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Isolation and Characterization of *Methanomicrobium paynteri* sp. nov., a Mesophilic Methanogen Isolated from Marine Sediments†

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A new mesophilic methanogenic bacterial species isolated from marine sediments collected in the Cayman Islands is described. Cells are small rods occuring singly without filaments, are not motile, and do not possess flagella. Colonies are semitransparent and off-white in color. After 2 weeks of incubation at 37°C colonies are 1 to 2 mm in size, circular, and have entire edges. Only hydrogen-carbon dioxide is a substrate for growth and methane formation. Cells can tolerate a variety of organic secondary buffers (bicarbonate-CO_2 being the primary buffer). Cells do not require yeast extract or Trypticase, but do require acetate, for growth. The optimum growth temperature is 40°C. The optimum sodium concentration is 0.15 M. The optimum pH for growth is 7.0. The minimum generation time is 4.8 h. The DNA base composition is 44.9 mol% guanine plus cytosine. The name *Methanomicrobium paynteri* is proposed in honor of M. J. B. Paynter. The type strain is G-2000 (=ATCC 33997, =DSM 2545).

Current investigations on inoculum development for biomass conversion systems concern bacterial species involved in the various stages of methane fermentation as it occurs in a variety of habitats. Marine fermentation systems may play an integral role in the production of methane gas for use as a supply of energy. Knowledge of the marine bacterial species that are involved in the terminal stages of the fermentation process will be a valuable tool in improving the efficiency of the conversion of biomass to methane gas.

In 1979, Balch et al. (1) described the characteristics and phylogenetic relationships of 13 species of methanogens, of which 4 species were isolated from marine or near-shore environments. The isolation of *Methanococcus vannielii* from San Francisco Bay by Stadtman and Barker in 1951 (16) is the first reported isolation of a methanogen from a marine environment. This motile irregular coccus frequently appears in pairs and utilizes formate as well as hydrogen-carbon dioxide for growth and methane production. A marine methanogenic coccus was isolated by J. M. Ward (M.S. thesis, University of Florida, Gainesville, 1970) from Waccasassa Estuary, Fla. This bacterium, *Methanococcus voltae*, is a regular to irregular coccus, is highly motile, has a lower and more restrictive pH requirement than does *M. vannielii*, and requires NaCl, whereas *M. vannielii* does not require NaCl (1). *M. voltae* also utilizes formate and hydrogen-carbon dioxide for growth and methane production. Romesser et al. (13) describes the genus *Methanogenium* and two species, *M. cariaci*, from the Cariaco trench, and *M. marisnigri*, from the Black Sea. These organisms are irregular cocci that occur singly, require 1.5 to 3.0% NaCl for growth, and utilize formate and hydrogen-carbon dioxide for growth and methane production (1, 13).

Since the review by Balch et al. (1), two additional marine methanogens have been isolated and characterized. *Methanococcus thermolithotrophicus* (5) was isolated from geothermally heated sea sediments near Naples, Italy. It is a motile coccus that requires 1.8 to 8.3% NaCl, has an optimum temperature of 65°C for growth, and utilizes formate and hydrogen-carbon dioxide for growth and methane production (5). Rivard and Smith (12) isolated *Methanogenium thermophilicum* from marine sediments receiving heated effluent from a coastal nuclear power plant. This marine methanogen is an irregular nonmotile coccus with an optimum temperature of 55°C for growth and utilizes formate and hydrogen-carbon dioxide for growth and methane production.

To date, all methanogens isolated from marine or near-shore environments have been coccoid

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and utilize formate as well as hydrogen-carbon dioxide for growth and methane production. In this paper we describe a mesophilic methanogen isolated from marine sediments that is rod shaped and does not utilize formate for growth or methane production. This species is named in honor of M. J. B. Paynter for his contributions to the microbial ecology of anaerobic environments.

MATERIALS AND METHODS

Sample collection. Sediments cores were taken along the edges of the mosquito control canals within mangrove swamps located southeast of Georgetown, Grand Cayman, British West Indies. A 3.0-cm by 1.0-m Plexiglas tube was inserted into the sediment to 20 cm, plugged at the top with a rubber stopper, and retrieved. Sediment cores were removed from the tube, and subcores were taken at depths of 4 and 12 cm. These subcores were placed into 125-ml serum bottles containing 25 ml of sterile anaerobic medium (medium no. 3 of Balch et al. [1]). The sediment enrichments were transported to the laboratory where they were flushed and pressurized to 170 kPa (10 psig) with sterile oxygen-free 80% H_2–20% CO_2 gas mixture (Matheson, Morrow, Ga.) (2).

Enrichment and isolation. Sediment enrichments were shaken at 37°C for 1 week, after which time the sediment enrichments positive for methane were serially diluted (1:10) in Balch et al. no. 3 medium (1). Anaerobic roll tubes were prepared from these dilutions by using Balch et al. no. 3 medium supplemented with agar (2%, final concentration). The roll tubes were incubated at 37°C with sterile oxygen-free 80% H_2–20% CO_2 as the gas phase at 170 kPa. After 2 weeks of incubation, roll tubes were analyzed for methane. The highest dilution roll tubes that contained methane were examined by epifluorescence microscopy. Isolated colonies that autofluoresced (indicating the presence of F420 [4, 8]) were picked and serially diluted; roll rubes were prepared from the serial dilutions. The process was repeated until a pure culture was obtained.

Media and anaerobic preparations. All media were prepared by the principles of the Hungate anaerobic technique (6) modified to utilize serum tubes and bottles (2). The composition of Balch et al. no. 3 medium used in the sediment enrichments is as described previously (1). Secondary organic buffers analyzed for isolate tolerance were as follows: *N*-(2-acetamido)-2-aminoethanesulfonic acid; *N*,*N*-bis(2-hydroxyethyl)-2-aminoethanesulfonic acid; *N*-2-hydroxyethylpiperazine-*N*'-2-ethanesulfonic acid (HEPES); morpholine propanesulfonic acid (MOPS); *N*-tris(hydroxymethyl)methyl-2-aminoethanesulfonic acid; Tris; and *N*-[tris(hydroxymethyl)methyl]glycine. All buffers were obtained from Sigma Chemical Co. (St. Louis, Mo.) or Eastman Kodak Co. (Rochester, N.Y.). After a pure culture was established, the following defined medium (designated G-2000 medium) was developed and used throughout the characterization studies. The medium was as follows: mineral solution d (1), 500 ml; trace mineral solution e (1), 10 ml; trace vitamin solution f (1), 10 ml; NaCl, 6.3 g; $Fe(NH_4)_2(SO_4)_2$, 0.002 g; $NaHCO_3$, 5.0 g; NH_4Cl, 1.25 g; $CH_3COONa\ 3H_2O$, 4.15 g; cysteine-hydrochloride, 0.5 g; $Na_2S \cdot 9H_2O$, 0.25 g; MOPS, 20.9 g; resazurin, 0.001 g; distilled water to 1,000 ml. G-2000 medium was prepared by adding all components, except MOPS, sulfide, and cysteine-hydrochloride, in a total volume of 890 ml of distilled water. The solution was deoxygenated, and the pH was adjusted to 7.1 with 2 M KOH. MOPS was separately dissolved in 90 ml of distilled water and deoxygenated, and the pH was adjusted to 7.1 with 2 M KOH. The MOPS solution was added to the salts solution, cysteine-hydrochloride was added, and the medium was anaerobically transferred to 500-ml serum bottles with a gas phase of oxygen-free 80% N_2–20% CO_2. The medium was autoclaved at 121°C for 20 min, and upon cooling to room temperature the final pH was 7.1.

Gas mixtures were made oxygen free by passage through heated (350°C) copper turnings.

Growth rate measurements. Anaerobic growth flasks were constructed by joining an 18- by 150-mm serum tube (no. 2048-00150; Bellco Glass Inc., Vineland, N.J.) to a 125-ml serum bottle (no. 223748; Wheaton Scientific, Millville, N.J.). Growth flasks were flushed with oxygen-free 80% H_2–20% CO_2, and anoxic distilled water was added (0.2 ml) to assure steam sterilization upon autoclaving. The flasks were closed with butyl rubber stoppers (no. 2048-11800; Bellco) that were retained with aluminum seals (no. 224193, Wheaton), and the flasks were autoclaved at 121°C for 20 min.

For growth measurements, 14.0 ml of sterile G-2000 medium and 0.3 ml of sterile sodium sulfide solution (final concentration, 0.25 g/liter) were aseptically added to each sterile growth flask. The anaerobic growth flasks were either inoculated with 0.5 ml from an early log phase culture (experimental flasks) or 0.5 ml of sterile medium (uninoculated controls). The growth flasks were pressurized to 170 kPa with oxygen-free 80% H_2–20% CO_2 and incubated at 37°C on a gyratory shaker (200 rpm). Variations in the assay conditions were as follows: 0.2 M MOPS buffer (optimum pH experiment); shaking water baths (optimum temperature experiment); and all G-2000 medium components normally added as the sodium salt were added as the potassium salt (optimum sodium ion concentration experiment). Optical density measurements were determined at 610 nm with a Perkin-Elmer model 35 spectrophotometer. After each optical density determination, the gas phase was evacuated and pressurized to 170 kPa with oxygen-free 80% H_2–20% CO_2 for three cycles. A standard curve relating viable cell numbers to optical density measurements was determined by diluting broth cultures of known optical density and preparing roll bottles (125 ml), from which colonies were counted after 2 weeks of incubation at 37°C with a 170-kPa 80% H_2–20% CO_2 substrate. Specific growth rate calculations were based on cells per milliliter. The minimum generation time was calculated from the maximum specific growth rate.

Gas mixtures were sterilized by passage through a sterile 2.5-ml syringe filled with cotton.

Gas chromatography. Methane concentrations were measured using a Hewlett-Packard model 5880A gas chromatograph. Gases were separated in a 1.8-m by 1.0-mm copper column packed with Porapak Q, mesh 80/100 (Supelco) and measured with a thermal conductivity detector. Helium was the carrier gas. Column and detector temperatures were maintained at 30 and

145°C, respectively. Methane concentrations were determined by comparison to standards prepared from ultra-high-purity methane (Matheson).

Microscopy and photomicroscopy. A Carl Zeiss Standard WL microscope equipped for epifluorescence was used for observation of wet mounts and fluorescence of the bacteria (wet mounts) and colonies (roll tubes). Light in the 420-nm wavelength range was provided by a mercury light source (HBO 50 DC 3) and a filter set comprised of an exciter filter (BP 390-440), a chromatic beam splitter (FT 460), and a barrier filter (LP 475). A Nikon FM camera back was attached to the microscope for photomicroscopy. Colonies were observed with an Olympus model SZ-Tr stereo microscope.

Electron microscopy. Midlog-phase cells were prepared at 37°C as described (12). Fixed cells were embedded in Spurr's low-viscosity medium (15) and stained by the method of Reynolds (11).

DNA isolation and analysis of base ratio. DNA was isolated by the method of Marmur (7), and base ratios were determined by the procedures of Preston and Boone (10) and the calculations of Schildkraut et al. (14) as described previously (12).

RESULTS

An obligately anaerobic mesophilic methanogenic bacterium was isolated from marine sediments obtained in the Cayman Islands. The presence of a pure culture was determined on the basis of similar cellular morphology in wet mounts, Gram stain reaction, similar colony morphology in roll tubes, and fluorescence of all cells and colonies upon excitation with 420-nm light. Growth was not observed upon the addition of glucose (4.0 g/liter) to the complex Balch no. 3 medium when methanogenic substrates were not present.

Cells were gram-negative, irregular rods, 0.6 by 1.5 to 2.5 μm in size, that occurred singly and without filaments (Fig. 1). Neither motility (wet mounts) nor flagella (electron micrographs) were observed. Thin sections examined with the electron microscope revealed a central region of fibrous material resembling condensed DNA, an outer granular region resembling a ribosome-rich region, and a defined membrane and cell wall (Fig. 2A and B). Cells were easily lysed by treatment with 0.1% sodium dodecyl sulfate, sodium desoxycholate, or Triton X-100.

Upon substrate deprivation, cells became more irregular (Fig. 3A) and finally coccoid (Fig. 3B). These coccoid cells could survive substrate starvation for more than 6 months.

Colonies are 1 to 2 mm in diameter, circular with entire edges, and off-white in color after 2 weeks of incubation at 37°C in roll tubes. Colonies are initially semitransparent and became more opaque with age.

The isolate required neither yeast extract nor Trypticase (BBL Microbiology Systems, Cockeysville, Md.) for growth, but did require acetate. However, yeast extract and Trypticase were stimulatory to the growth rate and final cell concentration. G-2000 medium reduced with cysteine-hydrochloride alone supported growth of the isolate; thus, added sodium sulfide was not required for growth. Various buffers were analyzed for the ability of the isolate to tolerate them at concentrations used for secondary buffering of the medium. *N*-(2-Acetamido)-2-aminoethanesulfonic acid, *N*,*N*-bis(2-hydroxyethyl)-2-aminoethanesulfonic acid, *N*-tris(hydroxymethyl)methyl-2-aminoethanesulfonic acid, Tris, and *N*-[tris(hydroxymethyl)methyl]glycine at both 0.05 and 0.1 M were not inhibitory as compared with controls without a secondary buffer. Phosphate was also not inhibitory, but produced substantial precipitation.

Various substrates were analyzed for the isolate's ability to metabolize them for energy and the production of methane. Only 80% H_2–20% CO_2 was utilized as a substrate for methane production and growth. Ethanol, methanol, methylamine, dimethylamine, trimethylamine, formate, acetate, pyruvate, propionate, glutamate, and glucose (0.5% final concentration) were not metabolized under an 80% N_2–20% CO_2 atmosphere or under an 80% N_2–20% CO_2 atmosphere with 1 ml of 80% H_2–20% CO_2 added. In all experiments on substrates, growth occurred when an 80% H_2–20% CO_2 gas phase was present, indicating that the added substrates were not toxic at those concentrations.

The optimum sodium ion concentration for growth of the isolate was 0.15 M (Fig. 4) with a range of 0 M to slightly greater than 0.8 M (data not shown). The optimum temperature for

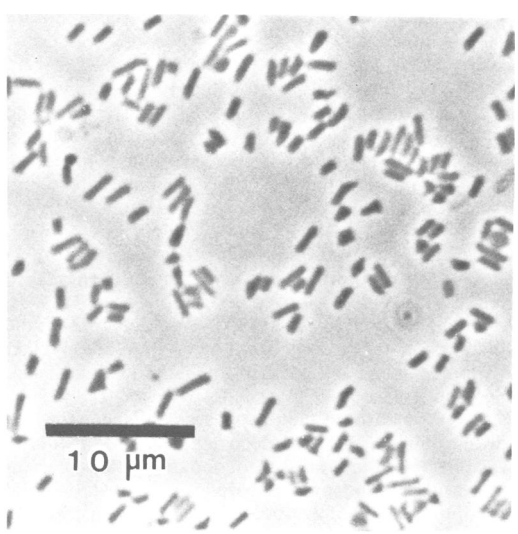

FIG. 1. Phase-contrast photomicrograph of *M. paynteri* showing rod-shaped morphology.

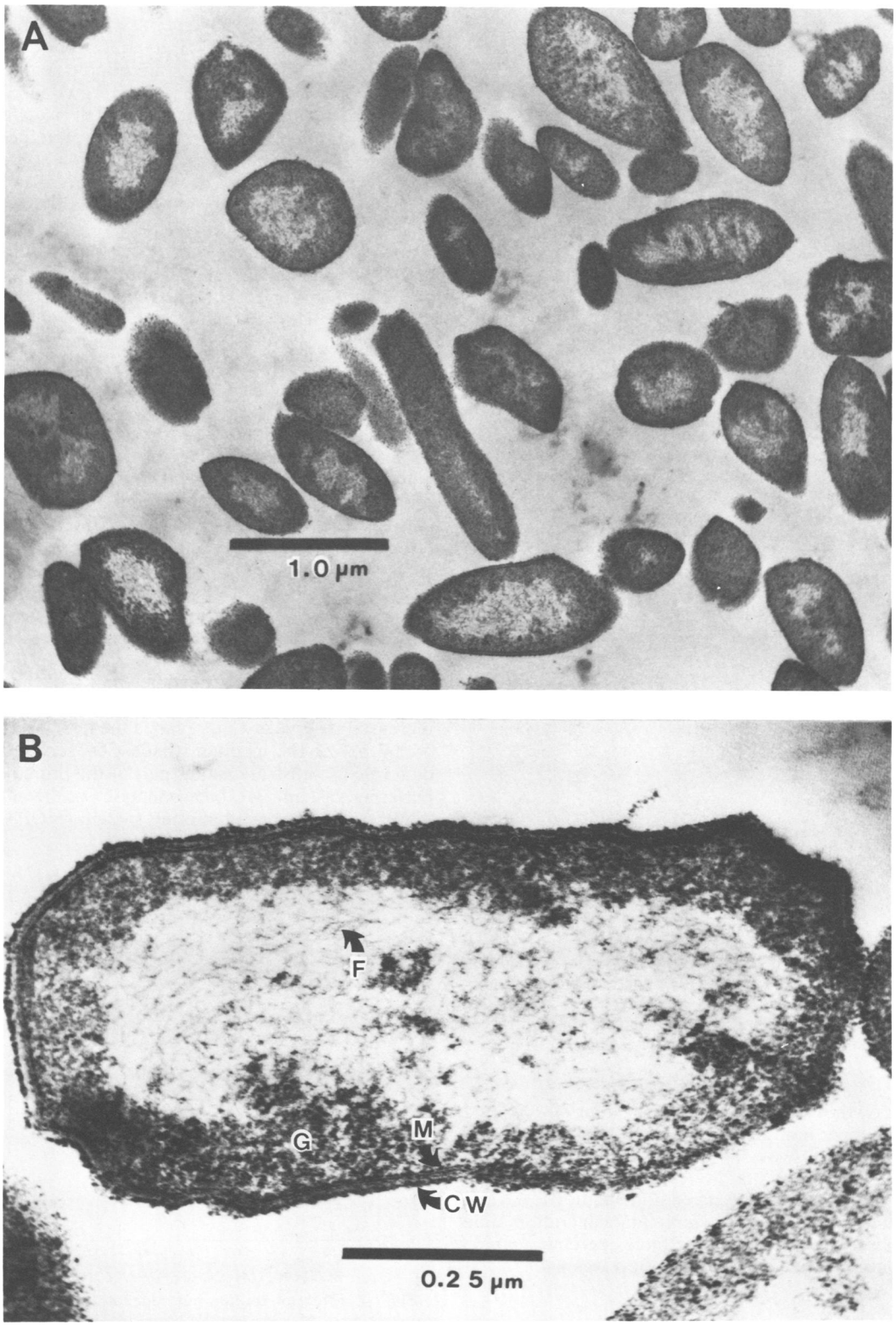

FIG. 2. Electron photomicrographs of *M. paynteri* cells. (A) Group of typical cells. (B) Single cell detailing internal structures such as (F) fibrous material, (G) granular region, (M) membrane, and (CW) cell wall.

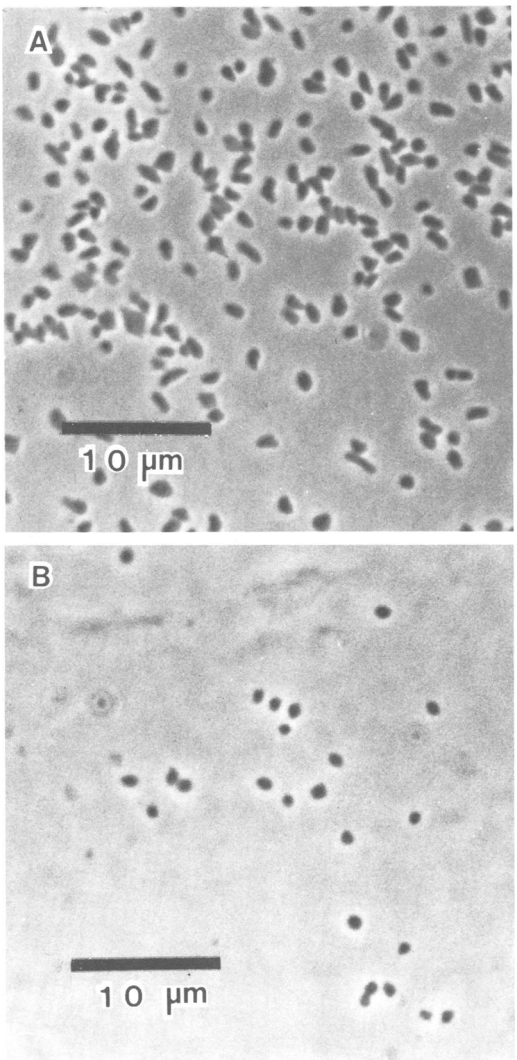

FIG. 3. Phase-contrast photomicrographs of *M. paynteri* showing the effect of substrate deprivation. (A) Intermediate stage with a decrease in the number of rod-shaped cells and the appearance of coccoid shaped cells. (B) Complete deprivation with cells primarily coccoid in morphology.

growth was 40°C with a lower limit of 20°C and an upper limit of 45°C (Fig. 5). The optimum pH for growth was 7.0 (Fig. 6) with a bicarbonate-CO_2 (primary) and MOPS (secondary) buffering system. Growth occurred both below pH 6.6 and above pH 7.3. The minimum generation time observed was 4.8 h. The moles percent guanine plus cytosine (G+C) base ratio was 44.9.

DISCUSSION

We propose that this new mesophilic methanogenic bacterium isolated from marine sediments in the Cayman Islands be placed in the family *Methanomicrobiaceae* described by Balch et al. (1) because cells are gram-negative rods that oxidize H_2 as the sole energy source for growth and methane production. This isolate belongs in the genus *Methanomicrobium* because its cells are small, irregular rods that occur singly without filaments, are gram negative, and have an optimum growth temperature of 40°C. The mol% G+C base ratio (44.9) is close to that of *M. mobile* (48.8) (1). The isolate differs from *M. mobile* in that cells are nonmotile, do not utilize formate for growth and methane production, have a pH optimum of 7.0 (higher than that in *M. mobile* [6.1 to 6.9]), and do not require complex organic nutrients (1, 9).

Immunological fingerprint data (E. Conway de Macario, personal communication) obtained by the indirect immunofluorescence technique (3) shows that the new isolate weakly reacts only with the anti-*Methanomicrobium mobile* BP S probe. Nucleotide cataloging (16S RNA) (17, 18) places this new isolate in the family *Methanomicrobiaceae* (R. S. Wolfe and C. R. Woese, personal communication).

Anaerobic growth flasks were constructed to increase the reservoir of hydrogen-carbon dioxide. This decreased the number of times the substrate had to be replenished. Therefore the substrate should not become limiting, and chances of contamination were reduced. The increased reserve of CO_2 reduced the fluctuation of the pH of the medium. Since CO_2 serves a dual role as substrate and as part of the primary buffering system, i.e., bicarbonate-CO_2, the addition of a secondary buffering system (MOPS)

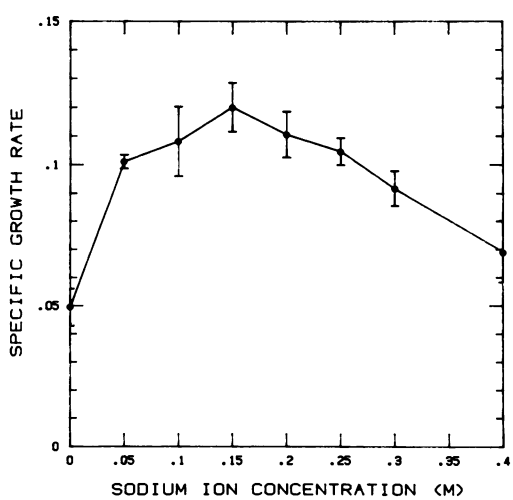

FIG. 4. Effect of sodium ion concentration on the growth rate of *M. paynteri*. Points represent means of triplicate determinations. Bars represent standard deviations.

brought about a more stable pH during the experiments (±0.1 pH units).

Until now, all methanogens isolated from marine environments have had a coccoid morphology and have utilized formate in addition to hydrogen-carbon dioxide for growth and methane production. This isolate is distinct because of its rodlike morphology and its inability to utilize formate. It does show the same sensitivity to low levels of detergents that lyse other marine methanogens.

The ability of the isolate to withstand periods of substrate depletion may afford the isolate with a unique mechanism for survival. This characteristic is currently being studied further.

We propose that this isolate be accorded species status, for which we propose the name *Methanomicrobium paynteri* sp. nov. paynter. i, M.L. gen. nov. *paynteri* of Paynter.

The cells are gram-negative, irregular rods that occur singly without filaments, 0.6 by 1.5 to 2.5 μm in size; they undergo a morphological change to a coccoid shape during substrate deprivation and remain viable at room temperature for months.

Colonies in agar are off-white and circular with entire edges.

Only hydrogen-carbon dioxide served as a growth substrate. Neither growth nor methane production was detected after 5 weeks when this substrate was replaced by ethanol, methanol, formate, acetate, propionate, pyruvate, monomethylamine, dimethylamine, or trimethylamine, glutamate, or glucose.

The organism can tolerate a variety of organic secondary buffers. The organism does not require yeast extract or Trypticase, but does require acetate.

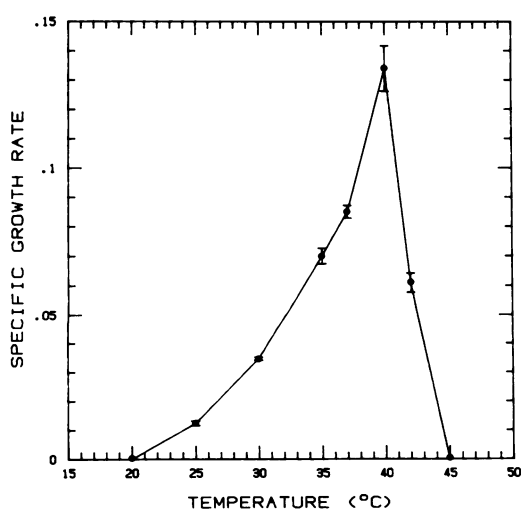

FIG. 5. Effect of temperature on the growth rate of *M. paynteri*. Points represent means of triplicate determinations. Bars represent standard deviations.

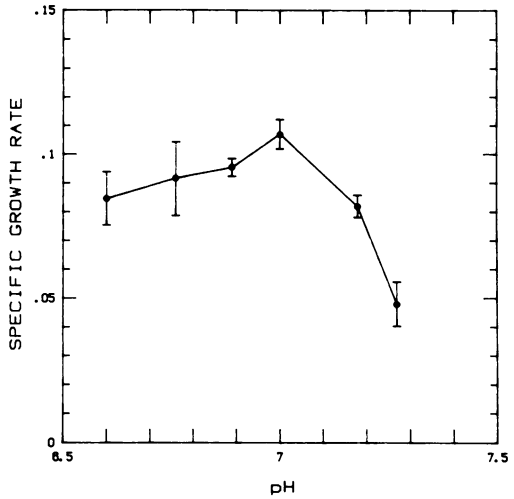

FIG. 6. Effect of pH on the growth rate of *M. paynteri*. Points represent means of triplicate determinations. Bars represent standard deviations.

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