath the striated surface structure (Fig. 3b). Bladen et al. (2) have postulated a pore some 30 to 40 nm in diameter in the cell surface (2). Negatively stained cells of *Methanobacterium* sp. strain M. o. H. exhibited similar patterns, which were referred to as baglike inclusions (5).

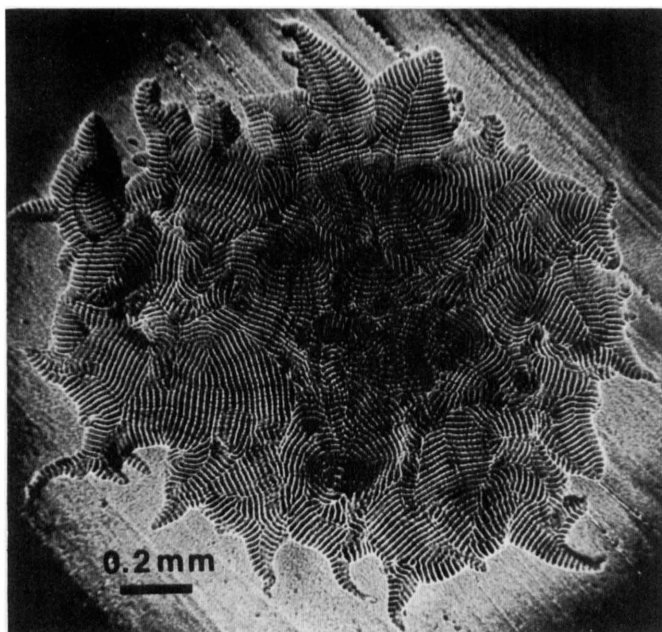

FIG. 1. *Surface colony of M. hungatii sp. nov. exhibiting ridges two cell lengths (16 μm) apart.*

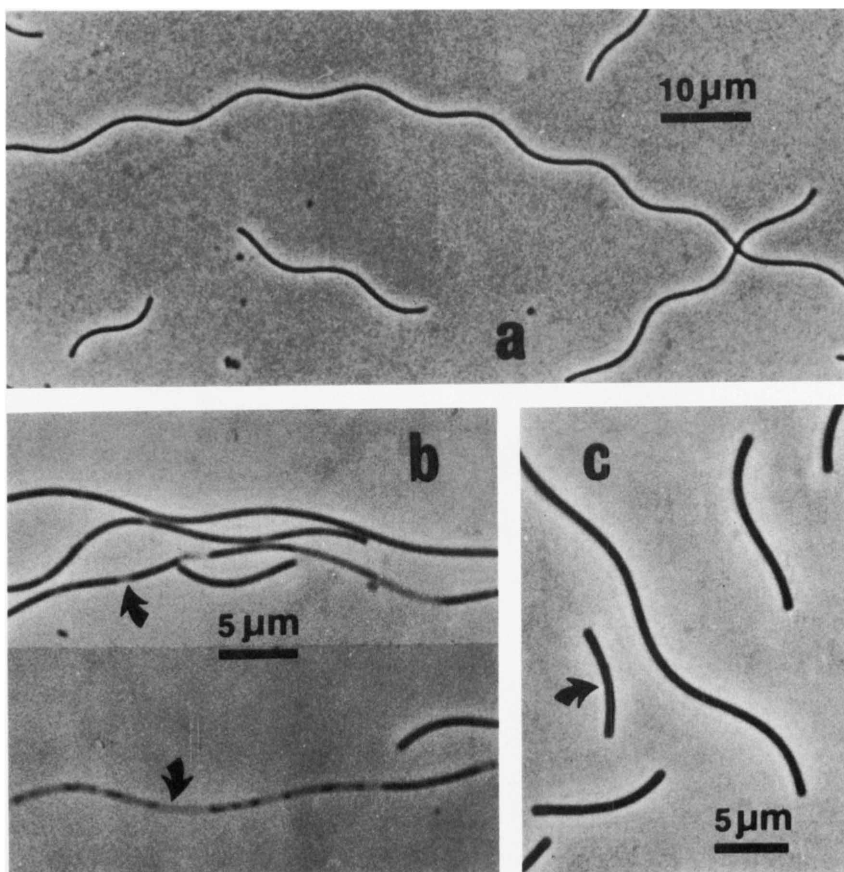

FIG. 2. *Phase-contrast micrographs of M. hungatii sp. nov. (a) Filaments of a young culture; (b) lysed filaments of older cultures exposing the sheathlike structure (arrows); (c) a single cell (arrow).*

Broth cultures of the new microorganism, which were briefly exposed to air, were unable to continue growth or production of methane when returned to an atmosphere of 80% H_2-20% CO_2. These results emphasize the extreme sensitivity of this organism to oxygen, a general characteristic of the methane bacteria.

Table 1 lists the results obtained in an experiment designed to determine substrates for methane formation by strain JF1. Tubes of liquid maintenance medium (in triplicate; final volume, 5 ml) were supplemented with 0.4% of the substrate listed. Pyruvate was sterilized by filtration. After inoculation, each tube (3 through 9) was injected with 7.1 µmol of H_2 as an internal control to confirm that the inoculum was active. After 30 days of incubation, tubes were assayed for methane production. Formate and H_2-CO_2 were the only substrates which yielded methane. No methane was formed in the presence of CO_2 alone. In the tubes which received an 80% H_2-20% CO_2

TABLE 1. *Effect of added substrates on methane formation by growing cultures of M. hungatii sp. nov.*

Tube no.	Substrate tested	CH_4 formed[a] (µmol)
1	80% N_2-20% CO_2 atmosphere	0.0
2	80% H_2-20% CO_2 atmosphere	209.0
3	80% N_2-20% CO_2 atmosphere +7.1 µmol of H_2	0.6
4[b]	+ acetate	0.7
5	+ benzoate	0.5
6	+ ethanol	0.6
7	+ methanol	0.6
8	+ pyruvate	1.0
9	+ formate	48.2

[a] Mean value of total CH_4 from three replicate 5-ml cultures after 30 days.

[b] Tubes 4 through 9 were identical to tube no. 3 except for addition of each substrate, as indicated, to a final concentration of 0.4%.

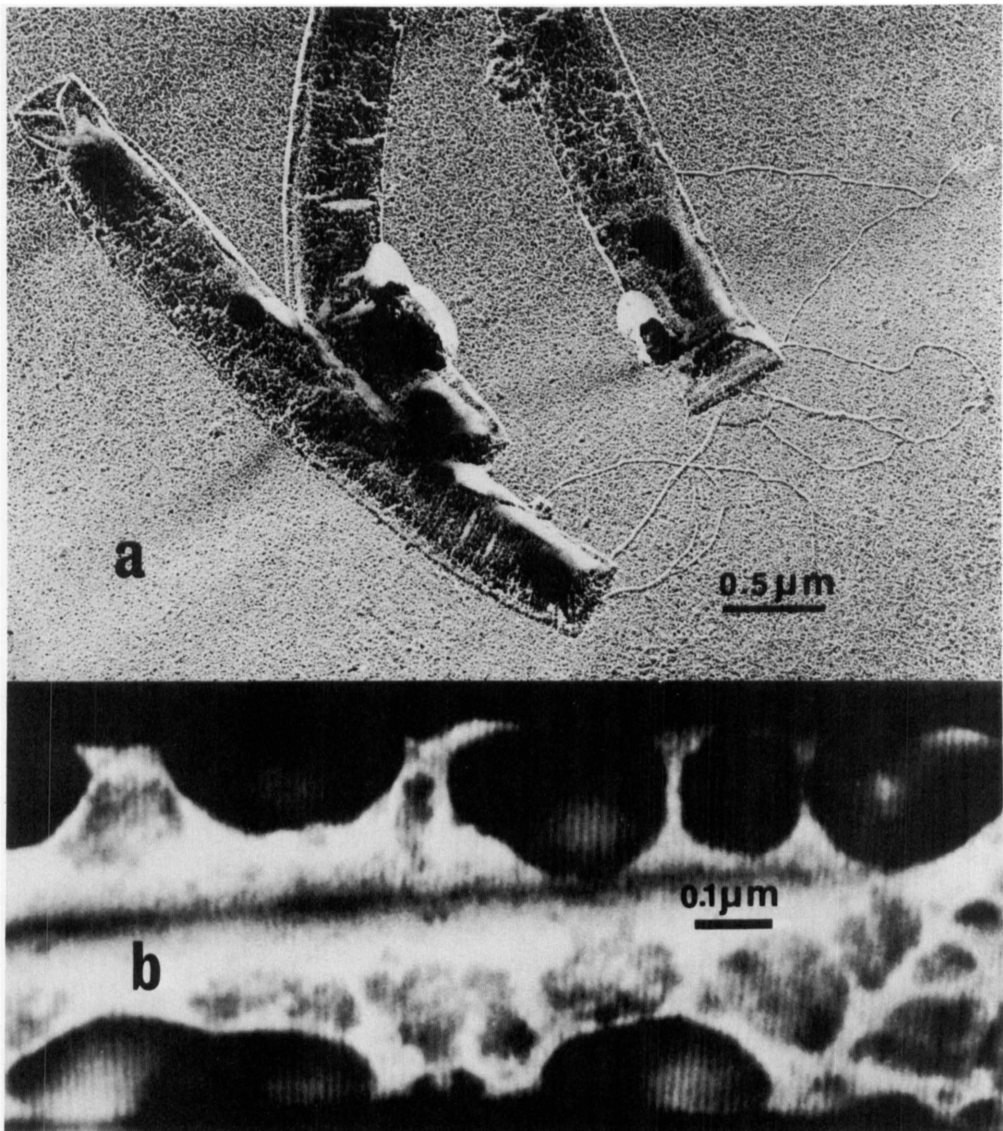

FIG. 3. *Electron micrographs of M. hungatii sp. nov. (a) Shadowed whole cells showing tufts of polar flagella (prepared by Jon Mielenz); (b) negatively stained cells showing dark patches and surface striations (prepared by Charles Eller).*

atmosphere, 840 µmol of H_2 was available and 209 µmol of methane was produced in agreement with the equation 4 H_2 + CO_2 → CH_4 + $2H_2O$. Failure of the organism to produce methane from other substrates tested is not due to unsatisfactory conditions, since methane was produced from the 7.1 µmol of injected hydrogen. Both strains 3-PS and BM used formate and H_2-CO_2 as substrates for methane formation. A formate-utilizing strain of spiral-shaped methanogenic bacteria also has been isolated by Sin Fu Tzeng (M. P. Bryant, personal communication).

Sodium dodecyl sulfate was used to break cells for the isolation of DNA, since lysozyme was ineffective. The DNA base composition for the new microorganism was determined to be 45 mol% guanine plus cytosine (G + C). This compares with previously published values of 38 mol% G + C for *Methanobacterium* strain M. o. H. and 52 mol% G + C for *Methanobacterium thermoautotrophicum* (11). Cells of young and old cultures were gram negative. This result was confirmed when the organism was mixed and stained with cells of *Staphylococcus albus* and *Escherichia coli.*

DISCUSSION

Based on the description of the family *Methanobacteriaceae* Barker (1) appearing in the eighth edition of *Bergey's Manual of Determinative Bacteriology* (M. P. Bryant, personal communication), the strains described herein are included in this family. Due to their spiral morphology, these strains belong to a new genus of methane-producing bacteria, for which the name *Methanospirillum* is proposed. The type species of this genus is *M. hungatii* sp. nov., named in honor of R. E. Hungate.

A combined generic and specific description follows.

Methanospirillum gen. nov. and *M. hungatii* sp. nov.

Morphology: Curved rods measuring 7.4 by 0.5 μm. Cells most often occur in filaments from 15 to several hundred μm in length. Gram negative. Previously, strain 3-PS was observed to give a gram-positive reaction (9). A reexamination reveals that the cells are gram negative. Other gram-negative properties include resistance to lysozyme and a striated cell surface. Motile by means of polar, tufted flagella. Endospores are absent.

Colony characteristics: Light yellow, circular, convex with lobate margins, uniquely striated when observed under low-power magnification.

DNA base composition: 45 mol% G + C.

Temperature relationship: Optimum, 30 to 37 C.

pH relationship: Optimum, 6.6 to 7.4.

Source: Sewage sludge.

Physiology: Fastidious anaerobe; formate or H_2-CO_2 serves as a substrate for methane production; benzoate, ethanol, methanol, pyruvate, and acetate do not.

Nutrition: Best growth is obtained in a complex medium containing yeast extract and Trypticase. Rumen fluid is not required.

Type strain: JF1. This strain has been deposited in the American Type Culture Collection (ATCC), Rockville, Md. under the number 27890. The description of the type strain is the same as that given above for the genus and species.

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REPRINT REQUESTS

Address reprint request to: R. S. Wolfe, Department of Microbiology, 131 Burrill Hall, University of Illinois, Urbana, Ill. 61801.

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